DEVELOPMENTAL CHANGES IN GONADOTROPIN RELEASING HORMONE NEURONS IN THE BRAIN OF THE FEMALE RABBIT (*Oryctolagus cuniculus*)

By

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DEVELOPMENTAL CHANGES IN THE GnRH NEURON

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ABSTRACT

This thesis examined developmental changes in morphology of the GnRH neuron in the female rabbit brain during sexual maturation. In the adult virgin rabbit approximately 1,000 GnRH cells were counted in half the hypothalamus. Two morphologically distinct populations of GnRH neurons were found. Fusiform cells with relatively smooth contours (smooth) accounted for 34% of the total. Cells with irregular contours (rough) represented 64% of the immunoreactive cells counted. In a subsequent experiment GnRH cell types were quantified in rabbits treated with Tamoxifen (TAM, 10 mg/kg/day), vehicle, and pregnant mare serum (PMS, 50 IU on postnatal days (PND) 25 and 28). Sexual maturity was considered achieved when rabbits attained a body weight of 3.0 kg. The proportion of rough cells increased while the smooth cells decreased with sexual maturation. This change was advanced by PMS treatment and prevented by TAM treatment compared to controls. Sexual maturity was advanced by PMS treatment (92 PND) versus controls (108 PND) and delayed by TAM treatment (128 PND). Mean plasma estradiol was significantly (P= 0.01) elevated in PMS rabbits versus controls between PND 25 and 34 and again at PND 75 (P= 0.05). Since the total number of immunoreactive cells remained constant, it is concluded that smooth cells are transformed to rough cells. In another experiment chronic ovariectomy did not change the total number of GnRH cells counted when compared to

sham operated rabbits. However, the developmental shift of smooth cells to rough cells was prevented ($p<0.05$) in ovariectomized rabbits. These results suggest that the developmental change in GnRH cell morphology is functionally related to puberty onset. Moreover, estradiol seems to induce these changes through indirect mechanisms. It is proposed that estradiol augments the growth neural imputs to GnRH cellls.

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LIST OF ABBREVIATIONS USED

ARCN	arcuate nucleus
CNS	central nervous system
DA	dopamine
DAB	diaminobenzidine
E	epinephrine
EB	estradiol benzoate
EOP	endogenous opioid peptides
FSH	follicle stimulating hormone
GABA	gamma-amino-butyric acid
GH	growth hormone
GHRH	growth hormone releasing hormone
GnRH	gonadotropin releasing hormone
GnRH-IR	gonadotropin releasing hormone immunoreactivity
h	hour
hpg	hypogonadal
IGF-I	insulin like growth factor I
i.v.	intravenous
LH	luteinizing hormone
MBH	medial basal hypothalamus
ME	median eminence
min	minute
mRNA	messenger ribonucleic acid
mths	months
NAL	naloxone
NE	norepinephrine

NGS	normal goat serum
OVLT	organum vasculosum of the lamina terminalis
OVX	ovariectomized
PBS	phosphate buffered saline
PGE_2	prostaglandin E_2
PMS	pregnant mare serum
PND	postnatal day
POMC	pre-pro-opio-melanocortin
s.c.	subcutaneous
Tris-HCl	Tris (hydroxymethyl) aminomethane-hydrochloric acid buffer
$[^3H]$-	tritiated
6-OHDA	6-hydroxydopamine
TAM	Tamoxifen citrate

CHAPTER I

INTRODUCTION

Despite extensive study of the hypothalamic-pituitary-gonadal axis (Fig. 1) the mechanisms regulating sexual development and puberty onset remain poorly understood. Numerous theories have been proposed, the most popular of which is the "Gonadostat Theory" (Hohlweg and Junkmann, 1932; Ramirez and McCann, 1963). In the gonadostat theory puberty is considered to be the consequence of decreased hypothalamic sensitivity to the inhibitory influences of gonadal steroids. However, it has been shown (Odell and Swerdloff, 1974) that changing hypothalamic sensitivity to gonadal steroids is a consequence of puberty rather than its cause. Moreover, this theory fails to account for the dual (inhibitory and excitatory) effects of gonadal steroids on the hypothalamus. An alternative hypothesis, to the gonadostat theory, suggests that sexual development is dependent on maturation of the neuroendocrine apparatus regulating the secretion of gonadotropin releasing hormone (GnRH) (Terasawa et al., 1984). Moreover, it has been proposed that puberty is restrained by temporary disconnection of GnRH neurons from excitatory inputs (Ruf, 1982). Indeed, synapse formation in the arcuate nucleus (ARCN) is reported to be accelerated in rats with an induced precocious puberty (Matsumoto and Arai, 1976). However, neurochemical identification of the pre- and postsynaptic neurons remains unknown. Bhanot and Wilkinson (1983) suggest a physiological role for endogenous opioid peptides in the mechanism of puberty onset. In their study opioidergic

Fig. 1. Classical Model Of The Hypothalamic-Pituitary-Ovarian Axis

Positive Feedback

Hypothalamus

Negative Feedback

GnRH

Pituitary

Estrogen/Progesterone

LH/FSH

Estrogen/Progesterone

Ovary

inhibition of luteinizing hormone (LH) secretion was observed to decrease with sexual maturation. These authors suggest that at puberty opioidergic inhibition of GnRH secretion is reset to a higher level of sensitivity. Furthermore, in the rat, puberty is viewed as the result of decreased opioidergic inhibition coupled with the emergence of an excitatory drive for GnRH secretion. However, the issue requires investigation.

Precocious puberty has been induced by pulsatile infusion of GnRH in the immature rhesus monkey (Wildt et al., 1980; Abeyawardene et al., 1989), guinea pig (Loose and Terasawa, 1985), ewe (Pirl and Adams, 1987) and rat (Urbanski and Ojeda, 1987). When pulsatile GnRH infusion was suspended the rhesus monkeys promptly reverted to the immature state. In the prepubertal rat (Urbanski and Ojeda, 1987) and rhesus monkey (Gay and Plant, 1988) premature activation of the GnRH neuron was achieved and precocious puberty produced with N-methyl-DL-aspartate: an analog of the neuroexcitatory amino acid aspartate (Urbanski and Ojeda, 1987). These results suggest that GnRH neurons of immature animals are capable of responding in an adult manner but in the prepubertal state are in some way restrained, perhaps by temporary disconnection from excitatory inputs, or by inhibitory influences of endogenous opioids.

Hormonal modulation of the hypothalamic circuitry has previously been proposed as the mechanism limiting the rate

of puberty onset (Gorski, 1982). Temporary disconnection of GnRH neurons from excitatory inputs, most notably norepinephrine (NE), or modification of inhibitory inputs such as dopamine (DA) and opioids to GnRH neurons by hormonal influences have only recently been proposed.

Finally, morphological and functional studies have shown that gonadal steroids, while inhibiting gonadotropin secretion, appear to play a facilitating role in the maturation of the neural mechanisms which limit puberty onset. The facilitatory role of estrogen has been shown in experiments in which injections of estrogen (Ramirez and Sawyer, 1975) and median eminence implants of estradiol (Motta et al, 1968) induce precocious puberty in the rat. Morphological studies (Matsumoto and Arai, 1976; Arai and Matsumoto, 1978; Clough and Rodriguez-Sierra, 1983) have demonstrated changes in the number of synapses formed in the hypothalamic arcuate nucleus of rats as sexual maturation progresses to puberty. In these studies it was demonstrated that precocious puberty could be produced in immature female rats treated with pregnant mare serum (PMS) or estradiol benzoate (EB) accompanied by augmented synaptogenesis compared to control rats (Matsumoto and Arai, 1977; Arai and Matsumoto, 1978; Clough and Rodriguez-Sierra, 1983). Additionally, synaptogenesis has been produced in the completely deafferented medial basal hypothalamus (MBH) of EB treated ovariectomized female rats (Matsumoto and Arai, 1979). Based

on the above findings it is suggested that estrogen plays a facilitatory role in synaptogenesis in the ARCN, leading to puberty. However, to date it is not known if these morphological changes in any way directly involve the GnRH neuron. Transplantation studies with hypogonadal (hpg) mice have provided some insight. Hpg mice possess an autosomal recessive gene mutation (Cattanach et al., 1977) resulting in failure to elaborate GnRH and therefore have reproductive systems which are functionally immature. Brain grafts to the third ventricle of hpg mice produce physiological reversal of signs (Gibson et al., 1983; 1984; 1987; Kokoris et al., 1988). It would appear that at least some of the synapses involve GnRH neurons since transplants to the lateral ventricle of GnRH containing grafts were found to be unsuccessful in restoring normal reproductive function (Gibson et al., 1987). Kokoris and colleagues (1988) suggested that the graft must be innervated by fibers from the median eminence to regulate GnRH neuron behaviour. Since estrogen and PMS, which presumably act via induction of ovarian estrogen synthesis, induce precocious puberty and morphological changes in the rat brain, it is hypothesized that estrogen augments the development of GnRH afferents which regulate GnRH secretion.

In this thesis, the hypothesis that estrogens advance puberty onset in the female rabbit by inducing developmental changes in the GnRH neuron cytoarchitecture was tested. If changes in GnRH neuron cytoarchitecture suggestive of

increased input are indeed functionally related to puberty onset as promulgated by Wray and Hoffman (1986a; 1986b: 1986c), then these changes should be advanced in precocious puberty and delayed in retarded puberty onset. This hypothesis is tested in the female rabbit treated with either PMS or Tamoxifen (an antiestrogen), and ovariectomized rabbits.

The rabbit was selected as a model due to its extensive use in biomedical research (Fox, 1984). The fact that the rabbit is a reflex ovulator is perhaps the most serious limitation to the use of this species as a model. However, it has been observed that in many species of spontaneous ovulators, LH release can be induced by copulation (Marion, 1950; Zarrow and Clark, 1968). This observation has prompted the suggestion that the mechanisms underlying reflex ovulation are common among mammalian species while the mechanism of spontaneous ovulation represents a specialization (Conaway, 1971). Also, it is important to learn if morphological changes in GnRH neurons reported by Wray and Hoffman (1986a; 1986b; 1986c) in the rat, a spontaneous ovulator, also occur in the rabbit.

In using the rabbit it is assumed that the basic mechanisms underlying sexual development are conserved across mammalian species. The most notable mechanisms have been identified by Ojeda and Urbanski (1988) and include: 1) regulation of GnRH secretion by neurotransmitters, 2)

initiation of gonadotropin secretion, and 3) control of ovarian follicular development. The female rabbit is a very useful model of human sexual development since ovarian follicle maturation which occurs between 15 and 20 weeks gestation in the human fetus, occurs entirely postnatally in the rabbit. Oogenesis commences after birth in the rabbit (YoungLai and Byskov, 1983) and is complete within the first 2 postnatal weeks (Peters et al., 1965). Ovarian follicle maturation begins at approximately 50 days of age in the rabbit (de Turckheim et al., 1983). Consequently, use of the female rabbit permits evaluation of the role of the immature ovary in sexual development. Additionally, use of this model will extend findings in the rat (Wray and Hoffman, 1986a; 1986b; 1986c) to another mammal, providing insight into mechanisms of puberty common to both reflex and spontaneous ovulators.

CHAPTER II

THE RABBIT AS A MODEL FOR STUDIES IN REPRODUCTIVE NEUROENDOCRINOLOGY

2.1 INTRODUCTION:

Ojeda and colleagues (1980a) state that "defining puberty is as complex as the process of puberty itself". It is generally accepted that sexual development is a process which has its beginning in fetal life and continues past puberty into the post fertile years. Evidence suggests that puberty is the consequence of a complex series of interrelated events involving developmental changes in the pituitary and ovary, orchestrated by hypothalamic factors and direct neural inputs. For reviews of the endocrine changes associated with sexual maturation and puberty the reader is referred to papers by Ruf (1973), Odell and Swerdloff (1974), Gorski (1979), Chipman (1980), Cutler and Loriaux (1980), Frisch (1980), Grumbach (1980), Ojeda et al. (1980b), Ryan and Foster (1980), Wuttke et al. (1980), Reiter and Grumbach (1982), Bronson and Rissman (1986), Foster et al. (1986), Ojeda et al. (1986), Kinder et al. (1987), Ojeda and Urbanski (1988), and Wilkinson and Landymore (1988).

To date, the mechanism by which puberty is triggered has remained resistant to simple explanation. Numerous factors such as height, body weight (Glass et al., 1984; Barker, 1985; Arsenijevic et al., 1989), body composition (Frisch and Revelle, 1971; Wilen and Naftolin, 1977; Hansen et al., 1983; Frisch, 1980), environment (Bronson and Rissman, 1985; Wilson et al., 1988), diet (Hulot et al., 1982; Hansen et al., 1983; Bronson, 1985; Foster and Olster, 1985; Foster et al., 1986;

Landenfeld et al., 1989), photoperiod (Lincoln, 1981; Hansen et al., 1983; Kamwanja and Hauser, 1983; Rivest et al., 1985; Foster et al., 1986; Sisk and Bronson, 1986; Lang, 1986), adrenarche (Cutler and Loriaux, 1980) and social factors (Bronson and Rissman, 1985) have been inculpated as participants in the mechanism by which puberty is initiated. However, the exact mechanism(s) initiating sexual development and puberty remain(s) elusive.

The purpose of this review is to examine the literature concerning puberty in the female rabbit. Since the rat is the most frequently studied model of human sexual development, the major differences between the rat, rabbit, and human will be discussed where appropriate. Additionally, since GnRH plays a pivotal role in sexual development and puberty, hypothalamic localization of GnRH and the factors regulating GnRH secretion will be reviewed.

2.2 PUBERTY IN THE FEMALE RABBIT:

2.2.1 Physical Signs Of Puberty:

Investigations of the factors regulating puberty in the rabbit have been few in contrast to that of rat and man. Moreover, sexual maturity is difficult to determine in the female rabbit in comparison to the female rat. Vaginal opening is the most frequently used indicator of sexual maturity in the female rat (Ramirez and Sawyer, 1965; Motta et al., 1968; Clough and Rodriguez-Sierra, 1983). In

contrast, maturity in the female rabbit cannot be characterized by any readily observable external signs. Therefore, different indices of sexual maturity have been employed by different investigators. de Turckheim and colleagues (1983) used the age of first conception (110-120 days) to estimate maturity. YoungLai (1986) suggested that a body weight of greater than 3 Kg is the best indicator of sexual maturity. Hulot and coworkers (1982) found that while a body weight of 3 Kg was necessary for successful matings, a weight of 3.3 Kg was required before matings consistently resulted in ovulation. Since age at which female rabbits are found to be sexually competent varies widely, it is suggested that a weight of between 3.0 and 3.3 Kg may be the best indicator as it is correlated with both receptivity and ovulation.

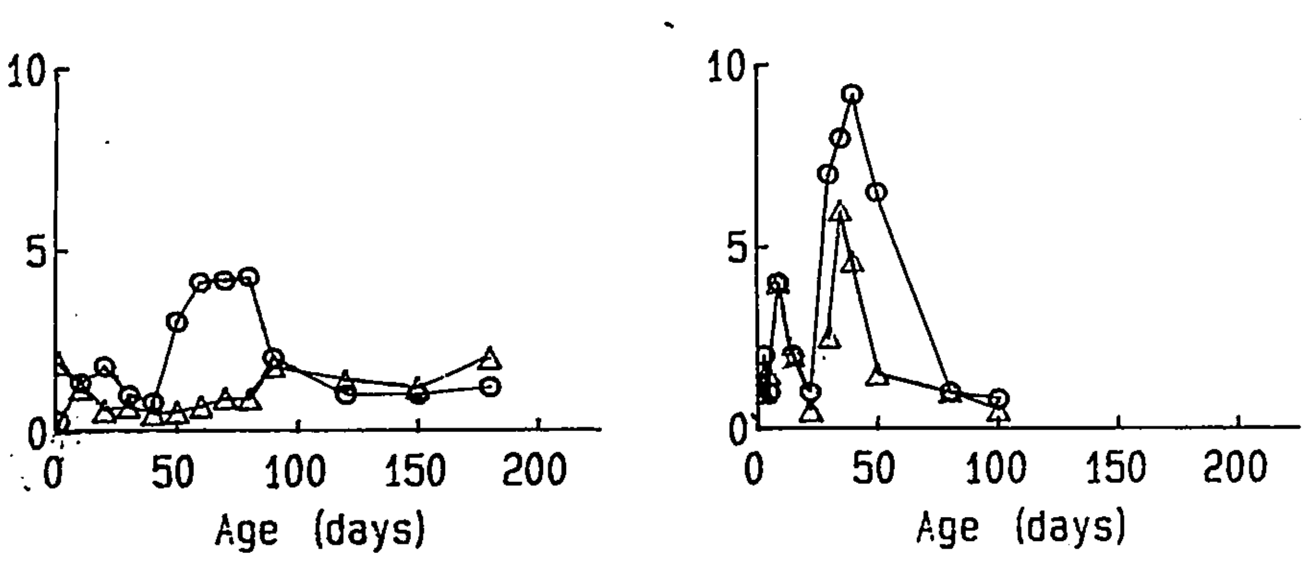
Identification of developmental stages offers the opportunity for insight into the mechanisms underlying sexual development. Distinct developmental periods have been identified in the rat, based on serum gonadotropin levels and alterations in steroid feedback mechanisms (Ramirez, 1973). Later, Ojeda and coworkers (1980) introduced a classification system for the rat based on both morphological and physiological factors. Stages of sexual development have also been described for man (Marshall and Tanner, 1970; Boyar et al., 1972; Chipman, 1980) based on secondary sexual characteristics and measurements of peripheral gonadotropins.

Stages of prepubertal development based on morphological and physiological factors and behaviour have also been described for the male rabbit (Berger et al., 1982) and female rhesus monkey (Terasawa et al., 1984). But to date, stages of sexual development have not been reported for the female rabbit. A need exists for identification of developmental stages in the female rabbit due to their frequent use in biomedical research (Fox, 1984) and reproductive biology.

2.2.2 Circulating Gonadotropins:

Although developmental stages have not been described for the female rabbit, postnatal changes in serum gonadotropins, and gonadal steroids (YoungLai et al., 1981; Berger et al., 1982; de Turckheim et al., 1983; YoungLai, 1986), have been reported. However, early studies have provided equivocal results and therefore make it difficult to identify critical stages of reproductive development in the female rabbit. de Turckheim and colleagues (1983) propose that puberty in the female rabbit is initiated around 50 to 60 days of age and was characterized by ovarian follicular maturation, accelerated ovarian growth, increased ovarian estrogen content and decreased circulating follicle stimulating hormone (FSH) levels. Serum FSH concentrations increased from birth to a plateau between day 10 and 40 of life (Fig. 2), peaked around day 50, then declined to adult levels by 100 days of age. In another study (YoungLai, 1986) circulating concentrations of

Fig. 2. Comparison of developmental changes in plasma gonadotropins reported by (A) de Turckheim et al., 1983 and (B) YoungLai, 1986.



FSH are reported to have two distinct peaks (Fig. 2) occurring on day 9 and day 40 of life.

Serum LH concentrations paralleled serum FSH levels (YoungLai, 1986) whereas de Turckheim and coworkers (1983) found no significant elevations. The differences reported in these studies may be due to multiple maternal influences which cannot be excluded during the pre-weaning ages. A protein which is functionally and immunologically similar to native GnRH has been demonstrated in the maternal milk of rats, cows and man (Baram et al., 1977) and may be present in the rabbit and other mammals. This GnRH-like factor is chromatographically indistinguishable from the native decapeptide (Smith et al., 1984). Moreover, it is suggested that "milk GnRH" has a physiological role in inducing elevated circulating gonadotropin levels in the neonatal rat since GnRH immunoreactivity is found in the stomach and plasma of suckling pups (Baram et al., 1977). Smith et al. (1984) have also demonstrated that available ovarian GnRH receptors decrease following suckling and this effect was blocked by passive immunization to GnRH. The occurrence of "milk GnRH" may account for the determinations of YoungLai (1986) of peak LH and FSH on day 9. While the use of different standards may account for some of the differences, the pattern of gonadotropin secretion described by de Turckheim et al., (1983) is significantly different from that described by YoungLai (1986) to warrant further study.

It is interesting that the period of elevated serum gonadotropins (35 - 50 days of age) reported by YoungLai (1986) is correlated with decreased [^{3}H] - Naloxone (an opioid antagonist) binding (Wilkinson and Younglai, 1986). Decreased binding coincided with increased serum gonadotropins, suggesting that GnRH secretion had escaped opioid inhibition during this period. Alternatively, it is feasible that increased circulating gonadotropins may be the result of increased numbers of GnRH receptors, enhanced pituitary responsiveness to GnRH or to developmental changes in GnRH receptors at this age. Further studies must be performed before the significance of these findings is understood.

It is suggested (YoungLai, 1986) that the prepubertal gonadotropin elevations may be related to oogenesis and ovarian maturation. In the rabbit oogenesis does not begin until after birth (YoungLai and Byskov, 1983) and is completed within the first two weeks of life (Peters et al., 1965). Ovarian follicular maturation begins around 50 days of age (de Turckheim et al., 1983).

Circulating levels of gonadotropins have been determined (Ojeda and Ramirez, 1972; Meijs-Roelof et al., 1973) for the sexually immature female rat. Plasma levels of FSH had a monophasic pattern with a peak on postnatal day 15. This is in contrast to the biphasic peak described by YoungLai (1986) for the female rabbit. Plasma levels of rat LH were also monophasic with a peak on postnatal day 10 and a modest

peak between postnatal days 20 and 30 (Ojeda and Ramirez, 1972).

Circulating levels of gonadotropins have also been reported for sexually immature girls and boys (Boyar et al., 1972; 1973) and summarized for each stage of puberty in a review by Chipman (1980). Both plasma gonadotropins have monophasic patterns in the immature state. Peak levels are found between 2 and 4 months of age. Circulating levels of gonadotropins begin to increase around 7 to 8 years of age and attain adult levels coincident with the onset of puberty.

2.2.3 Pituitary Responsiveness To Exogenous GnRH:

The ontogeny of pituitary responsiveness to exogenous GnRH in vivo has recently been investigated in the female rabbit (YoungLai et al., 1989). Significant increase in LH secretion occurred within 15 min of injection in all age groups. FSH secretion, in contrast, was significantly increased on days 22, 29 and 72 only. These data suggest that the pituitary of prepubertal rabbits is functional and that LH and FSH secretion may be differentially regulated. YoungLai and colleagues (1989) suggest that a distinct ovarian releasing factor for FSH may exist, or that other ovarian factors such as inhibin and 3-α-hydroxypregn-4-en-20-one are elaborated to selectively inhibit FSH secretion at sexual maturity. However, pulse frequency of GnRH secretion may be

the final common step in the pathway resulting in differential regulation of gonadotropin secretion as evidenced from studies in the rat reviewed by Chappel, 1985. Recently, Dalkin et al. (1989) showed that the frequency of GnRH pulse differentially regulates gonadotropin subunit messenger ribonucleic acid (mRNA) expression in the rat. Nevertheless, the pituitary of immature female rabbits is functionally responsive to GnRH. Based on circulating gonadotropin levels in the female rabbit (de Turckheim et al., 1983; YoungLai, 1986) it can be inferred that GnRH secretion is inhibited after 72 days of age.

Pituitary responsiveness to exogenous GnRH has also been examined in the sexually immature female rat (Debeljuk et al., 1972; Ojeda et al., 1977; Urbanski and Ojeda, 1987). Debeljuk and colleagues (1972) found that a bolus s.c. injection of GnRH resulted in a significant release of LH at all ages studied with a maximum response in 15 to 25 day olds which corresponds to the period of peak pituitary LH concentration (Dohler et al., 1977). A significant release of FSH was reported only in 15, 25 and 30 day old rats with maximum responses at 15 and 25 days of age (Debeljuk et al., 1972). These data suggest that the immature pituitary is functionally mature but the pituitary responsiveness to exogenous GnRH is diminished after about 30 days of age. In contrast, Ojeda and coworkers (1977) found that maximum pituitary responsiveness to an intravenous (iv) injection of GnRH was between days 10 and 15 of life after which

circulating levels of gonadotropins declined. It would appear that the method of GnRH delivery (s.c., or iv) plays a significant role in determining pituitary responsiveness to exogenous GnRH.

In addition to circulating levels of gonadotropins, pituitary gonadotropin concentration has also been described. Pituitary FSH concentration in the sexually immature female rat was described (Dohler et al., 1977) as monophasic with a peak between days 21 and 31. Pituitary LH concentration, however, was biphasic (Dohler et al., 1977). A peak at days 19 to 21 was found, followed by a second peak occurring on days 31 to 33. Thereafter, pituitary LH concentration declined until vaginal opening occurred on approximately postnatal day 38. Pituitary FSH storage appears to be increased only after the period of increased circulating levels found on day 15 (Ojeda and Ramirez, 1972; Meijs-Roelof et al., 1973). Pituitary LH concentration, however, seems to be unrelated to circulating levels. These data suggested that gonadotropin secretory granule is immature until approximately 3 weeks of age. Similar work has yet to be performed in the rabbit.

The pituitary response to GnRH in vitro has also been studied in the rabbit (Todoroff et al., in preparation) and confirmed the in vivo studies (YoungLai et al., 1989). A circadian rhythm in pituitary responsiveness to GnRH was also observed in the immature rabbit. Additionally, in immature

girls a circadian rhythm in circulating gonadotropins has been observed. However, increased secretion of LH and FSH occurred during sleep beginning in mid-puberty; the difference was lost in late puberty and adulthood. In the sexually immature rat, however, i.v. administration of GnRH showed no circadian rhythm in gonadotropin response (Ojeda et al., 1977). In contrast, pituitary glands in vitro did have a circadian rhythm (Wilkinson and Moger, 1981). Evidence of a circadian rhythm of gonadotropin secretion has also been found in the rhesus monkey (Terasawa et al., 1984). As in the human, the circadian rhythm is lost in the late pubertal stage (50 to 60 mths; after first ovulation) or adulthood. Push pull perfusion of the rhesus monkey median eminence showed a developmental increase in GnRH secretion with increased pulse amplitude and nocturnal GnRH output during the mid pubertal stage (Watanabe and Terasawa, 1989).

Under in vivo conditions the rabbit pituitary was responsive to GnRH at all ages studied up to sexual maturity (YoungLai et al., 1989). In contrast, pituitary sensitivity to GnRH has been shown to decline prior to the onset of puberty in the rat (Wilkinson and Moger, 1981; Naish et al., 1986) and rabbit (Todoroff et al. In preparation). In superfused pituitaries LH secretion in response to GnRH was greatest in 15 to 20 day old rats and in first proestrous animals compared to rats 30 day old (Naish et al., 1986). These authors speculate that changes in LH secretory dynamics

may be due to maturation of the LH secretory granule. However, changes in pituitary GnRH receptor dynamics must also be considered. In addition, the manner in which GnRH is delivered (bolus vs pulse, and route) must also be considered.

2.2.4 Ontogeny Of Pituitary GnRH Receptors:

It has been demonstrated that GnRH regulates the number of pituitary GnRH receptors in a dose dependent manner. To date, there have been no published reports of the ontogeny of pituitary GnRH receptors in the sexually immature female rabbit. However, results from a number of rat studies indicate that the receptor number increases early in sexual maturation (Dalkin et al., 1981; Marian et al., 1981; Duncan et al., 1983). Duncan and colleagues (1983) demonstrated that GnRH receptor number in the pituitary rises to a plateau between days 15 and 30 of age before rising to a peak at 50 days. Simultaneous measures of circulating gonadotropins and pituitary GnRH receptor number showed that the maximum number of pituitary GnRH receptors occurs when the pituitary is maximally responsive to GnRH between days 10 and 15 (Dalkin et al., 1981).

2.2.5 Pulsatile Secretion Of Gonadotropins:

In the female rabbit, LH and FSH secretion is described as being pulsatile (YoungLai and Byrne, 1989) between the ages of 36 and 60 days. These results suggest that the GnRH pulse

generator is active prepubertally in the female rabbit. Similarly, in immature boys and girls gonadotropins are released in a pulsatile fashion (Chipman, 1980; Hale et al., 1988; Wu et al., 1990) beginning nocturnally in early-mid-puberty. Frequency of LH pulses were found to increase significantly between the prepubertal and peripubertal stages and to increase further in the pubertal group (Wu et al., 1990). Pulses of gonadotropins also become apparent in both wake and sleep periods and become increased in amplitude in mid to late puberty. Consequently, it appears that the GnRH pulse generator is also active prepubertally in both boys and girls. Wu and coworkers (1990) conclude that 1 to 2 years prior to the clinical onset of puberty the nocturnal pulse frequency of LH, and by inference GnRH, increases.

2.2.6 Pulsatile GnRH Secretion:

To date, pulsatile GnRH secretion has not been measured directly in the sexually immature rabbit. The hypothesis that sexual development and puberty depends on maturation of the neuroendocrine apparatus regulating the secretion of GnRH has been tested in other mammals. This hypothesis has gained support from both functional and morphological studies. In cattle it was found that episodic GnRH release precedes the appearance of pulsatile LH secretion (Rodriguez and Wise, 1989). Moreover, when LH pulses became apparent they were associated with an increase in the pulse frequency of GnRH.

Similarly, push pull perfusion of the rhesus monkey median eminence (Watanabae and Terasawa, 1989) revealed that a developmental increase in GnRH pulse amplitude and a decrease in interpulse interval precedes puberty. Also, in the sexually immature male rat it was observed (Bourgignon and Franchimont, 1984) that GnRH pulse frequency increases with age and attains the adult frequency prior to becoming sexually mature. It is proposed by these authors that accelerated GnRH pulse frequency may be responsible for pituitary activation at puberty. However, the question of what initiates the acceleration in GnRH pulse frequency remains unanswered.

In sheep, GnRH pulse frequency increases in the pubertal transition and becomes further accelerated when a critical body mass has been achieved (Foster et al., 1985). Plant (1984) proposes that in the male rhesus monkey the GnRH pulse generator is fully mature prior to puberty but is restrained until the animal becomes mature. The mechanism whereby the pulse generator is restrained remains a mystery. However, it has been hypothesized (Loose and Terasawa, 1985) that an increase in GnRH due to hypothalamic maturation is the significant factor regulating the onset of puberty.

2.2.7 Ontogeny Of Ovarian Estrogens In The Rabbit:

The role of estrogen in sexual development and puberty has been considered in a wide variety of studies in the rat. However, little information exists regarding the role of

estrogens in rabbit puberty. de Turckheim and co-workers (1983) report that immunoreactive estradiol was not measurable in the plasma of New Zealand female rabbits at any of the ages studied. In contrast ovarian estrogens were detectable at low levels from birth to 50 days of age, but from 50 days up to 6 months of age ovarian estrogens increased. Two sharp peaks were reported for estradiol between 50 days and 60 day nd then at 90 days of age. Estrone concentration in the ovaries followed the same pattern as estradiol. The sharp rise in ovarian estrogens at 50 days of age correlates well with peak levels of circulating FSH (de Turckheim et al., 1983; YoungLai, 1986) in the rabbit. On the basis of these studies it is difficult to determine if the pattern of gonadotropin and estrogen secretion behaves in a manner consistent with the gonadostat theory. According to the gonadostat theory decreasing gonadotropin secretion should be preceded by increased estrogen secretion. In order to test this hypothesis more frequent blood sampling from 40 to 60 days of age should be performed.

Changes in various endocrine measures during sexual maturation in the female rabbit are summarized in Table I.

2.2.8 The Gonadostat Theory:

The gonadostat theory has achieved considerable popularity since it is descriptive and the physiological principles of inhibition and disinhibition are well recognized

Table I Summary Of Reported Changes In Various Endocrine Measures In The Rabbit During Sexual Maturation.

Measure	Days Postpartum									
	25	35	40	45	50	60	75	85	95	100
Circulating GT's	1. 2 peaks of FSH; 1 at 9 days and the second at 40 days of age. from day 40 FSH levels decline to adult levels by age 100 days. 2. FSH rises to a plateau at 40 days of age and then rises to a peak at 50 days of age. from 50 days onward FSH level fall to adult levels by day 100. 3. LH peaks on days 9 and 35 before declining to adult levels by day 100. 4. LH never exceedes adult levels at any age.									
Response to exogenous GnRH	1. Significant increase in LH secretion at all ages. 2. FSH significant increase at day 25.						FSH significant increase at day 75.			
AM V.S. PM Plasma GT's										
Plasma estradiol	1. Not measurable throughout the prepubertal period.									
Ovarian [estadiol]					1. peak between days 50 and 60.				2. Second peak at day 90.	
Ovarian [Estrogens]	1. low to 50 days of age then increase. Follows estradiol pattern.									
Follicular maturation			1. begins between days 40 and 60.							
Ovarian weights			1. increase between days 40 and 60.							
Opiate binding		1. Binding of tritiated Naloxone is decreased between days 35 and 50.								

(Ruf, 1982). The gonadostat theory promulgated by Ramirez and McCann (1963) implies that the hypothalamus of sexually immature mammals is highly sensitive to gonadal steroids which inhibit secretion of GnRH and the gonadotropins. Coincidental with the onset of puberty, hypothalamic sensitivity to the inhibitory effects of gonadal steroids is reset to a higher level. Consequently, higher plasma concentrations of gonadal steroids are necessary to inhibit GnRH and subsequently gonadotropin secretion in pubertal and sexually mature individuals. Ramirez and McCann (1963) observed that prepubertal female rats were 2 to 3 times more sensitive to daily subcutaneous injections of estradiol benzoate (EB) than were mature females. The increased gonadal steroid levels are also responsible for the development of secondary sexual characteristics (Ruf, 1982) which begin to appear with the onset of puberty.

It is implied by the gonadostat theory that gonadotropin secretion is actively inhibited by gonadal steroids in sexually immature subjects. Moreover, desensitization to the inhibitory influences of the gonadal steroids is suggested as the trigger for the pubertal process. However, decreased hypothalamic sensitivity to gonadal steroids associated with puberty has been suggested to be a consequence rather than a cause of puberty (Odell and Swerdloff, 1974; Ojeda et al. 1980a and 1980b; Ruf, 1982). Ojeda and colleagues (1980a, 1980b) report that in female rats hypothalamic desensitization

to gonadal steroids does not occur until after the first ovulation. Additionally, hypothalamic desensitization to gonadal steroids was not demonstrable in any prepubertal animals. Therefore, these results must be viewed as being incompatible with the gonadostat theory. Finally, while the concept of disinhibition is well recognized, the physiological mechanisms are poorly defined. Desensitization of the hypothalamus to gonadal steroids may be due to a number of different factors such as changing estrogen receptor kinetics, post receptor processing of the signal, responsiveness of GnRH neurons and/or changes in opioid mediation of the gonadal steroid signal.

In contradiction to the gonadostat theory, gonadotropin secretion does not rise immediately prior to puberty in female rats but shows a steep fall (Odell and Swerdloff, 1974). Although the prepubertal period is longer in rabbits than rats, similar data has been described for prepubertal female rabbits (YoungLai, 1984, 1986). These findings must also be considered incompatible with the gonadostat theory, since the theory would predict that as the hypothalamus becomes less sensitive to the inhibitory influence of gonadal steroids, gonadotropin secretion should increase. However, this is contrary to the findings of de Turckheim et al. (1983) and YoungLai (1986) in the rabbit. Indeed, pituitary sensitivity to GnRH appears to decrease in synchrony with decreasing hypothalamic sensitivity to gonadal steroids (Ojeda et al.,

1980b; Naish et al., 1986). These results suggest that less GnRH is being released by the hypothalamus rather than more as would seem to be predicted by the gonadostat theory. However, Ojeda and colleagues (1980a) postulate that a subtle increase in basal LH levels may occur before the first preovulatory gonadotropin surge in the rat. A subtle increase in basal LH secretion could in theory contribute to the activation of the ovaries that precedes the gonadotropin surge. These authors speculate that more frequent sampling might detect such subtle changes in the basal LH plasma concentrations.

A final criticism of the gonadostat theory involves the paradoxical observation that throughout most of the prepubertal period agonadal children (hypergonadotropic hypogonadism) children are found to have elevated plasma gonadotropin levels (Winter and Faiman, 1972; Conte et al., 1976). Plasma gonadotropin concentrations are greater in agonadal children than in normal prepubertal individuals but are less than those found in adult castrates. The pattern of gonadotropin secretion is qualitatively the same as that of age matched normal prepubertal subjects (Reiter and Grumbach, 1982). However, after the normal age of puberty, there is a further increase in circulating gonadotropin levels in agonadal children (Winter and Faiman, 1972). Similar findings have been reported for the peripubertal and ovariectomized female rhesus monkey (Terasawa et al., 1984).

These findings are indeed paradoxical considering the absence of inhibition by gonadal steroids on the hypothalamus. Moreover, these findings suggest that normal pubertal changes in the hypothalamus and pituitary can occur in the absence of a functional gonad. Proponents of the gonadostat theory have advanced the concept of intrinsic central nervous system (CNS) restraint (Grumbach, 1980) in order to assure a sexually quiescent prepubertal period (Ruf, 1982). The concept of intrinsic CNS restraint essentially proposes that the CNS, governed by an endogenous cycle, restrains the activation of the hypothalamic-pituitary-gonadal axis independent of gonadal steroid secretion. However, like the gonadostat theory itself, the concept of intrinsic CNS restraint is descriptive and does not offer any explanation of the mechanism by which it operates. Consequently, the gonadostat theory does not adequately account for both clinical and recent experimental observations. Alternatively, Terasawa and colleagues (1984) and others (Reiter and Grumbach, 1982; Bourguinon and Franchimont, 1984) suggest that puberty is the consequence of maturational changes in the hypothalamus which alter the GnRH release pattern during maturation rather than any change in hypothalamic sensitivity to gonadal steroids. However, direct and conclusive evidence of what maturational changes must precede puberty has yet to be demonstrated.

2.2.9 Synaptogenesis Theory:

The possibility that puberty may be the result of extended brain plasticity and increasing stimulation of the hypothalamic areas governing gonadotropin secretion was advanced (Odell, 1979; Gorski, 1982; Ruf, 1982) as an alternative model to the gonadostat theory. The literature contains a number of interesting morphological reports which are consistent with such an hypothesis. In 1971 Loizou, using histochemical fluorescence methods, demonstrated that CNS neurocircuitry is not fixed at birth but subject to continued postnatal development. In this study, it was observed that amine-containing cell bodies in various hypothalamic areas and nerve endings in the median eminence develop entirely postnatally in the rat. The adult distribution of hypothalamic neuron cell bodies was not achieved until 3 to 5 weeks of age. Additionally, a catecholaminergic pathway originating in the lower brain stem was found to innervate the hypothalamus (Fuxe and Hokfelt, 1969). Recently, Wray and Hoffman (1986b), employing a double label, light microscopic immunohistochemical technique demonstrated catecholaminergic innervation of GnRH neurons of the rat hypothalamus.

The hypothesis of extended brain plasticity is further supported by morphological studies which have described developmental changes in the arcuate nucleus of female rats (Matsumoto and Arai, 1976; Arai and Matsumoto, 1978; Matsumoto, 1984). In these studies the number of axodendritic

and axosomatic synapses per unit area of the ARCN was reported to increase with age, with more synapses observed in sexually mature rats than in less mature subjects. Later, it was demonstrated that precocious puberty, induced by treatment with either PMS or EB was accompanied by an increase in the number of synapses observed per unit area of the ARCN (Matsumoto and Arai, 1977; Arai and Matsumoto, 1978; Matsumoto and Arai, 1979; Clough and Rodriguez-Sierra, 1983). Additionally, synaptogenesis has been produced in the completely deafferented medial basal hypothalamus of EB-treated, ovariectomized female rats (Matsumoto and Arai, 1978). Presumably, PMS acts by stimulating the ovaries and it is the subsequent increased secretion of estrogen by the ovaries which accounts for the similar effects of PMS and EB (Matsumoto and Arai, 1977). Consequently, it is suggested that sexual maturation entails synapse formation in the rat ARCN. Furthermore, it would appear that estrogen acts through a neural mechanism to accelerate maturation of the neuroendocrine apparatus regulating GnRH secretory patterns.

In a further series of studies it has been shown that grafts of medial basal preoptic area from fetal rats survive well when transplanted to the third ventricle of young and aged rats (Matsumoto et al., 1985a; 1985b; 1987). Not only do fetal grafts survive well in the aged brain but it was shown that the graft continues to develop as evidenced by a greater number of synapses per 10,000 um^2 in the graft than

in the neonatal brain (Matsumoto et al., 1985b). Increased dopaminergic activity was found in such grafts (Matsumoto et al., 1985b). This suggests that DA is involved in activating the GnRH producing neural system in the aged brain as determined by the finding of increased ovarian weights of grafted rats compared with intact rats and controls. This model is appealing because it focuses attention on the hypothalamus and the adult pattern of neural circuitry and therefore neural integration as the site for the rate limiting step affecting puberty.

While the concept of extended brain plasticity is an attractive alternative to the gonadostat theory, there are many questions which remain to be answered. For example, this new model suggests that the abnormal gonadotropin levels observed in agonadal children may be explained by the assumption that GnRH producing neurons are temporarily disconnected from their neuroregulatory inputs (Ruf, 1982). However, this assumption has yet to be seriously considered, let alone experimentally tested. Additionally, if sexual maturation is dependent on extended brain plasticity, then what are the factors which govern hypothalamic synaptogenesis and ultimately the adult pattern of hypothalamic circuitry? Moreover, morphological studies in the rat have shown that the ARCN is essentially devoid of immunoreactive GnRH cells (Clayton and Hoffman, 1979). What then is the nature of the pre- and postsynaptic neurons forming the synapses?

2.3 Hypothalamic Localization of GnRH:

Following isolation and purification of GnRH (Amos et al., 1971; Schally et al.,1971) the decapeptide (Fig. 3) has been synthesized (Matsuo et al., 1971) and specific anti-GnRH antisera prepared and GnRH demonstrated in the hypothalamus of a variety of mammalian species such as the guinea pig (Silverman, 1976; Silverman and Desnoyers, 1976; Silverman and Krey, 1978; Silverman et al., 1979; Silverman, 1984; Silverman and Witkin, 1985), hamster (Phillips et al., 1982), mouse (Kozlowski and Zimmerman, 1974; Zimmerman et al., 1974; Naik, 1975; Hoffman, 1976; Hoffman et al., 1978; Burchanowski et al., 1979), sheep (Kozlowski and Zimmerman, 1974), cattle (Lechin et al., 1988), rat (Watanabe and McCann, 1968; Crighton et al., 1970; Brownstein et al., 1974; Wray and Hoffman 1986a; 1986b; 1986c) and monkey (Zimmerman and Antunes, 1976; Hoffman,1976; Silverman et al., 1977; Marshall and Goldsmith, 1980) by a variety of techniques.

2.3.1 Radioimmunoassay:

Early efforts to localize GnRH in the hypothalamus involved in vitro bioassay (Watanabe and McCann, 1968; Crighton et al., 1970) and radioimmunoassay techniques (Brownstein et al., 1974; Araki et al., 1975; King et al., 1975; Wilber et al., 1976). Bioassay of samples from hypothalamic nuclei were used to quantify GnRH with area delimited regions of the hypothalamus (Brownstein et al.,

Fig. 3. Amino acid sequence of mammalian GnRH. The numerical position of each amino acid is shown below.

pGlu-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-NH2

1 2 3 4 5 6 7 8 9 10

1984). King and coworkers (1975) using a different approach also quantified the GnRH content of the rat hypothalamus. Sections of the adult rat brain in the frontal, transverse, and sagittal planes were made. Radioimmunoassays of 150 um thick sections were correlated with morphological findings in the adjacent section. In the adult diestrous rat the majority of GnRH was reported to be confined to the ventral and medial core of the hypothalamus "extending from the rostral to the caudal tip of the arcuate-ME complex" (King et al., 1975). GnRH content was variable in the arcuate nucleus. The highest concentration was found in the middle third of the nucleus. High concentrations of GnRH were also found in the anterior pole of the nucleus caudal to the supraoptic nucleus. A third region of high GnRH concentration was in the post-infundibular median eminence where the stalk is separate. Araki and coworkers (1975) using a radioimmunometric method observed a dramatic increase in hypothalamic GnRH content just prior to vaginal opening in the rat. However, similar studies of the rabbit hypothalamus have yet to be performed.

2.3.2 Immunohistochemical:

The majority of immunohistochemical investigations have used the rat as the experimental subject for both light (King et al., 1974; Kordon et al., 1974; Pelletier et al., 1974; Naik, 1975; Weiner et al., 1975; Pelletier et al., 1976; Setalo et al., 1976a, 1976b; Knigge et al., 1977; Flerko et

al., 1978; Krisch, 1978; McNeill and Sladek, 1978; Ajika, 1979; Krisch, 1980; Krisch and Leonhardt, 1980; Jennes et al., 1982; Terasawa and Davis, 1983; Wray and Hoffman-Small, 1984; Jennes et al., 1985; Wray and Hoffman, 1986a; 1986b; 1986c; Pelletier, 1987; Wray and Gainer, 1987) and ultrastructural (Jennes et al., 1985; Leranth et al., 1985; Pelletier, 1987; Wray and Gainer, 1987) studies. Immunoreactive GnRH neural elements have also been studied in mice (Zimmerman et al., 1974; Naik, 1975; Hoffman, 1976; Hoffman et al., 1978; Silverman et al., 1988), man (Barry, 1977), pigs and cattle (Zimmerman, 1976), subhuman primates (Barry and Carette, 1975; Zimmerman and Antunes, 1976; Hoffman, 1976; Silverman et al., 1977; Marshall and Goldsmith, 1980), sheep (Kozlowski and Zimmerman, 1974; Zimmerman, 1976) and the guinea pig (Silverman, 1976; Silverman and Desnoyers, 1976; Silverman and Krey, 1978; Silverman, 1984; Silverman and Witkin, 1985). A number of reviews of the subject have been published (Barry et al., 1973; Pelletier, 1976; Wilber et al., 1976; Goldsmith, 1977; Zimmerman, 1977; Kozlowski, 1982; Kalra and Kalra, 1983; Krieger, 1983; Halasz et al., 1989). In comparison to other mammalian species, studies involving the rabbit have been few (Barry, 1976; Flerko et al., 1978; Weindl and Sofroniew, 1980a). GnRH immunoreactivity in the rabbit hypothalamus is reviewed by Barry (1979), Weindl and Sofroniew (1980b) and Barry et al. (1985).

Preliminary data on the topography of gonadotropin

hormone-releasing hormone immunoreactivity (GnRH-IR) in the rabbit hypothalamus has been reported (Barry, 1976; Flerko et al., 1978; Weindl and Sofraniew, 1980a). But detailed distributional maps have not been reported in this species. Immunoreactive perikarya are scattered from the mammillary bodies to the paraolfactory area of the rabbit hypothalamus (Barry, 1976). Five main groups of cells were described: paraolfactory, pre- and pericommissural and subfornical; pre- and suprachiasmatic; tuberoinfundibular and premammillary; perimammillary and mesencephalic. Flerko et al. (1978) report that immunoreactive cells are limited to the preoptic area and tuberal regions with the majority of cells in the preoptic-suprachiasmatic areas (Flerko et al., 1978). Two main tracts of immunoreactive axons, the preoptico-terminal and hypothalamo-infundibular have been described (Barry, 1976). In contrast, four GnRH-IR fiber tracts were described in another report (Flerko et al., 1978). These tracts were the preoptico-infundibular, tuberoinfundibular, extrahypothalamic and subependymal of the anterior periventricular area. Data from the preliminary reports described above is difficult to interpret because the source of the primary antisera used is not adequately described nor is the method by which it was produced reported. Finally, these studies fail to report the number of animals studied, housing conditions, their age, sex, or reproductive histories. The topography of GnRH neural elements in the rabbit hypothalamus, therefore, deserves re-

evaluation due to discrepant results reported in the above studies and the availability of improved immunohistochemical techniques. Moreover, study of developmental changes in GnRH neuron morphology in the rabbit is necessary in order to determine if developmental changes reported for the rat (Wray and Hoffman, 1986 a; 1986b; 1986c) also occur in the rabbit.

2.3.3 In Situ Hybridization:

Molecular biological techniques have now been employed to localize GnRH-mRNA-containing cells of the rat hypothalamus (Pfaff, 1986; Rothfeld et al., 1987; Park et al., 1988). The distribution of GnRH cells reported agrees with those employing immunohistochemical methods. It was also demonstrated that hybridizable GnRH-mRNA is decreased in the gonadectomized rat compared to controls (Pfaff, 1986; Park et al., 1988) while the total number of GnRH-mRNA cells did not change (Rothfeld et al., 1987). Steroid replacement increased the quantity of measurable GnRH-mRNA. Consequently, it is proposed that cells immunoreactive for GnRH elaborate GnRH rather than simply store product which was produced elsewhere. Additionally, it would appear that gonadectomy causes a reduction in GnRH transcription which is augmented by gonadal steroids.

2.4 REGULATION OF GnRH SECRETION:

Numerous studies have brought forward voluminous evidence to show that catecholamines, opioids, and gonadal steroids affect GnRH secretion either directly or through interaction with each other. Other compounds which have been demonstrated to modify GnRH secretion include neuropeptide Y, melatonin, serotonin, kyurenin, prostaglandins and ionic calcium. The majority of studies are, however, the result of experiments using adult rats. Consequently, for the purposes of this review, a discussion of the effects of catecholamines, opioids and gonadal steroids on GnRH secretion in the adult will be presented. Where available, emphasis will, however, be given to reports concerning immature animals and where appropriate, in the rabbit. It should be noted that in each case substantive reviews could be written. The goal of this thesis is to provide a parsimonious review of the subject.

2.4.1. Catecholamines:

Norepinephrine and dopamine are the principal catecholamines, which on the basis of morphological, functional, and pharmacological evidence, have been shown to modify GnRH secretion. Numerous reviews (Fuxe and Hokfelt, 1969; Kordon and Glowinski, 1972; McCann et al., 1972; Fuxe et al., 1978; Vijayan and McCann, 1978; Sawyer and Clifton,

1980; Wuttke et al., 1980; Palkovits, 1981; Barraclough and Wise, 1982; Ramirez et al., 1984; Hokfelt et al., 1986) are available for the interested reader.

(i) Morphological studies: The topography of monoamines in the hypothalamus of the rabbit has been investigated using the formaldehyde-induced fluorescence technique (Carlson et al., 1962; Fuxe, 1964; Fink et al., 1975). Fink and coworkers (1975) state that the distribution of catecholamine containing neural elements in the hypothalamus of the adult female rabbit is similar to that of the rat. In the rat, noradrenergic nerves arise in the locus coeruleus, medulla, and pons (Fuxe and Hokfelt, 1969; Hokfelt and Fuxe, 1972; Palkovits, 1981). Processes from noradrenergic cells in these brain regions travel by either the ventral (medulla and pons) or dorsal noradrenergic nerve tract to the hypothalamus. Dopaminergic nerve cells are located in the arcuate nucleus, nucleus dorsomedialis, and preoptico paraventricular nucleus (Palkovits, 1981). A small number of studies have attempted colocalization of catecholaminergic and GnRH containing neural elements in the hypothalamus. To date these studies have been confined to the rat. Fluorescence histochemistry combined with immunohistochemistry (Ibata et al., 1979) demonstrated that catecholaminergic nerve fibers are anatomically related to GnRH nerve endings in the lateral portion of the middle part of the median eminence but not the organum vasculosum of

the lamina terminalis (OVLT). These findings led the authors to speculate that the association was close enough to have functional correlation. Jennes and coworkers (1982) have since brought forward evidence based on immunohistochemical double staining for GnRH and dopamine-beta-hydroxylase or serotonin, that showed close apposition of fibers containing serotonin or norepinephrine to GnRH producing neurons in the septo-preoptic region. In the OVLT and median eminence, GnRH and serotoninergic fibers overlap extensively while GnRH fibers have little anatomical overlap with noradrenergic fibers. However, the bulk of the morphological data suggests that catecholaminergic innervation of GnRH neural elements in the median eminence is via dopaminergic nerve fibers.

McNeill and Sladek (1978), using fluorescence immunocytochemistry demonstrated in the same section that two populations of dopaminergic nerve fibers exist in the lateral median eminence of the rat. One band of fibers overlapped GnRH terminals while the second band was juxtaposed to portal capillaries. Ajika (1979) demonstrated axo-axonic contact between dopaminergic and GnRH terminals in the perivascular region of the median eminence. Additionally, Jennes and coworkers (1983), using a double immunohistochemical staining technique for GnRH and tyrosine hydroxylase, found close spatial association in the septo-preoptic diagonal band complex and the median eminence of the rat brain. However, in the OVLT no close spatial relationship was found. In the

ewe, synaptic contacts between dopaminergic and GnRH containing profiles has been demonstrated (Kuljis and Advis, 1989). Moreover, incubation of the median eminence in the presence of dopamine resulted in reduced GnRH secretion (Kuljis and Advis, 1989). Consequently, it is proposed that dopamine affects GnRH secretion into the portal vessels by presynaptic inhibition.

Finally, it was shown that two populations of GnRH containing cells exist in the rat hypothalamus (Wray and Hoffman, 1986a). It was demonstrated that during the first 6 weeks of life the number of aspinous GnRH cells decreased while the number of irregular or spiny GnRH cells increased. In a subsequent study (Wray and Hoffman, 1986b), the number of catecholamine apposed aspinous GnRH cells remained constant while the number of catecholamine apposed irregular GnRH cells increased as a function of age. These data support the hypothesis that the GnRH subpopulations are differentially innervated. Furthermore, it is concluded (Wray and Hoffman, 1986b) that development of irregular cells represents newly innervated surfaces which are associated with the processing of incoming information relevant to sexual maturation. As yet, the hypothesis that development of irregular GnRH cells is functionally related to sexual maturation remains to be tested and confirmed in other mammals.

(ii) Functional Studies: In the adult female rabbit NE has been shown to induce GnRH secretion from the hypothalamus (Pau and Spies, 1986; Ramirez et al., 1986), to facilitate LH release (Sawyer et al., 1949; Sawyer et al., 1974; Pau and Spies, 1986), and to induce ovulation (Fink et al., 1975; Przekop and Domanski, 1976; Pau and Spies, 1986). While intraventricular infusion of NE (50 ug) was found to be effective in inducing an ovulatory LH surge, DA (5 to 50 ug) infusion was ineffective and at the higher dose blocked the NE effect (Sawyer et al., 1974). Using ovariectomized estrogen replaced does, Pau and Spies (1986) concluded that NE stimulated secretion of GnRH is estrogen dependent. However, results of in vitro experiments suggest that estrogen is not obligatory for NE induced GnRH secretion from explants of preoptic area-medial basal hypothalamus of ovariectomized or estrogen treated rats (Clough et al., 1988).

Lesioning the catecholaminergic system with 6 hydroxydopamine (6-OHDA; metabolic synthesis inhibitor) in rabbits (Przekop and Domanski, 1976) and rats (Martinovic and McCann, 1977) causes ovulatory blockade and inhibition of progesterone induced LH and FSH secretion. Intraventricular infusion of NE restored ovulation in 50% of rabbits studied compared to rare ovulations with epinephrine (E) and DA. In another study, bilateral lesions of the ascending noradrenergic pathway caused an 83% reduction in hypothalamic NE content and disrupted cyclicity for at least 3 weeks

(Clifton and Sawyer, 1979). Inhibition of NE synthesis was found to significantly reduce GnRH release without altering hypothalamic content (Drouva and Gallo, 1976; Kalra, 1977). These results suggest that NE is a potent stimulator of GnRH secretion and is capable of evoking gonadotropin secretion even when hypothalamic levels are very low.

In ovariectomized rhesus monkeys hypothalamic pulses of NE were found to be synchronous with pulses of GnRH (Terasawa et al., 1988). Hypothalamic infusion through push pull cannulae of NE or methoxyamine (an α-adrenergic receptor agonist) stimulated GnRH release in the same animals (Terasawa et al., 1988). Moreover, i.v. injection of prazosin (an α-adrenergic receptor antagonist) suppressed but did not block GnRH release. Similarly, phentolamine, an α-adrenergic receptor antagonist, either dampened or blocked GnRH and LH pulses in ovariectomized rhesus monkeys (Pau et al., 1989). The use of adrenergic receptor agonists, phenylepherine (α), and clonidine (α_2) has produced results which support the view that NE stimulatory effects on GnRH release are mediated primarily by α-adrenergic receptors (Leung et al., 1982). In contrast, NE interaction with ß receptors is primarily inhibitory with respect to GnRH release (Ramirez et al., 1984). Consequently, it is concluded that NE plays an important stimulatory role in the regulation of GnRH secretion mediated primarily through α-adrenergic receptors (Sawyer, 1975; Wilkinson et al., 1979).

DA, in contrast to NE, appears to have mixed effects on GnRH release. At high concentrations it is capable of blocking NE induced ovulation in the rabbit (Sawyer et al., 1974), while intraventricular infusion was shown to weakly induce ovulation (Przekop and Domanski, 1976). Moreover, in PMS-treated 30 day old rats, DA was found to augment GnRH release via receptors blocked by haloperidol (a DA receptor antagonist), and inhibited by receptors blocked by demperidone (Sarkar and Fink, 1981). Recently, it was shown that chronic activation of the dopamine receptor results in a precocious puberty in the rat (de Menzido et al., 1989). These authors suggest that the precocious puberty may be the result of DA receptor desensitization or to changes in gonadotropin secretion during and after receptor activation. The role of NE and DA in the preovulatory LH surge has been questioned in the rat (Kalra, 1985). In their study, adult female rats received intraventricular injections of NE, E, and DA in the morning and afternoon of proestrous. Both NE and DA were reported to have no effect on plasma LH levels, whereas E evoked a twofold increase in plasma LH levels. Consequently, E containing neurons of the hypothalamus play a significant role in the preovulatory LH release. However, the physiological role of E in the regulation of GnRH secretion has been less well studied than either NE or DA. From the preceding it is concluded that NE and E induces GnRH secretion while DA has mixed functions but primarily inhibits GnRH

secretion.

2.4.2 Opioid Peptides:

The role of opiates in the regulation of GnRH release is essentially one of inhibition. The anatomical localization of hypothalamic opiatergic neurons and the effect of endogenous opiates on GnRH release is covered in a review by Bicknell (1985). Additionally, the role of opiates in the regulation of pubertal onset is reviewed by Wilkinson and Landymore (1989).

(i) Morphological Studies: Three distinct opioid neuronal pools have been identified in the rat: a) opiocortin, b) enkephalin, and c) dynorphin (Knigge and Joseph, 1984), all three of which have been identified in the hypothalamus (Cuello, 1983; Khachaturian et al., 1985). Moreover, Knigge and Joseph (1984) report that opiocortin fibers colocalize with NE and serotoninergic fibers in the brainstem. However, direct contact between opioid and GnRH neural elements has not been observed. Results from functional studies suggest that endogenous opioid peptides (EOP) exert their influence on GnRH release indirectly. Indeed, it has been postulated that a functional axo-axonic interaction between EOP and catecholaminergic neurons occurs in close proximity to GnRH neurons of the hypothalamus (Kalra and Kalra, 1984).

(ii) Functional studies: Intraventricular infusion of EOP is known to inhibit LH release in the ovariectomized rat (Kalra and Kalra, 1984). Since it has been shown that the pituitary has few opioid receptors (Simantov and Snyder, 1977) and that EOP have no direct effect on pituitary gonadotropin secretion (Cicero et al., 1977) it is suggested that EOP act through inhibition of GnRH release.

Morphine treatment of ovariectomized steroid primed adult rats blocked intraventricular NE and E induced LH hypersecretion (Kalra and Gallo, 1983). Intraventricular infusion of dopamine had no effect. Additionally, E was found to reverse the effect of morphine on the progesterone induced LH surge of EB primed rats. Consequently, it is suggested that EOP did not block the ability of GnRH neurons to respond to excitatory inputs but EOP may decrease the influx of adrenergic stimuli to GnRH cells, resulting in diminished LH release.

Opioidergic inhibition of GnRH release has been further demonstrated in Naloxone (NAL)- treated ovariectomized rabbits (Orstead and Spies, 1987; YoungLai et al., 1988). NAL was found to induce a marked increase in GnRH release (Orstead and Spies, 1987) and LH (Orstead and Spies, 1987; YoungLai et al., 1988) in the short term ovariectomized rabbit. However, in intact does NAL was ineffective (Orstead and Spies, 1987), suggesting that gonadal steroid feedback modifies opioid effects. Treatment of ovariectomized does with EB 3 h before

NAL treatment abolished the LH increase (YoungLai et al., 1988). Therefore it is proposed that opioid inhibition of GnRH release in the female rabbit is modified by gonadal steroid feedback on the hypothalamus. In the female rat it has been demonstrated that ovariectomy reduces the ability of NAL to augment serum LH and FSH levels (Bhanot and Wilkinson, 1984). In the same study it was also shown that EB priming of long term ovariectomized rats produced reinstatement of opioidergic inhibition of GnRH release. Consequently, it would seem that estradiol modifies opioidergic inhibition of GnRH release in the rat also.

That opioidergic inhibition of GnRH release may be mediated by diminished catecholaminergic stimulation of GnRH secretion has been studied in the rat. In ovariectomized steroid primed adult rats NAL failed to stimulate LH release when NE or DA neurotransmission was blocked by either an alpha adrenergic receptor antagonist or a DA synthesis inhibitor (Kalra and Simpkins (1981). These results were reversed by treatment with clonidine, a NE agonist. The results of in vitro studies in which preoptic-medial basal hypothalamic fragments (Leadem et al, 1985) or median eminence fragments (Rasmussen et al., 1988) were incubated with morphine or NAL further suggest that opioid regulation of GnRH release may be mediated by catecholamines. By acting at the catecholaminergic neuron opiates appear to modify neuroexcitatory signals to GnRH neurons. Further support for

this hypothesis comes from in vivo studies in which it was found that in estrogen treated ovariectomized rats NE turnover was significantly increased concomitantly with increases in serum LH (Akabori and Barraclough, 1986a; 1986b). Morphine administration was shown to block NE turnover and the LH increase (Akabori and Barraclough, 1986b). Consequently, it is suggested that catecholaminergic induced GnRH release is modified by EOP.

Finally, it is proposed that negative feedback of gonadal steroids on GnRH release is mediated by opioidergic neurons. In the ovariectomized rat estrogen or testosterone propionate were reported to reduce the ovariectomy induced increase in circulating LH levels (Van Vugt et al., 1983). A single injection of NAL was able to reverse the effect of the gonadal steroids. Similarly, NAL injection in push pull perfusion experiments of metestrous rats 4 to 8 d post ovariectomy produced a significant increase in GnRH release (Karahalios and Levine, 1988). These results, together with the above, suggest that steroid inhibition of GnRH release is mediated by the opioid system. However, the possibility that gonadal steroids are capable of inducing both inhibitory and excitatory effects on GnRH secretion via catecholaminergic neurons cannot be excluded.

2.4.3 Gonadal Steroids

The gonadal steroids estrogen and progesterone have a

wide range of effects on reproductive functions. Additionally, effects of gonadal steroids in different mammalian species are variable. For example, estrogen in the rabbit is primarily inhibitory for gonadotropin release, while in the rat and primate estrogen exerts both positive and negative feedback effects. Furthermore, gonadal steroids are known to have effects on mRNA expression for GnRH and the common opiate precursor pre-pro-opiomelanocortin (POMC), alter catecholaminergic turnover, induce synaptic remodelling in the hypothalamus, and to normalize gonadotropin secretion in the ovariectomized animal. The literature is, however, too extensive to thoroughly review here. Consequently, a very brief overview of the salient findings will be presented.

(i) Morphological studies: Tritiated estradiol has been shown to be concentrated in neurons of the rat hypothalamus (Pfaff, 1968a; 1968b; Stumpf and Sar, 1977; Morrell and Pfaff, 1982; Pfaff and Keimer, 1983; Shivers et al., 1983; Brown et al., 1989). However, by combining autoradiography with immunohistochemistry, it was estimated that only about 0.2% of GnRH neurons concentrate estradiol (Shivers et al., 1983). Consequently, estrogenic effects on GnRH release must be mediated by another group(s) of neurons. Indeed, estrogens have been found to colocalize with catecholaminergic neurons (Grant and Stumpf, 1973; Sar and Stumpf, 1981; Sar, 1984). Sar and Stumpf (1973) found that catecholaminergic neurons

projecting to the hypothalamus concentrate estradiol. Sar (1984) later found that estradiol is also concentrated in the nuclei of tyrosine-hydroxylase containing neurons of the rat arcuate and paraventricular nuclei, and zona incerta. These presumptive dopaminergic neurons may be the route through which estradiol exerts inhibitory effects on GnRH release. Although a population of estrogen addressed adrenergic neurons were observed (Grant and Stumpf, 1973), many non-catecholaminergic but estradiol labelled neurons were also identified in the paraventricular and ARCN, surrounded by fluorescent adrenergic fibers. These data suggest that estradiol may exert its influence on sexual maturation and GnRH secretion via a variety of distinct neuronal populations. Catecholaminergic neurons apparently represent only a fraction of the estradiol labelled neurons. Other candidates might include opioidergic and GABA-ergic neurons. Other investigators (Morrell et al., 1984; 1985; Jerikowski et al., 1986) have demonstrated that opioid containing neurons of the medial basal hypothalamus of rats and mice also concentrate estrogens. Therefore, the effects of gonadal steroids on gonadotropin release and reproductive function may be mediated through genomic effects on both catecholaminergic and opioidergic neurons.

(ii) Functional studies: In the female rabbit estradiol inhibits GnRH release (Pau et al., 1986) and attenuates the

ovariectomy-induced increase in pulsatile GnRH and LH secretion (Orstead et al., 1985). The role of progestins, however, is unclear. Pau and coworkers (1986) demonstrated that progestins inhibit hypothalamic GnRH secretion but facilitate pituitary responsiveness and therefore LH secretion. Lin and Ramirez (1988) found that pulsatile progesterone infusion of the hypothalamus induced an increase in mean GnRH release without affecting spontaneous activity of the GnRH neural apparatus. 20-α hydroxyprogesterone was also found to stimulate GnRH release but was unable to override the inhibitory effects of estradiol (Orstead et al., 1985). However, in our laboratory, it was found that female rabbits bearing chronic implants of progesterone had circulating gonadotropin levels which were not significantly different from controls (YoungLai et al., 1990). In contrast, circulating gonadotropins were significantly suppressed in rabbits bearing estradiol implants. The mechanism of gonadal steroid inhibitory and stimulatory effects on GnRH release and subsequent gonadotropin secretion are not yet fully understood. From the above it is proposed that in the female rabbit progestins have mixed functions while estrogens are purely inhibitory.

In the rat, it has been found that estrogenic inhibition of LH secretion is primarily mediated by suppression of GnRH release (Mercer et al., 1988) which results in suppression of LH β mRNA expression (Mercer et al., 1988; Shupnik et al.,

1988). It has also been found that gonadal steroids decrease hypothalamic content of the EOP ß-endorphin (Wardlaw et al., 1982). Additionally, it has been shown that gonadal steroids play a permissive role in the inhibitory action of EOP on gonadotropin secretion (Masotto and Negro-Vilar, 1988). Moreover, in the castrated rat model, POMC mRNA expression is suppressed, an effect which is reversed by testosterone replacement (Chowen-Breed et al., 1989a; 1989b; Blum et al., 1989). The effects of other gonadal steroids have yet to be investigated, but it is speculated that estrogen would behave similarly.

With regard to catecholamines, gonadal steroids have been found to alter brain concentration and turnover rates (Barraclough and Wise, 1982; Kalra and Kalra, 1983). Additionally, hypothalamic adrenergic receptor levels are altered by estrogens (Wilkinson et al., 1979; Wilkinson et al., 1983). It has also been demonstrated in the rat that estradiol-induced GnRH release can be prevented by α-adrenergic receptor antagonists such as prazosin and phenoxybenzamine (Sarkar and Fink, 1981).

2.5 SUMMARY:

The female rabbit has some processes which are common to those of the rat and primate with regards to processes involved in sexual maturation and pubertal onset. Specifically, the pituitary appears to be functionally mature

prior to sexual maturity in the rabbit and rat. Moreover, preliminary evidence suggests that there is a circadian rhythm to gonadotropin secretion in the rabbit, albeit opposite to that found in immature girls and boys as well as rats and primates. Gonadotropins are also secreted in a pulsatile manner by the pituitary of the immature rabbit as in the rat and primate. It would also appear that the regulation of GnRH secretion by catecholamines and opiates are at least temporally common to rabbits, rats and primates.

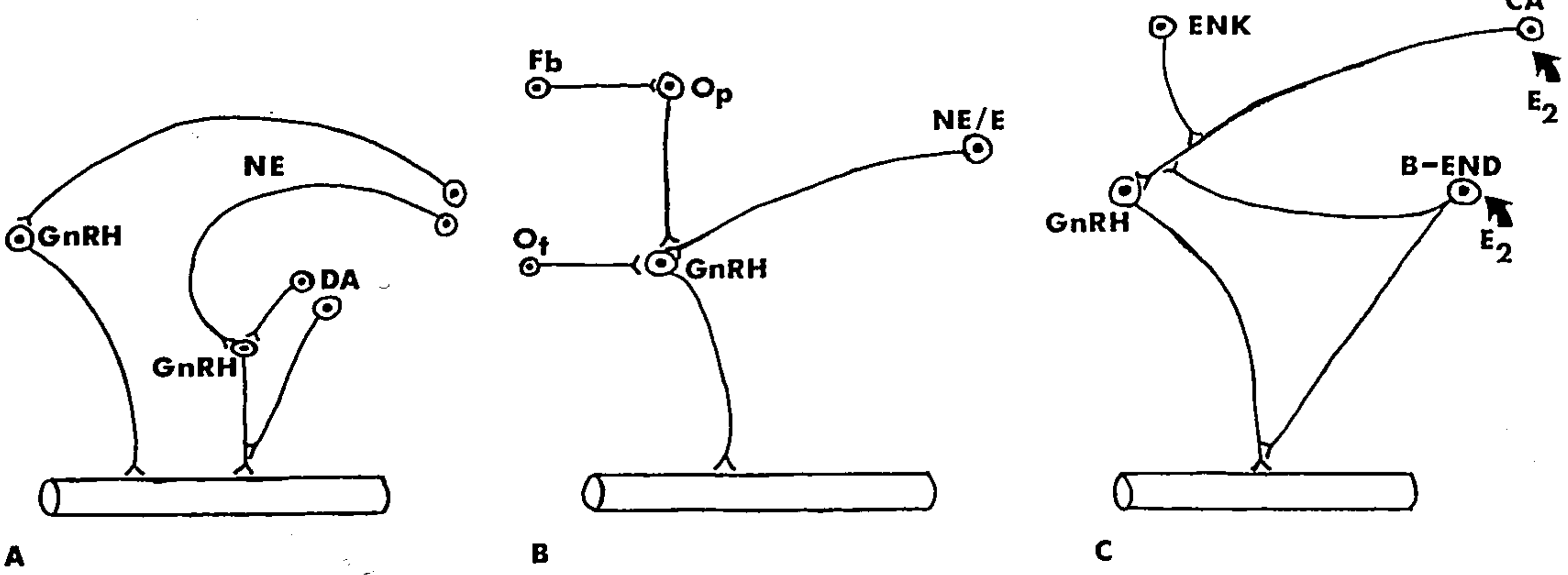
The ontogeny of gonadotropin secretion observed in the rabbit was biphasic for both LH and FSH in contrast to the monophasic pattern found in the rat and human. The significance of the peak levels of gonadotropins found between postnatal days 9 and 40 in the female rabbit (YoungLai, 1986) is as yet unknown. However it is suggested that this age period represents a critical period in sexual maturation of the female rabbit. Not only is it correlated with diminished [^{3}H]-NAL binding (Wilkinson and YoungLai, 1986) but this period is also followed by an increase in ovarian estradiol content (de Turckheim et al., 1983). Consequently, the suggestion (YoungLai, 1986) that this period, when gonadotropins are elevated, is related to ovarian maturation is supported but it also is proposed that this period is functionally related to changes in the mechanisms regulating GnRH secretion.

While estradiol seems to have only an inhibitory role in

the regulation of GnRH secretion in the female rabbit, it has both stimulatory and inhibitory effects in the rat and primate. Regardless, the development of positive feedback effects of estradiol in the rat and primate may occur late in the process of sexual maturation and may be separable from the fundamental process of puberty onset.

It is clear that for the mammal, profound developmental changes in the CNS precede sexual maturity and culminate in the adult pattern of GnRH secretion. Regulatior of GnRH secretion is affected by direct action of catecholaminergic and possibly opioidergic neurons on the GnRH cell. The evidence brought forward to date, however, suggests that estrogens exert their effects indirectly. A number of models of the adult pattern of hypothalamic neurocircuitry have been advanced and are shown in Figure 4. It has been proposed by Ruf (1982) that puberty results when the GnRH neuron is connected with its excitatory inputs. This hypothesis would seem to be supported by the findings of Wray and Hoffman (1986a; 1986b; 1986c), who demonstrated that catecholaminergic innervation of spinous GnRH neurons increased with age. An alternative hypothesis holds that puberty is the consequence of the removal of inhibitory influences rather than the establishment of excitatory inputs (Bhanot and Wilkinson, 1983; Wilkinson and Landymore, 1988). It is possible that both hypotheses are correct and that puberty is the consequence of the developing brain achieving the adult

Figure 4. Models suggested for the adult pattern of hypothalamic neurocircutry. Gonadotropin Releasing-Hormone (GnRH) neurons of the preoptic area and arcuate nucleus receive input from norepinephrine (NE) containing nerve cells from the brain stem in model A, modified from Ojeda and McCann (1978). Dopaminergic (DA) cells terminate on the soma and processes of GnRH cells on the arcuate nucleus. In the Kalra and Kalra (1984) model B GnRH cells of the arcuate nucleus receive input from NE and epinephrine (E) cells. Opioid cells (Op) innervate NE/E processes. Feedback (Fb) inputs are proposed to be through Op neurons. Other (Ot) regulators of GnRH secretion are also symbolized. The Bicknell (1985) model C has estrogen feedback on GnRH secretion modulated through catecholaminergic (CA) neurons of the brain stem and extrahypothalamic Beta-endorphin (B-END) containing cells. B-END and enkephalin (ENK) cells are shown to terminate on CA neurons prior to CA termination on GnRH containing cells. GnRH containing cells in these models are shown to terminate on portal blood vessels.


GnRH
NE
GnRH
DA
A
Fb
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NE/E
Of
GnRH
B
ENK
CA
E2
GnRH
B-END
E2
C

pattern of neurocircuitry and the resultant mechanisms which regulate the interaction of the various neurotransmitters that ultimately regulate GnRH secretion.

2.6 HYPOTHESIS:

It is the hypothesis of this thesis that the induction of increased circulating levels of estradiol via premature ovarian activation with PMS will produce developmental changes in the cytoarchitecture of the GnRH neuron of the female rabbit. Removal of estrogen by ovariectomy or estrogen receptor blockade is hypothesized to cause impairment of developmental changes in GnRH neurons. The developmental changes in the GnRH neuron are further hypothesized to be functionally related to the development of inhibitory control of GnRH secretion.

2.7 EXPERIMENTAL QUESTIONS:

1. What are the morphological characteristics of the GnRH neuron in the adult virgin female rabbit?

2. What is the topography of GnRH neural elements in the rabbit hypothalamus?

3. Does treatment with PMS induce estradiol secretion in immature female rabbits and produce developmental changes in the hypothalamic GnRH neuron?

4. Can sexual maturation and developmental changes in the GnRH neuron be delayed by an estrogen receptor blocker?

5. Is estrogen negative feedback operative in the

early postnatal female rabbit?

6. Does ovariectomy and thus removal of all gonadal influences prevent the developmental changes in GnRH neurons?

CHAPTER III

MATERIALS AND METHODS

3.1 Animals:

New Zealand White female rabbits (n= 122) were purchased from a local breeder and housed in individual cages on a 12-h light:12-h dark schedule (lights on at 7:30 EST). Food and water were available ad libitum.

3.2 Experimental Design:

One hundred and twenty-two female rabbits were used in three separate experiments (Table II).

3.2.1 Experiment I:

Thirty New Zealand white rabbits (female, n=24; male, n=6) were used in 4 studies to localize hypothalamic nuclei, determine the most favourable methods for immunostaining, and the optimum dilution of the primary antibody. Of the thirty rabbits, 14 (female, n=10; male n=4) were used to study the morphology and topography of immunoreactive GnRH neural elements in the hypothalamus.

3.2.1.1 Localization Of Hypothalamic Nuclei:

Four female and 2 male adult rabbits were used to localize hypothalamic nuclei with cresyl violet staining (Humason, 1979). The animals were anaesthetized with 3 cc Sodium Pentobarbitol (Somnotol, MTC Pharmaceuticals, Mississauga, Ont.). The skull was opened with rongeurs, the brain removed and placed in cold Bouin's fixative (2 h.). The

Table II. Summary Of Experiments Performed.

Experiment number	Study ID	Number of rabbits	Age	Purpose	Outcome measures
I	1.1	6	180 days	Localize hypothalamic nuclei.	
	1.2a	5	229 days	To determine the ideal primary antibody dilution.	Immunoreactive cells and proceeses in the hypothalamus.
	1.2b	5	240 days		
	1.2c	14	200 days	1. To determine the best immunohistochemical methods to use. 2. To demonstrate the topography of GnRH neural elements in the rabbit hypothalamus.	1. Immunoreactive cells and proceeses in the hypothalamus. 2. Cell counts.
II	2.1	72	22 to 108 days	To determine the effect of estradiol on sexual maturation of the GnRH cytoarchitecture at the light microscopic level.	1. Immunoreactive cell counts. 2. RIA of plasma estradiol and gonadotropins. 3. Body, pituitary, ovary, and uterine weights. 4. Follicular maturation.
	2.2	16	22 to 110 days	To determine the effect of ovariectomy on sexual maturation of the GnRH cytoarchitecture at the light microscopic level.	1. Immunoreactive cell counts. 2. Body weights. 3. RIA of plasma gonadotropins.
III		4	160 days	To evaluate the effect of Tamoxifen on estradiol negative feedback in the adult female rabbit.	RIA of plasma gonadotropins.

hypothalamus was dissected out and returned to fresh Bouin's fixative overnight. Hypothalami were dehydrated in a graded series of alcohols, cleared with Alpha Terpineol (Fisher Scientific Co., Fair Lawn, NJ.) and embedded in paraffin (Paraplast plus, Fisher Scientific Co., Fair Lawn, NJ.). Paraffin blocks were serially sectioned at a thickness of 10 um in either the frontal or sagittal plane using a "820" rotary microtome (American Optical Co., Buffalo, NY.). Sections were mounted on albuminized glass slides, and air dried overnight on a warming tray (45 C). Slides were dewaxed with xylene, rehydrated, and stained with Cresyl violet (Humason, 1979). Sections were then dehydrated, washed in xylene, mounted with Permount (Fisher Scientific Co., Fair Lawn, NJ), and studied using a Zeiss Universal Research microscope using objectives 10, 25 and 100. Location of hypothalamic nuclei was confirmed by comparison with an atlas of rabbit neuroanatomy (Ramirez and Sawyer, 1975).

3.2.1.2 Immunohistochemistry:

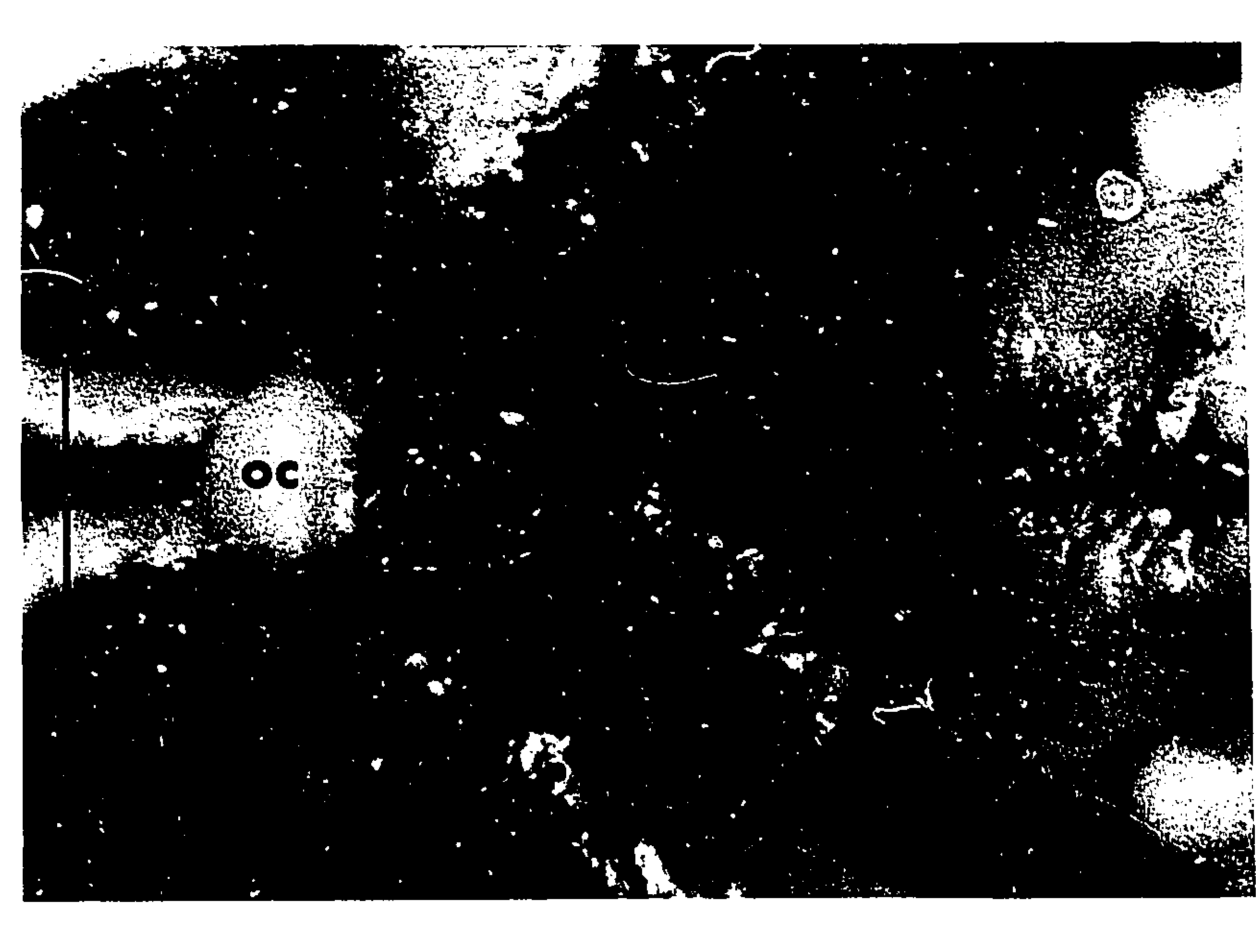
3.2.1.2a Paraffin Technique:

In the first study 5 adult female rabbits were anaesthetized with 3 cc Sodium Pentobarbitol. The thorax was opened by a midline incision, the descending aorta ligated, and Bouin's fixative was perfused through the left ventricle. An exit port was made in the right ventricle. Perfusion was continued until the egress from the right ventricle was clear.

The brain was then removed and the hypothalamus dissected out and placed in fresh fixative and left overnight. The hypothalamus was dissected by means of two longitudinal cuts at the lateral margins of the median eminence (Fig. 5). The caudal margin was defined by a cut caudal to the mammillary bodies. Rostrally, the anterior hypothalamus was cut through the olfactory region at a site approximately 1.5 cm rostral to the optic chiasm. The dorsal margin of the hypothalamus was defined by a horizontal cut superior to the rostral commissure, giving the tissue block a height of 2 cm.

Hypothalami were processed through a series of graded alcohols, cleared with alpha Terpineol, and embedded in wax. Serial sections were made at a thickness of 10 um in the sagittal plane. Sections were mounted onto albuminized glass slides and air dried overnight. Sections were cleared of wax with xylene and rehydrated to buffer (0.1 M Phosphate Buffered Saline (PBS), pH 7.4). Sections were incubated at room temperature overnight in a humidified chamber with a polyclonal primary antibody (rabbit anti-GnRH, LR_1 7-5-79, donated by Robert Benoit, Montreal General Hospital; Silverman, 1976) at dilutions of 1:100, 1:1000, and 1:10000. Sections were gently washed several times with PBS (2 h.) prior to incubation with goat anti-rabbit gamma globulin (Jackson Immunoresearch Laboratories Inc., West Grove, PA.) diluted 1:50 in PBS (2 h.). Sections were washed as above and then incubated with rabbit peroxidase anti-peroxidase

Figure 5. Ventral view of the adult rabbit brain. The rostral direction is to the left. The boxed area contains the hypothalamus and surrounding tissue which was dissected from the brain and trimmed for subsequent sectioning and immunostaining. Anatomical landmarks include the optic chiasm (OC), third ventricle (arrow), brain stem (BS), mamilliary bodies (MB), median eminence (ME), and the pyriform lobes (P).


OC

(Jackson Immunoresearch Inc., West Grove, PA.) at a dilution of 1:800 in PBS for 2 h. Several changes of PBS were used to wash the sections over a 2 h. period before being replaced with 0.05 M Tris-HCl buffer (pH 7.6). Incubation for 10 min. with 0.05% 3,3'- diaminobenzidine HCl (DAB) (Sigma Chemical Co., St. Louis, Mo.) and 0.02% hydrogen peroxide (Sigma Chemical Co., St Louis, Mo.) in 0.05 M Tris-HCl (pH 7.6) was performed to demonstrate immunoreactive sites in the tissue sections. A final series of washes was performed before the sections were dehydrated, mounted with permount, and studied with the light microscope.

3.2.1.2b Thick Sections:

In an alternative method 5 adult female rabbits were anaesthetized with 3 cc Sodium Pentobarbitol. The thorax was opened by a midline incision, the descending aorta ligated, and the primary fixative (4% paraformaldehyde, 0.25% glutaraldehyde, and 0.1% acrolein in 0.1 M Sorensen's phosphate buffer containing 1% sucrose, pH 7.3) was gently infused through the left ventricle. An exit port was made in the right ventricle. Perfusion was continued until the egress from the right ventricle was clear. Following complete perfusion with the primary fixative the brain was removed and the hypothalamus was dissected as described above and placed in vials containing fresh primary fixative for 1 h. The secondary fixative (4% paraformaldehyde in 0.05 M sodium

bicarbonate buffer containing 1% sucrose, pH 10.5) was slowly added to the vials over 1 h until it completely replaced the primary fixative. Hypothalami were left in the secondary fixative overnight at 4 C.

Following fixation, tissues were transferred through a graded fixative series to rinse buffer (0.1 M Sorensen's sodium phosphate buffer containing 0.25 M NaCl, 0.25 M KCl, 0.2 M $CaCl_2$ and 3% sucrose, pH 7.3) and then to rinse buffer containing 30% glycerol. Hypothalami were frozen at -70 C, thawed and transferred back to rinse buffer without glycerol. Residual free aldehyde groups were quenched with 1% sodium borohydride (Fisher Scientific Co., Fair Lawn, NJ.) in rinse buffer for 1 h containing 0.003 % hydrogen peroxide. Hypothalamic slices approximately 0.5 mm thick were prepared according to the methods of Eldred et al. (1983) and stored in rinse buffer with 1% normal goat serum (NGS) (Jackson Immunoresearch Laboratories Inc., West Grove, PA) until required for immunostaining. Tissue strips were incubated for 24 h in the polyclonal primary antiserum described previously. Dilutions used were 1:100, 1:1000, 1:3500, 1:15000, and 1:30000 in rinse buffer containing 1% normal goat serum and 0.05% Triton X-100 (Sigma Chemical Co., St. Louis, Mo.). Several changes of rinse buffer were used to wash the tissues (4 h) before incubating with the secondary antibody diluted 1:50 in rinse buffer containing 1% NGS (8h). After a 2 h wash in several changes of rinse buffer, tissue was

incubated for 4 h in rabbit peroxidase anti-peroxidase at a dilution of 1:800 in rinse buffer containing 1% NGS. Tissues were washed with several changes of rinse buffer (2 h). The rinse buffer was replaced by slowly adding 0.05 M Tris-HCl buffer (pH 7.6). Immunoreactive product was demonstrated with 0.05 % DAB and 0.003 % hydrogen peroxide in 0.05 M Tris-HCl (pH 7.6) for 10 min. The reaction was arrested by an additional incubation of 10 min in 0.05 M Tris-HCl buffer (pH 7.6) without DAB and hydrogen peroxide. All incubations were performed at room temperature.

Tissues were washed twice in 0.15 M sodium cacodylate buffer (pH 7.3, 2 x 15 min) and post fixed in cacodylate-buffered 2 % osmium tetroxide (4 h). Hypothalami were dehydrated by passing through a graded ethanol series to propylene oxide and embedded in TAAB low-viscosity resin (Marivac Ltd., Halifax, NS.). Tissue blocks were then sectioned serially at a thickness of 40 um using a heated microtome blade and a "820" rotary microtome. Sections were then flat embedded in TAAB resin on glass slides and studied with a light microscope at magnifications between 10 and 100 times (oil immersion).

3.2.1.2c Comparison Of Thick Sections Technique With Vibratome And Cryostat Methods:

In a final trial the methods of Eldred et al. (1983) were compared with sections prepared with a Vibratome (Carl Zeiss

Canada, Don Mills, Ontario), and Cryostat (Bright Instrument Co. Ltd., Huntingdon, England). In this trial 14 adult New Zealand white rabbits (female n=10 and male n=4) were randomly assigned to one of the three different procedures. All animals were treated alike and hypothalami recovered as above. Four hypothalami were processed following the methods described by Eldred et al. (1983) as detailed above. An additional four rabbits were prepared as above but the hypothalami were sectioned with a vibratome. Sections were 30 um thick and cut in the sagittal plane. Once cut, sections were floated in rinse buffer and stored in vials at 4 C until needed for immunostaining. Rabbits in the cryostat group were obtained from Animal Quarters following aesthesia as above and euthanasia by exsanguination. Their brains were rapidly recovered without perfusion with fixatives. Brains were immediately placed in cold primary fixative before returning to the laboratory. Hypothalami were dissected out as described and processed as above up to but not including thawing from -70 C. These hypothalami were maintained at -70 C until required for sectioning with the cryostat. Tissues were transferred from -70 C to the cryostat and allowed to reach -50 C before being mounted onto aluminum chucks with O.C.T. compound (Miles Laboratories Inc., Naperville, Il.) for sectioning. Sections in the frontal (n=4) and sagittal (n=2) planes were made at 20 um and mounted on subbed (Humason, 1979) glass slides. Sections were kept frozen until used for

immunostaining.

Immunostaining was performed exactly as described above with the exception that Cryostat sections were stained in a humidified chamber and all staining was carried out on the glass slides rather than in vials.

Of the 14 rabbits in this study 4 of 6 rabbits in the cryostat group were excluded from analyses because no immunoreactive material could be found (n=2), and because of incomplete cryopreservation (n=2).

3.2.1.3 Controls:

Controls included processing tissue as above, but the primary antiserum was pre-absorbed by prior incubation (24 h) with synthetic GnRH (Factrel, Ayerst Laboratories, Montreal, Que.; 3.0 ug/0.1 ml). Additional controls used included substitution of the primary antiserum with normal rabbit serum or omission of the secondary antibody. Another set of controls was performed in which tissues (cervical spinal cord and fragments of the frontal lobe including the corpus callosum), believed to be devoid of GnRH neural elements, were immunostained along with experimental tissues.

3.2.1.4 Quantification Of Immunoreactive GnRH Neurons:

Slides were coded so as to blind the observer to the treatments employed. Tissue sections were examined with a Zeiss Universal research microscope using objectives between

10 and 100 (oil immersion). To be recorded as a positive immunoreactive cell, immunoreaction product had to be present in any part of the cell soma. Only part of the soma had to be present in order to be scored.

Two morphologically distinct cell types were recorded. Rough cells possessed processes with small bumps, blebs or spikes while smooth cells had a smooth soma and processes. Total cell counts were also recorded along with the number of cells which could not be classified as either rough or smooth. All cell counts were recorded with the assistance of a Laboratory Counter (Clay Adams, Parsippany, N.J.) and recorded in coded form until all slides had been counted. In order to minimize the error of counting the same cell in adjacent sections the procedures for performing cell counts in histological sections described by Abercrombie (1946) were used. This necessitated determining mean cell diameters. The mean cell diameter for each procedure above was determined by measuring the maximum and minimum diameters (Dmax and Dmin respectively) for a minimum of 100 cells for each histological method used. Cell diameters were measured with either an ocular reticule or a Bioquant digitizing system (R & M Biometrics, Toronto, Ont.) coupled by a video camera to a Leitz research microscope. The mean cell diameter was calculated by obtaining the mean of the Dmax and Dmin. A correction factor for cell counts was calculated by the following formula: M/M+1 where M is the section thickness and

L is the mean diameter of the cell. Raw cell counts were multiplied by the correction factor to give a corrected cell count for each cell type and each treatment.

3.2.1.5 Topography Of GnRH Neural Elements In The Hypothalamus:

Immunoreactive GnRH neural elements were mapped in the hypothalamus of the rabbit by examining serial sections in the frontal plane using a Zeiss Universal Research Microscope with a coupled camera lucida. Tracings of immunoreactive neural elements in sections approximately every 150 to 300 um apart were made with the camera lucida system. Location of hypothalamic nuclei in camera lucida tracings was assisted by using a rabbit neuroanatomical atlas (Sawyer et al., 1954) and cresyl violet stained sections.

3.2.2. Experiment II

3.2.2.1 Design:

In the second experiment, female rabbits (n= 88) were obtained at 22 days of age and used in one of 2 different studies.

3.2.2.1a Evaluation Of Estradiol Effects On Puberty And GnRH Cytoarchitecture:

In the first study 78 rabbits were randomly assigned to one of three treatment groups and cared for as previously

described. Treatment groups were subdivided into 4 age blocks of six rabbits each. In the first group (n= 24) rabbits received Tamoxifen citrate (TAM, Stuart Pharmaceuticals, Wilmington, DE) 10 mg/kg/day emulsified in sesame oil (Sigma Chemical Co., St Louis, Mo.), by s.c. injection over the course of the study. Control rabbits (n= 24) were given the vehicle (sesame oil) only s.c. while the remainder of the animals (n= 24) received pregnant mare serum (PMS, Sigma Chemical Co., St. Louis, MO) 50 IU s.c. on postpartum days 22 and 25, and vehicle on the remaining days. Blood was collected weekly from the middle ear artery for measurements of plasma gonadotropins and estradiol by radioimmunoassay. Puberty was considered to have been achieved when rabbits had attained a body weight of 3.0 Kg. Puberty was confirmed if rabbits successfully mated with a proven buck on at least one of two trials and a plasma LH spike was demonstrable 90 min after mating behaviour was observed.

Six rabbits from each treatment group were anaesthetized on days 25, 40, 75, and 108 with 3 cc Sodium Pentobarbitol injected through the ear vein. One ovary was removed through a midline incision and placed in Davidson's fixative for routine histology and follicular morphometric analysis. The other ovary was removed, weighed and frozen in liquid nitrogen until needed for determination of estradiol levels. The rabbit was then sacrificed by perfusion with primary fixative (described in section 2.2). The hypothalami were collected

and processed for GnRH immunohistochemistry as described previously. The pituitary and adrenal glands were removed by dissection and weighed. The uterus of 75 day old control and TAM treated rabbits were also collected and weighed.

3.2.2.1b Effect Of Ovariectomy On GnRH Cytoarchitecture:

In the second study 20 female rabbits were obtained and cared for as described previously. The rabbits were randomly assigned to 1 of 2 treatment groups. In the first treatment group (n=10) rabbits were anaesthetized with Ketamine (42 mg/kg; Rogar/STB Inc., Montreal, Que.) and Atravet (2.6 mg/kg; Ayerst Laboratories, Montreal, Que.) and then were bilaterally ovariectomized (ovx) at 25 days of age. The second treatment group (n=10) served as controls. These rabbits were anaesthetized as above and were sham operated at 25 days of age. This surgery involved the isolation of the fallopian tubes and ovaries before both were returned to the abdominal cavity and the incision closed. Weekly blood samples were collected for radioimmunoassay of plasma gonadotropins. Body weights were measured and the rabbits were sacrificed once they had attained a mature weight of 3.0 Kg. Fixation, collection of hypothalami and processing for GnRH immunohistochemistry was as described for vibratome sections. Cell counts were performed on coded slides as described previously.

3.2.2.2 GnRH Immunohistochemistry:

All rabbits were anaesthetized with 3 cc Sodium Pentobarbitol. The thorax was opened by a midline incision, the descending aorta ligated, and the primary fixative (4% paraformaldehyde, 0.25% glutaraldehyde in 0.1 M Sorensen's phosphate buffer containing 1% sucrose, pH 7.3, 20 C) was gently infused through the left ventricle. An exit port was made in the right ventricle. Perfusion was continued until the egress from the right ventricle was clear. Following complete perfusion with the primary fixative the brain was removed and the hypothalamus was dissected out as above and placed for 1 h in vials containing fresh primary fixative. The secondary fixative (4% paraformaldehyde in 0.05 M sodium bicarbonate buffer containing 1% sucrose, pH 10.5) was slowly added to the vials over 1 h until it completely replaced the primary fixative. Hypothalami were left in the secondary fixative overnight at 4 C.

Following fixation, tissues were transferred through a graded fixative series to rinse buffer (0.1 M Sorensen's sodium phosphate buffer containing 0.25 M NaCl, 0.25 M KCl, 0.2 M $CaCl_2$ and 3% sucrose, pH 7.3) and then to rinse buffer containing 30% glycerol. Hypothalami were frozen at -70 C, then thawed and transferred back to rinse buffer without glycerol. Residual free aldehyde groups were quenched with 1% sodium borohydride in rinse buffer for 1 h containing 0.5 % hydrogen peroxide. Tissues were then transferred back to

rinse buffer.

Half the hypothalamus was mounted onto a metal chuck and sectioned at 30 um with a vibratome. Sections were floated on the rinse buffer and stored in glass vials until required for immunostaining. Immunostaining was performed as described in section 1.2 above. The primary antiserum was used at a dilution of 1:15000.

3.2.2.3 Radioimmunoassays:

Plasma LH and FSH were measured by established techniques (Moor and YoungLai 1975; Armstrong et al. 1978). The LH standard was WP 360A (Dr A. F. Parlow), 1 ng of which was equivalent to 30 pg pure rabbit pituitary LH (EX 130 GB, Dr L. E. Reichert, Jr.) and guinea pig anti-rabbit LH,7F GPaLH (Dr R. Scaramuzzi). Ovine LH (LER-1056-C2, Dr L. E. Reichert, Jr.) was used for iodination. The sensitivity of the assay was 42 pg of the pure pituitary LH standard. All determinations below the level of sensitivity of the assay were arbitrarily assigned the lowest limit of sensitivity of the assay for data analysis. Intra- and inter-assay coefficients of variation were 4.8% and 18% respectively.

Reagents for FSH assays were provided by Dr A. F. Parlow. Rabbit FSH (AFP-9688-C) was used for iodination and standards. The antisera (AFP-4-7-21-76) was raised in the guinea pig. The sensitivity of the assay was 80 pg of the pure FSH standard. Values obtained that were below the level of

sensitivity of the assay were arbitrarily assigned the lowest limit of sensitivity for data analysis. Intra- and inter-assay coefficients of variation were 13.2% and 21% respectively.

Estradiol standard used for radioimmunoassay was obtained from Steraloids, Inc., Wilton, NH. Antibodies to estradiol were raised by immunizing rabbits to estradiol-6-oxime conjugated to bovine serum albumin (Wielgosz et al., 1980). Tritiated estradiol, 100 Ci/nmole (New England Nuclear, Boston, Mass.) was used as tracer for competitive binding. Bound steroid was separated from free by dextran coated charcoal (Norit A, Fisher Scientific Co., Fair Lawn, NJ) extraction. Plasma estradiol concentration was determined on dried ether (BDH Inc., Toronto, Ont.) extracts of 1 ml aliquots of plasma. Ovarian homogenates were also extracted with ether. The sensitivity of the assay was 10 pg of the standard used. Values below the sensitivity of the assay were assigned the lower limit of sensitivity. Intra- and inter-assay coefficients of variation were 6.33% and 6.91% respectively.

Cross reactivity of the estradiol antisera with estriol, estrone, progesterone, 20 α-hydroxy progesterone, TAM, and testosterone was determined. Each steroid was dissolved in 100% ethanol and used in place of unknowns in the assay procedure. The concentrations used ranged between 10 pg/100 ul and 10,000 pg/100 ul. Cross reactivity was determined by

comparing the binding obtained for each steroid with that of the standard curve.

3.2.2.4 Follicular Morphometry:

One ovary from each rabbit was collected, fixed in Davidson's fixative (24 h), transferred to 70% ethanol, and processed for light microscopy. Paraffin sections (10 um thick) were floated onto glass slides and subsequently stained with Ehrlich's Haematoxylin (BDH Chemicals, Toronto, Ont.) and Eosin (BDH Chemicals, Toronto, Ont.). Follicle counts were performed on every fifth section using a Bioquant image analysis system coupled by a video camera to a Leitz research microscope following the methods of Jarrell and Belbeck (1985). Only follicles in which the plane of section passed through the oocyte nucleus were recorded.

3.2.2.5 Data Analysis:

Data for study 2.1a was analyzed by two way analysis of variance using the Stats Plus program (Human Systems Dynamics, Northridge, CA.) for the Apple IIe computer. Comparisons of treatment means with control means was performed by Dunnett's Test (Steel and Torrie, 1980). Mean uterine weights and the mean number of corpora lutea were compared by T-tests (α = 0.05) for independent samples. Body weight data was analyzed by two way analysis of variance and treatment means compared with controls by Dunnett's procedure also. In addition,

simple linear regression was performed on data for each treatment and control using the Complete Statistical System (StatSoft, Tulsa Oklahoma). The slopes and intercepts for the three lines were calculated and compared (Steel and Torie, 1980) before concluding that the lines were not parallel.

Data for study 2.1b was compared by one way analysis of variance using the Number Cruncher Statistical System (Dr. J.L. Hintze, Kayseville, Utah). The mean cell counts for ovx rabbits were compared with the means of the control group by the least significant difference method (Steel and Torrie, 1980).

3.2.3 Experiment III

In the third experiment 4 mature virgin female rabbits (160 days of age) were bled weekly from the central ear artery for two weeks to establish baseline gonadotropin values. Then these rabbits were injected subcutaneously with TAM (10 mg/Kg/day) for 4 weeks. Weekly blood samples were collected for measurements of plasma gonadotropins as described previously. Means were compared by paired t-tests.

CHAPTER IV

RESULTS

4.1 Experiment I:

4.1.1 Immunohistochemistry:

4.1.1.1 Paraffin Technique:

Immunostaining of paraffin embedded and serially sectioned hypothalami was unsuccessful at any of the primary antibody dilutions used. High background staining was found at all dilutions of the primary antibody used, consequently, specific binding could not be determined accurately.

4.1.1.2 Thick Sections:

Both immunoreactive perikarya and neural processes were observed using this procedure.

4.1.1.2a Specificity of Antisera:

Immunoreactivity was diminished in sections incubated with primary antibody at dilutions above 1:15,000. Consequently, the final dilution of primary antibody used was 1:15,000. Immunoreactivity was eliminated by pre-adsorption of the primary antiserum with synthetic GnRH (3 ug/0.1 ml) or substitution with normal rabbit serum or omission of the secondary antibody. Additionally, immunostaining was not observed in tissues from the cervical spinal cord which is believed to be devoid of GnRH.

4.1.1.2b Immunoreactive GnRH Cells:

Immunoreactive cells were found individually, or in small to large clusters of cells (Fig. 6 and 7) throughout the hypothalamus. Product of the immunoreaction was observed in

the cytoplasm of both unipolar and bipolar cells (Fig. 8). The nucleus of immunostained cells was round, centrally located, and unstained. Processes were 3 to 4 um in diameter proximal to the cell and tapered to 0.5 to 1.0 um distally.

Two distinct populations of immunoreactive cells were observed. One group of cells had comparatively smooth contours (Fig. 9). The second group of cells were characterized by irregular surfaces. Multiple small bumps or blebs were observed on the surface of these cells (Fig. 10 and 11). Such bumps were clustered proximal to the cell soma and were estimated to be between 1/2 to 2 times the diameter of the process on which they were found. Some of the cells in this group also possessed contours with spike like processes (Fig. 12 and 13). These spikes projected a distance of approximately 2 times the diameter of the processes on which they were observed. Spikes were observed individually on processes or in multiples (Fig. 13). Similar blebs or spikes were not found at their terminals.

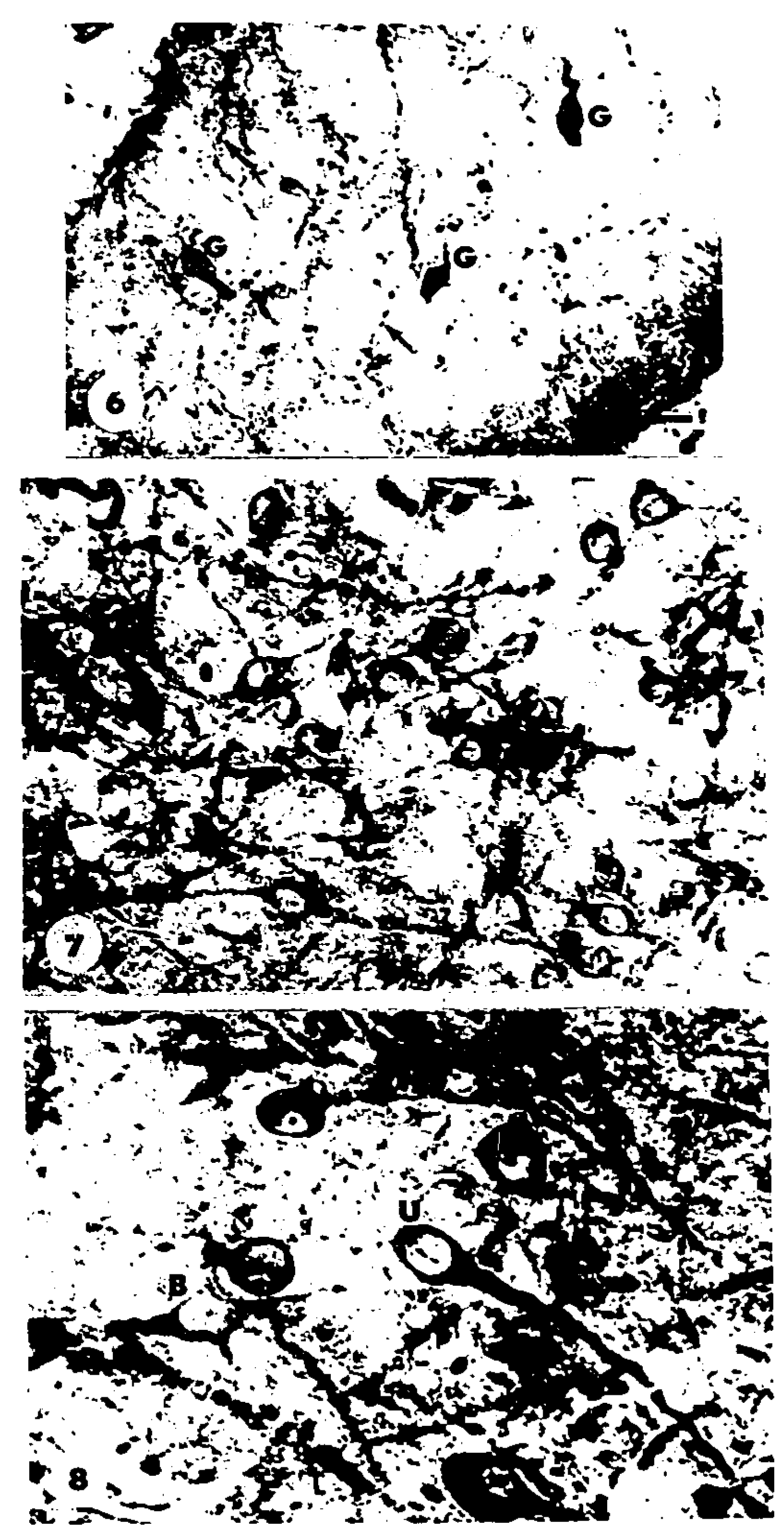
4.1.1.3 Comparison of Thick vs. Vibratome and Cryostat Techniques:

Immunoreactive cells were found in sections prepared by all three methods. However, the cryostat prepared sections were found to be unreliable for the purposes of performing cell counts. In two cryostat sectioned hypothalami, large areas of sections had a honeycombed appearance with just the

Fig. 6. A vibratome section (40 um) through the preoptic area showing immunoreactive GnRH cells (G) and beaded processes (arrows).

Fig. 7. A frontal vibratome section through the retrochiasmatic area of the hypothalamus showing clusters of immunoreactive GnRH cells.

Fig. 8. A high power magnification through the same area as in Figure 7 showing both unipolar (U) and bipolar (B) GnRH cells.


G
G
G
6
7
U
B
8

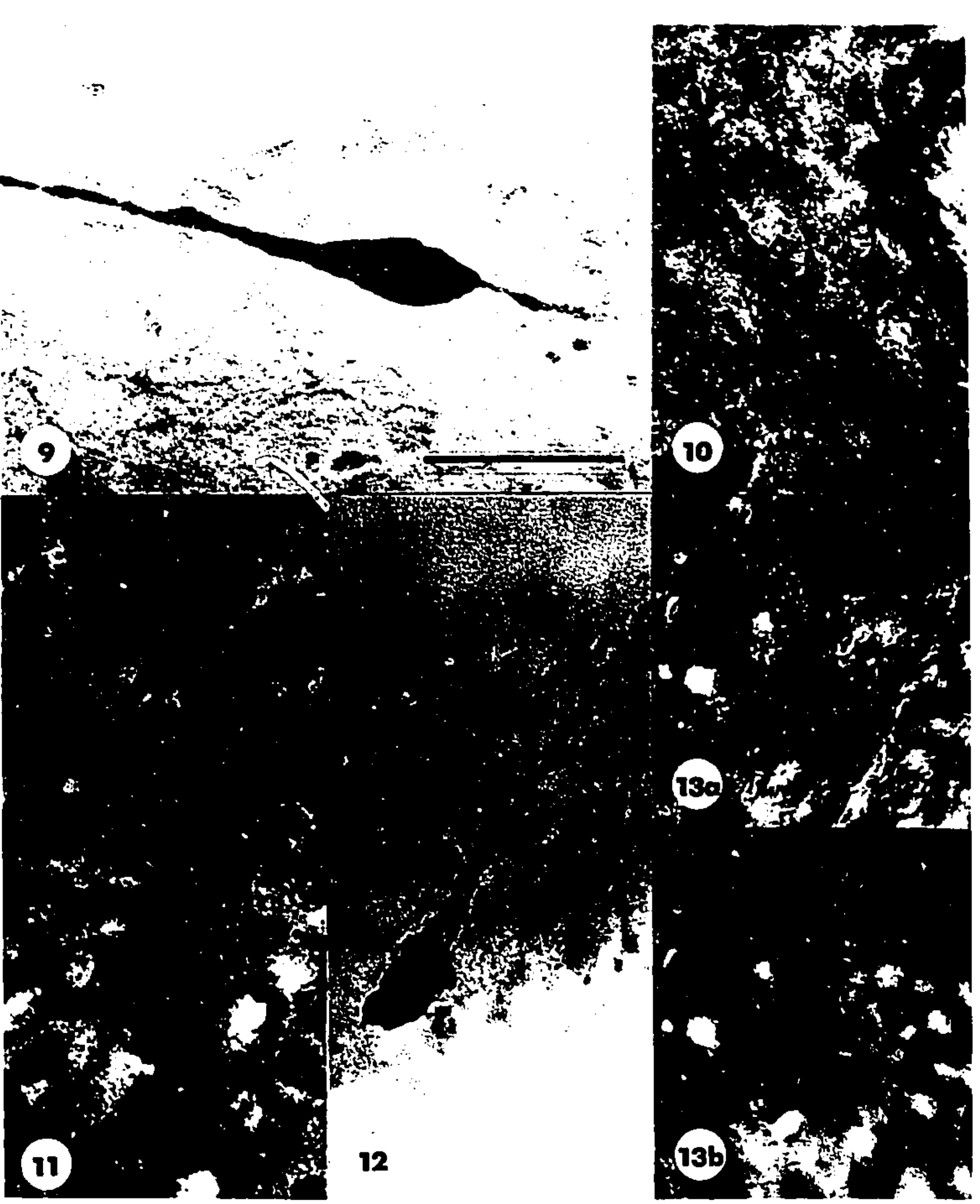
Figures 9 to 13. Immunoreactive GnRH cells. Magnification bar equals 10 um, fig. 9-12; 100 um, fig. 13.

Figure 9. A bipolar GnRH cell with relatively smooth contours of the soma and processes.

Figure 10 and 11. Two rough type GnRH cells are shown. Both possess small bumps or protruberances (arrow heads) on their processes.

Figure 12. A rough cell with a large spike (S) on its process.

Figure 13. A rough GnRH cell with multiple spikes (arrow heads) from processes and soma.
Fig. 13a. Normarski optics
Fig. 13b. Bright field micrograph of the same cell.


9
10
13a
11
12
13b

outline of cells remaining, indicating incomplete cryopreservation. In another two specimens no immunoreaction product could be found in one brain and in the other only processes were stained. Consequently, cell counts were performed on sections from the thick and vibratome methods only.

Serial sections collected from half the hypothalamus were examined and the number of immunoreactive cells in each of the two distinct cell types (rough and smooth) were recorded. The raw cell counts for both methods were multiplied by a correction factor (Abercombi, 1946) in order to minimize the effect of counting the same cell twice in serial sections. The number of sections in half the hypothalamus of thick sectioned animals was 105.00 +/- 7.64 (n=4) and the correction factor 0.76 was used (Table III). The number of sections counted from hypothalami sectioned with the vibratombe was 182.00 +/- 25.77 (n=4) and the correction factor was 0.65.

Unpaired t-tests of the corrected cell counts for rough, smooth, and total immunoreactive cells showed that vibratome sectioned material resulted in larger counts being determined. The total number of immunoreactive cells counted in vibratome sections (989.62 +/- 13.31) was significantly (p=0.0078) greater than that observed in thick sectioned brains (783.56 +/- 19.98). Rough cells counted in vibratome sections were also significantly (p=0.0249) greater (632.29 +/- 16.51 vs.

Table III Comparison Of Cell Counts Obtained By Different Techniques. The Number Of Immunoreactive GnRH Neurons Counted In Half The Hypothalamus Of Mature Female Rabbits.

Animal #	Raw Counts Rough	Raw Counts Smooth	Raw Counts Total	Corrected Counts; CF=0.76 Rough	Corrected Counts; CF=0.76 Smooth	Corrected Counts; CF=0.76 Total
Con-1	651	273	968	494.76	207.48	735.68
Con-2	722	307	1037	548.72	233.32	788.12
Con-3	641	353	1023	487.16	268.28	777.48
Con-4	720	351	1096	547.20	266.76	832.96
Mean				519.46	243.96	783.56
S.E M.				16.53	14.59	19.98
						CF=0.65
Vib-1	1040	480	1537	676.00	312.00	999.05
Vib-2	936	488	1472	608.40	317.20	956.80
Vib-3	931	621	1569	605.15	403.65	1019.85
Vib-4	984	508	1512	639.60	330.20	982.80
Mean				632.29[a]	340.76[b]	989.62[c]
S.E.M.				16.51	21.31	13.31

1. 0.5 mm or less thick sections were used for immunostaining in this group.
2. 0.40 to 50 um thick sections were prepared with a vibratome and then immunostained.
Statistical comparisons were made by means of unpaired t-tests.
a p=0.0249
b p=0.0111
c p=0.0078
CF = correction factor

519.46 +/- 16.53). Similarly, the number of smooth cells counted in vibratome sections (340.76 +/- 21.31) was much greater (p=0.0111) than those determined from thick sections (243.96 +/- 14.59). Cells which could not be classified as rough or smooth cells (error) was 2.57% in the thick section method compared to 1.67% in vibratome sections. Consequently, the vibratome method was employed for all subsequent cell count determinations.

4.1.1.4 Topography of Immunoreactive Neural Elements:

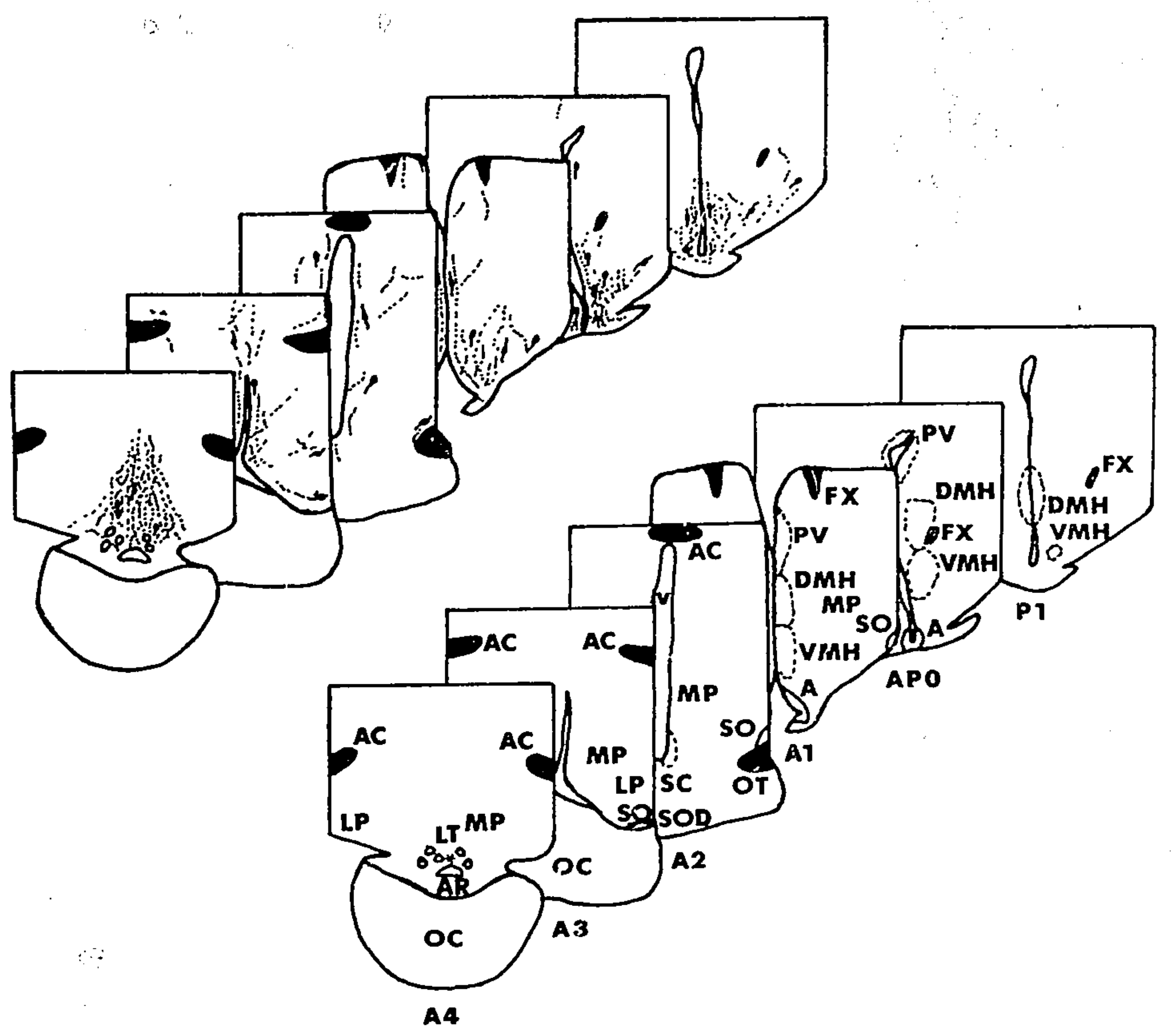
Immunoreactive nerve cells were observed in the anterior and medial basal hypothalamus and were not limited to defined nuclear groups (Fig. 14). Hypothalamic nuclei are shown in Figure 15. GnRH cells were found clustered (Fig. 7 and 8) or in a non clustered format (Fig. 6). Smooth and rough immunoreactive cells were homogeneously distributed throughout the anterior and medial basal hypothalamus. Both rough and smooth cells did not show any evidence of preferential localization to any area delimited region of the hypothalamus.

Immunoreactive processes appeared as thin fibers with numerous varicosities (Fig 16 and 17). Rarely, immunoreactive processes were followed from the medial preoptic area to the ependymal lining of the third ventricle (Fig. 18). The beaded swellings were of variable sizes (Fig. 19) joined by a very thin process which in some cases was not visible in the plane of section. Immunostained-processes were traced for variable

Figure 14. Camera lucida drawings of frontal sections of the rabbit hypothalamus demonstrating the topography of immunoreactive cells and their processes. The anterior or rostral border is to the left. Sections are labeled according to Sawyer (1954).

Figure 15. Hypothalamic landmarks are shown in this figure. Abbreviations used:

A	Arcuate nucleus	AC	Anterior commissure
AR	anterior reccess of the third ventricle		
DMH	n. dorsomediali hypothalami		
FX	fornix	LT	lamina terminalis
LP	lateral preoptic area	MP	medial preoptic area
OC	optic chiasm	OT	optic tract
*	n. periventricularis preopticus		
PV	n. paraventricularis	SC	n. suprachisamaticus
SO	n. supraopticus	SOD	n. supraopticus diffusus
V	third ventricle	VMH	n. ventromedialis hypothalami



PV
FX
DMH
VMH
P1
DMH
FX
VMH
A
AP0
FX
PV
DMH
MP
SO
VMH
A
A1
AC
MP
SO
OT
SC
SOD
A2
AC
AC
MP
LP
SO
OC
A3
AC
AC
LP
LT
MP
AR
OC
A4

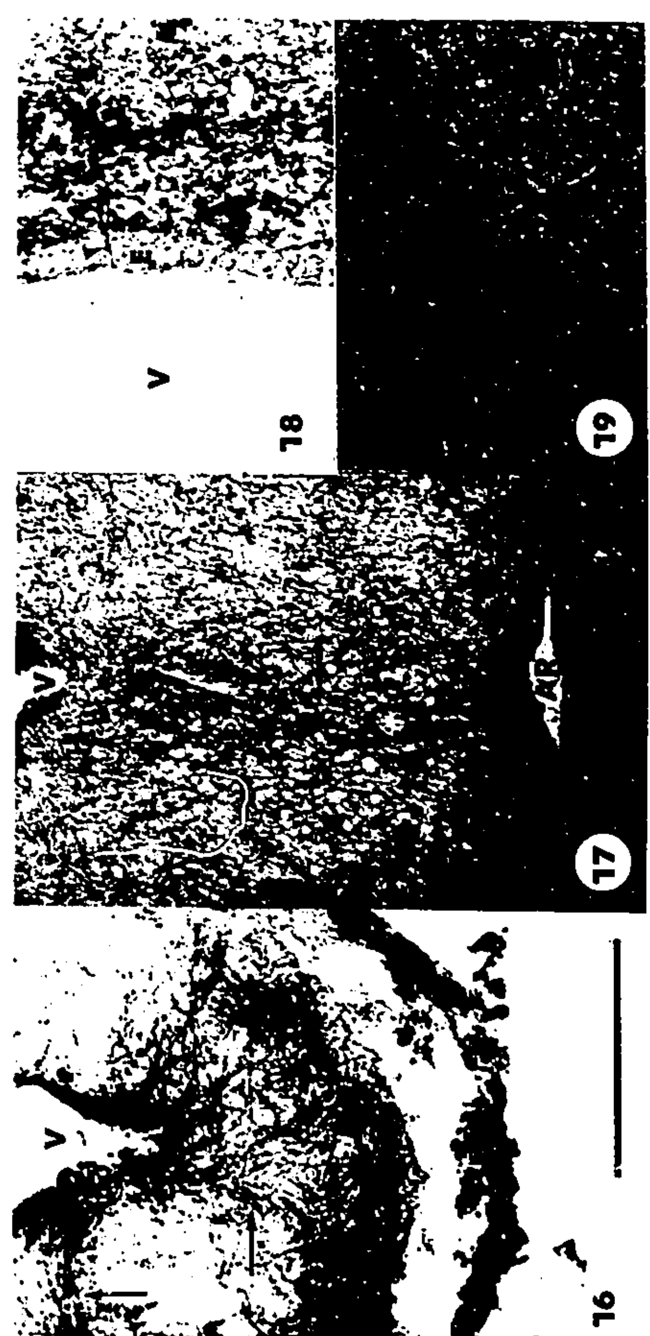
Figures 16 to 19. Frontal sections through the rabbit hypothalamus. Magnification bar represents 100 um, fig. 16 and 17; 10 um; fig. 18 and 19.

Figure 16. Frontal section at the A2 plane (Sawyer, 1954) showing immunoreactive fibers (arrows) which travel to the third ventricle (V), as well as to capillaries of the medial basal hypothalamus.

Figure 17. A frontal section in the A4 plane showing immunoreactive fibers (arrows) that can be followed from the ventral margin of the third ventricle to the dorsal surface of the anterior recess of the third ventricle (AR).

Figure 18. An immunoreactive fiber (arrow head) in the periventricular region travels to and then between ependymal (E) cells of the third ventricle (V).

Figure 19. Immunoreactive fibers showing the variability in diameter of beaded swellings (arrow heads). Note the process is nearly indistinguishable between beaded swellings.


16
V
17
V
18
V
19

distances, dorsoventrally in the medial preoptic area to capillaries of the OVLT and lateral median eminence (ME). Immunoreactive fibers were also observed in the rostral periventricular area and around the rostral commissure forming a subependymal pathway. Some of these fibers were found to exit the hypothalamus. Fibers in the paraventricular region were observed running parallel to the third ventricle.

Immunoreactive processes from the ARCN and retrochiasmatic area travelled to capillaries of the lateral median eminence. Also, GnRH-IR processes were followed from the medial POA and rostral commissure to extrahypothalamic sites. Immunoreactive-processes were also observed originating in the olfactory and suprachiasmatic regions and terminating in the vicinity of capillaries of the OVLT (data not shown).

4.2 Experiment II:

Over the course of the study a number of rabbits from all experimental groups died and were therefore lost to further analysis. Three rabbits were lost from the Tamoxifen group, 9 from the control, and 2 from the PMS group.

4.2.1 Immunoreactive Cell Counts:

Immunoreactive cells were counted in vibratome sections of half the hypothalamus of treated and control rabbits. The mean number of sections counted was 179.80 +/-

6.80 (n=72). A correction factor of 0.65 was applied to the raw cell counts. Treatments did not produce any significant changes in the total number of immunoreactive cells. The number of immunoreactive cells with smooth processes were found to decrease ($p=0.0160$) in response to treatment as the rabbits matured (Fig. 20 to 22). Significant differences were also observed within treatment groups. In the PMS group the number of smooth GnRH cells decreased significantly ($p<0.001$) from 736.28 +/- 6.84 on PND 25 to 374.11 +/- 35.47 on PND 75. A similar change was observed only in the control group at 108 days of age. However, it was not until day 75 that the number of smooth cells in the PMS group (374.11 +/- 35.47; n=4) became significantly less ($p<0.01$) than in the control group (613.70 +/- 24.40; n=4). At 108 days of age there were significantly ($p=0.0085$) fewer smooth cells in the control brains (340.95 +/- 12.19; n=3) than that found in the TAM group (721.76 +/- 50.11; n=5). The number of immunoreactive cells with rough processes was not significantly different among treatment groups. At PND 108 (Fig. 23) however, the number of rough cells were significantly ($p=0.0256$) greater in the control group than in the TAM group. The total number of immunoreactive cells counted in each of the two treatment groups did not differ significantly. However, significant differences ($p<0.001$) were found within treatment groups as the rabbits developed. The number of rough cells in the PMS group increased from 305.87 +/- 25.05 on PND 25 to 577.01 +/-

Fig. 20 to 23. The number of immunoreactive GnRH rough, smooth and total cells counted in the right half of the hypothalamus of Tamoxifen (Tam) and Pregnant Mare Serum (PMS) treated rabbits vs Controls (Con). A correction factor of 0.65 was applied to the raw cell counts. Comparisons between treatment and control means were made using Dunnett's test. A * indicates a significant difference at the 0.01 level. Each bar represents the mean ± sem of between 4-6 animals.

Rough cells

Smooth cells

Total cells

Fig. 20. Day 25 cell counts.

Number of GnRH cells

2500
2000
1500
1000
500
0

Tam Con PMS

Fig. 21. Day 40 cell counts.

2500
2000
1500
1000
500
0

Tam Con PMS

Fig. 22. Day 75 cell counts.

Number of GnRH cells

2500
2000
1500
1000
500
0

Tam Con PMS

Fig. 23. Day 108 cell counts.

2500
2000
1500
1000
500
0

Tam Con

39.01 on PND 75. A similar pattern was observed for the control group.

4.2.2 Plasma and Ovarian Estradiol Measurements:

4.2.2.1 Specificity of Estradiol Antiserum:

The specificity of the estradiol antiserum was tested by measuring percent specific binding of estriol, estrone, estradiol, progesterone, 20-α-hydroxy-progesterone, testosterone, and TAM in place of unknowns in the assay procedure. Only estradiol demonstrated an appreciable ability to displace binding of tritiated estradiol (Fig. 24). Estriol produced a negligible degree of cross- reactivity with the antiserum used. The other steroids did not cross react with the antiserum in this assay.

4.2.2.2 Developmental Changes in Mean Plasma Estradiol:

Two way analysis of variance showed that mean plasma estradiol was significantly ($P= 0.003$) affected by treatments. In the PMS group, plasma estradiol was 2.47 and 3.11 times control levels ($p<0.05$) between 25 and 34 days of age respectively (Fig. 25). At 60 days of age, plasma estradiol concentrations in the PMS group were 3.04 times those of controls. Circulating levels of estradiol in the PMS group were elevated throughout the study. No difference could be found between the control and TAM-treated rabbits at any age studied.

Fig. 24. Graphic demonstrating the specificity of the antiserum E2B9 used in the steroid assay. Percent specific binding of Estradiol (+), Estrone (·), Estriol (O), Progesterone (X), 20-alpha-hydroxy-progesterone (□), Tamoxifen (△), and Testosterone (*) versus the logarithm of steroid concentration.

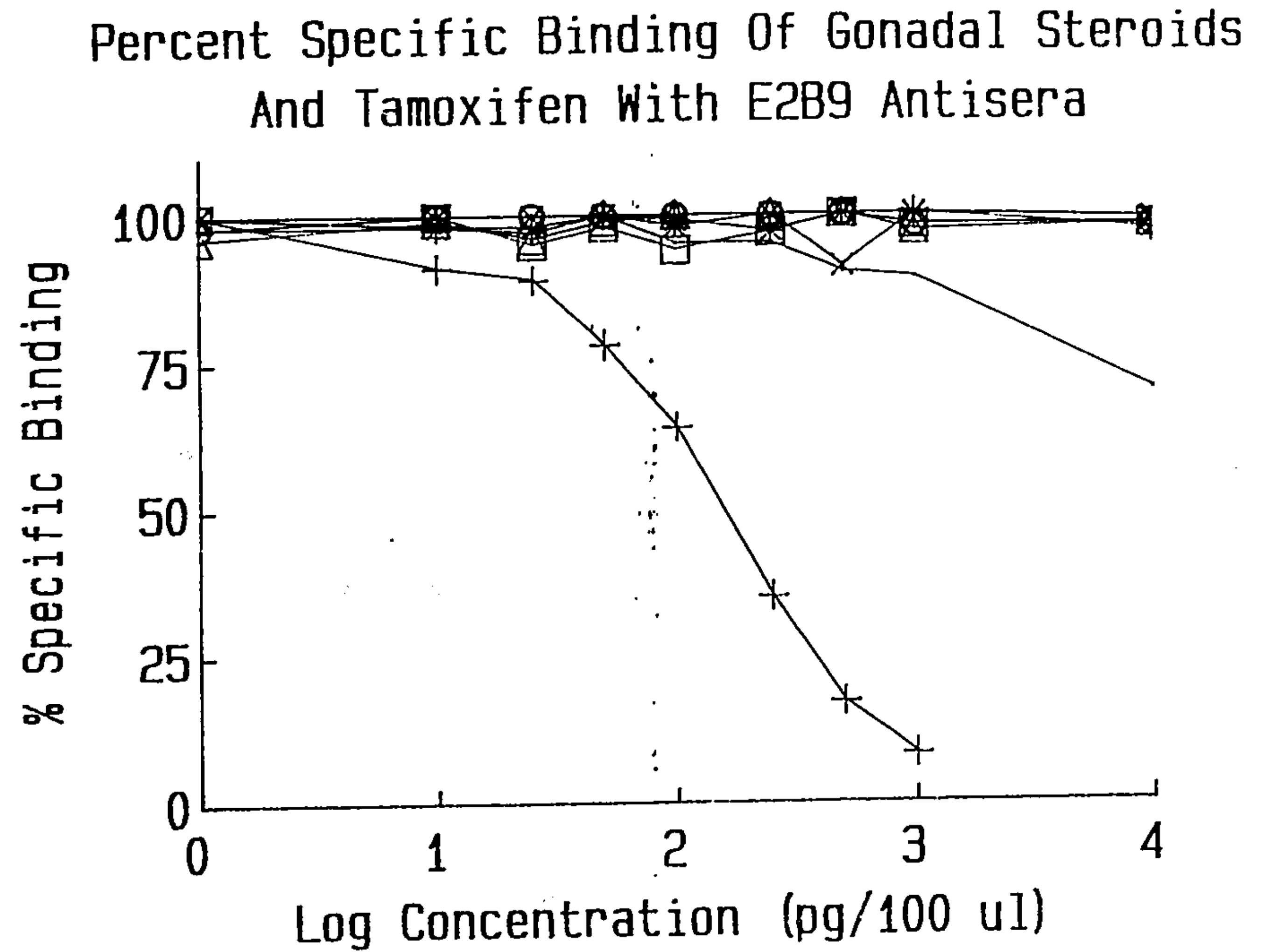
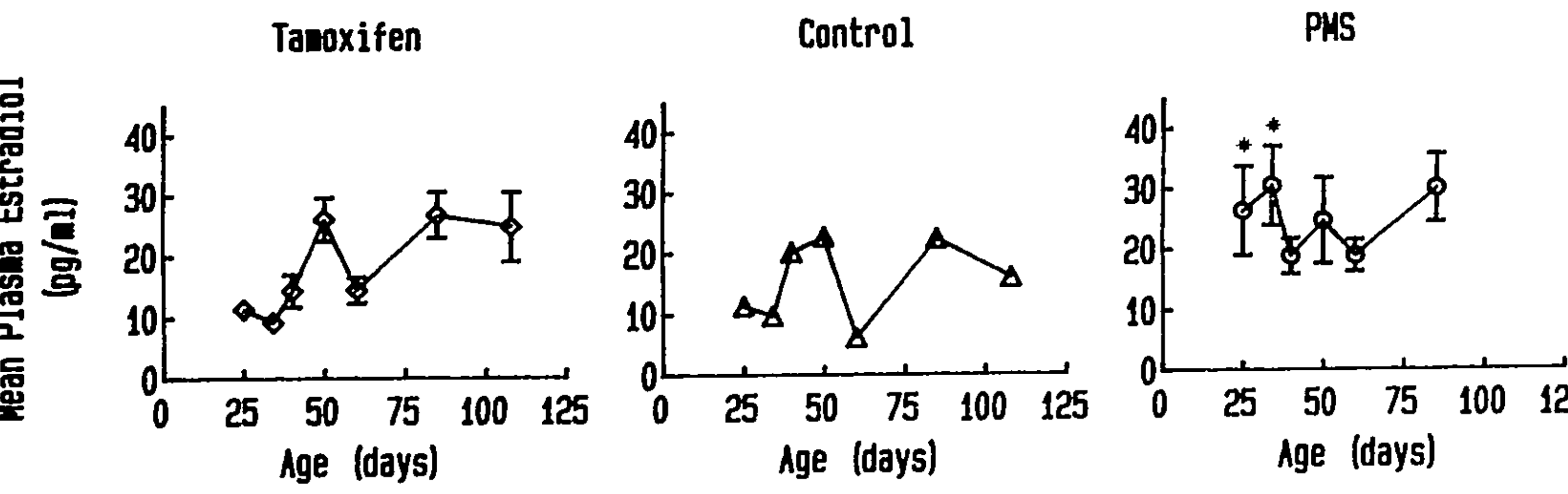

Percent Specific Binding Of Gonadal Steroids
And Tamoxifen With E2B9 Antisera
% Specific Binding
100
75
50
25
0
0
1
2
3
4
Log Concentration (pg/100 ul)

Fig. 25. Developmental changes in mean plasma estradiol concentration of Tamoxifen and PMS treated rabbits vs controls. Comparisons between control and treatment means were made using Dunnett's test, α=0.05. Each data point is the mean ± sem of between 4-6 animals.



4.2.2.3 Developmental Changes in Ovarian Estradiol:

Two-way analysis of variance demonstrated that ovarian estradiol content rose dramatically ($p = 0.029$) in PMS and control groups between postpartum days 25 and 75 while levels in TAM rabbits were suppressed (Fig. 26). No differences were found between the PMS and control groups except at 40 days of age when PMS levels were significantly ($p<0.05$) higher than controls. Mean ovarian estradiol content in the TAM group was significantly less than controls ($p<0.05$) on PND 75 and 108. In contrast, mean ovarian estradiol concentration was not significantly different between treatment groups ($p= 0.66$) when compared by two way analysis of variance.

4.2.3 Developmental Changes in Plasma Gonadotropins:

4.2.3.1 Plasma FSH

Plasma FSH were significantly different ($P< 0.001$) for the three treatment groups studied (Fig. 27). The pattern of circulating FSH was qualitatively similar for both the PMS and control groups. In both cases, FSH levels were monophasic with a peak at 40 days of age for control rabbits (17.98 +/- 1.87) compared to 50 days for the PMS group (34.20 +/- 5.04). Although FSH did not achieve a peak until 10 days after that of the control group, the levels were not significantly different at 40 days of age. Mean plasma FSH (ng/ml) in the PMS treated group (20.25 +/- 4.02 to 24.56 +/- 6.88; mean +/- standard error of the mean) was significantly different (P=

Fig. 26. Developmental changes in mean ovarian estradiol content of Tamoxifen and PMS treated rabbits vs controls. Comparisons were made using Dunnett's test, α=0.05.

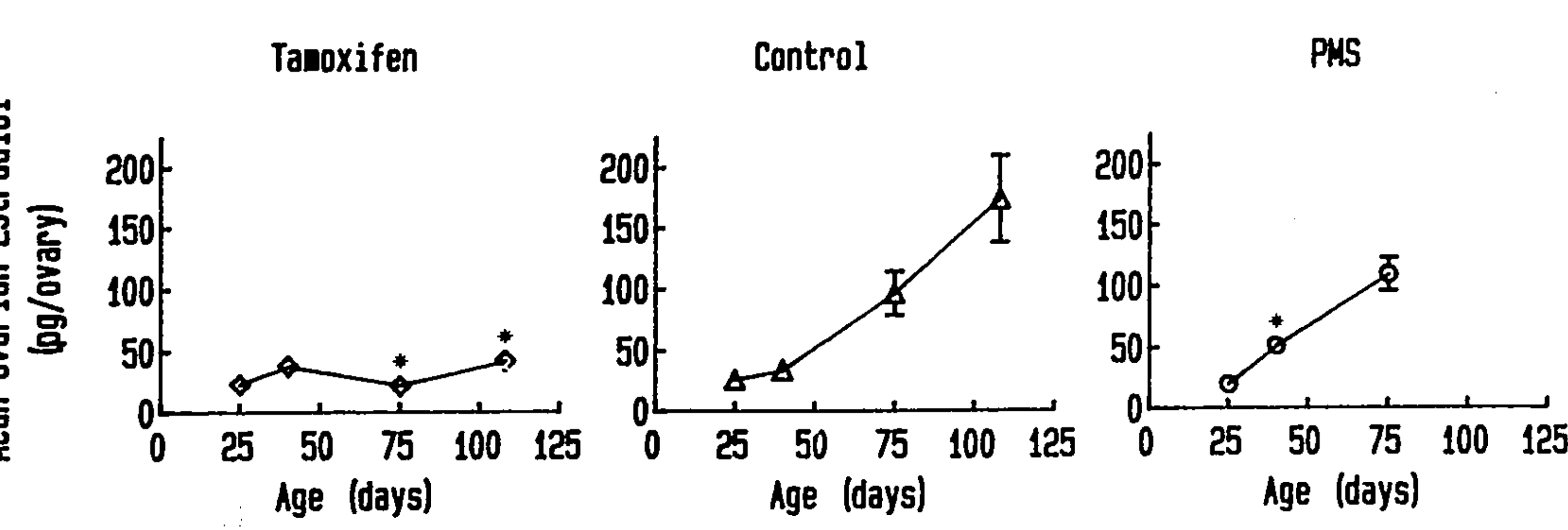
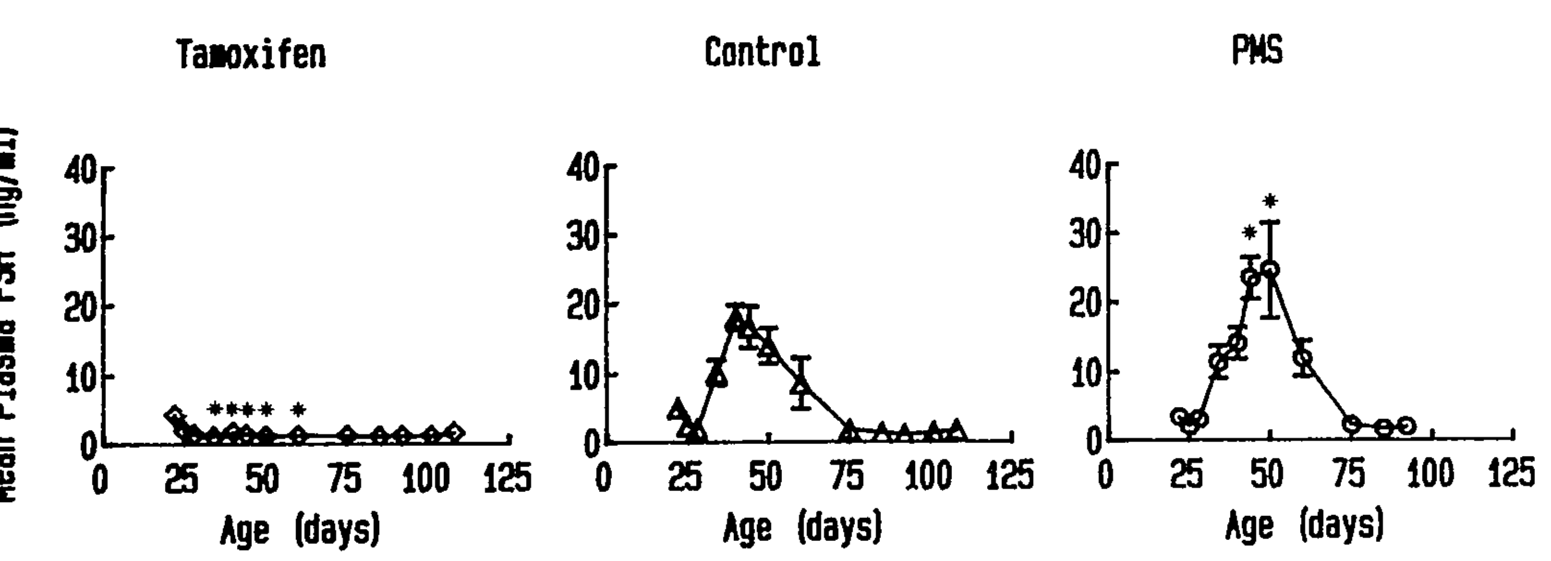


Fig. 27. Developmental changes in mean plasma FSH concentration of Tamoxifen and PMS treated rabbits vs controls. Comparisons between control and treatment means were made using Dunnett's test, α=0.05. Each data point is the mean ± sem of between 4-6 animals.

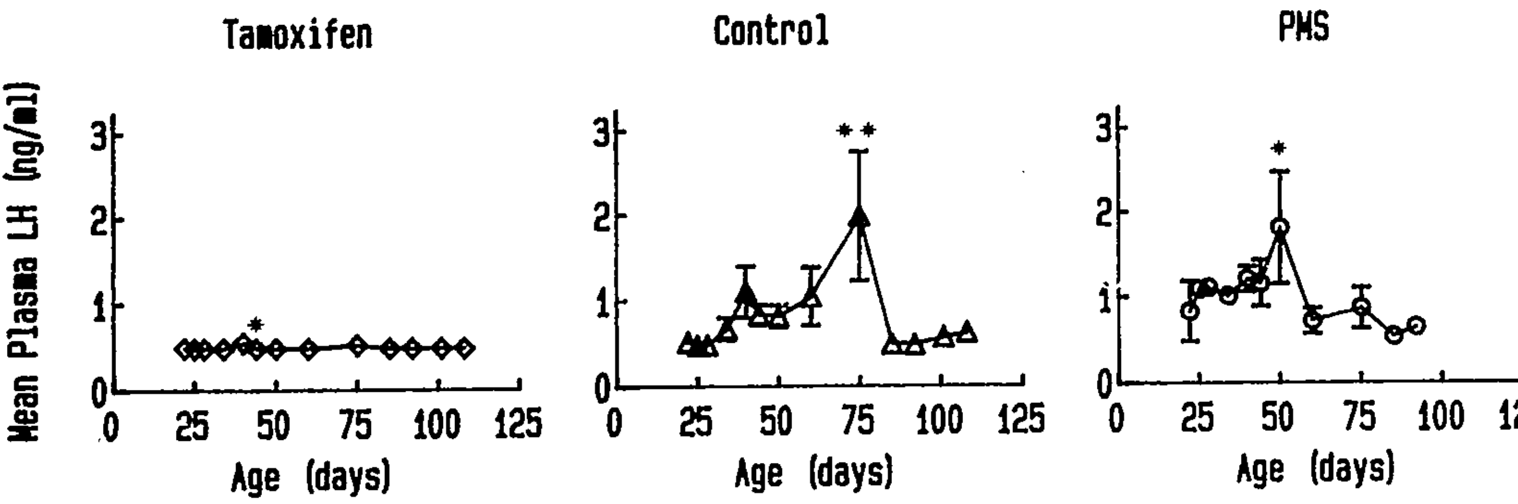


0.01) when compared to the control group (13.90 +/- 3.82 to 13.96 +/- 2.50) between postpartum days 44 and 50. In TAM-treated rabbits, FSH levels fell from 4.25 +/- 0.97 ng/ml at 22 days of age to levels under 2.0 ng/ml for the remainder of the study. Between 44 and 50 days of age TAM rabbits had approximately 10 times less circulating FSH (1.38 +/- 0.18 to 1.13 +/- 0.16) than controls ($p<0.01$). Indeed, mean plasma FSH was significantly greater ($P= 0.01$) for control rabbits when compared to TAM treated animals between days 34 and 60.

4.2.3.2 Plasma LH:

Two-way analysis of variance of mean plasma LH revealed significant treatment effects ($P< 0.001$). The pattern of circulating LH concentrations for the three treatment groups were quite different. Mean plasma LH showed a trend towards a precocious rise in the PMS treated rabbits (Fig. 28). Increased plasma LH in this group may be due to cross reaction of PMS within the radioimmunoassay used. Since this increase was not significantly different from that found in the control group it was not investigated further. In the PMS group, mean plasma LH concentration had a peak at 50 days of age (1.81 +/- 0.13 ng/ml; n=6) and then a much smaller peak at 75 days of age (0.87 +/- 01.24; n=5). A significant difference between mean plasma LH for the PMS treated group and controls was observed on postpartum days 44 ($P= 0.01$) and 50 ($P= 0.05$). In the control group a biphasic pattern was

Fig. 28. Developmental changes in mean plasma LH concentrations of Tamoxifen and PMS treated rabbits vs controls. Comparisons between treatment means were made using Dunnett's test. α=0.05 (*), α=0.01 (**). Each data point is the mean ± sem of between 4-6 animals.



observed with peaks on postpartum days 40 (1.10 +/- 0.30; n=4) and 75 (1.98 +/- 0.76; n=4). Both PMS and control groups had adult levels (<1 ng/ml) of circulating LH by day 85 of age. Unlike PMS or control rabbits, circulating levels of LH in the TAM group were suppressed throughout the study. The mean plasma LH for control animals was significantly different (P= 0.05) from PMS and TAM treated animals on day 75.

4.2.3.3 FSH/LH Ratio

FSH/LH ratios were calculated for each age studied and are shown in Table IV. A similar pattern was found for both PMS and control groups. The FSH/LH ratio was high at day 22 for all three experimental groups and decreased during the first week of the study. However, the ratio was 4:1 in the PMS group compared to 9:1 for controls and TAM treated rabbits. At 34 days of age the FSH/LH ratios rose by 73% for both control and PMS groups while the ratio remained low for the TAM group. Between ages 34 and 60 the FSH/LH ratio in both the PMS and control groups was very high, ranging between 8:1 and 20:1. From 60 days to the end of the study the FSH/LH ratio was approximately 3:1 in the PMS and CON groups. The TAM group had a FSH/LH ratio of 2:1 to 3:1 for 34 days of age onward to 108 days of age. At 8 days following the last TAM treatment the FSH/LH ratio in the TAM group increased by 50% to 4:1.

Table IV. Comparison Of FSH/LH Ratios For The Treatments, Pregnant Mare Serum (PMS) And Tamoxifen (TAM) versus Control (CON) during sexual maturation.

Age (days)	TAM	CON	PMS
22	9:1	9:1	4:1
25	4:1	5:1	2:1
28	3:1	4:1	3:1
34	2:1	15:1	11:1
40	3:1	16:1	12:1
44	3:1	20:1	20:1
50	2:1	19:1	14:1
60	3:1	8:1	16:1
75	2:1	1:1	3:1
85	2:1	3:1	3:1
92	2:1	2:1	3:1
101	2:1	2:1	
108	4:1	3:1	

4.2.4 Physical Measurements:

4.2.4.1 Body Weights:

Weight gain between the three treatment groups was found to be significantly different ($P < 0.001$) (Fig. 29). PMS treated rabbits were significantly heavier than control rabbits on day 34 ($P < 0.05$) and days 44 to 92 ($P = 0.01$). TAM treated rabbits weighed significantly less ($p < 0.05$) at 50 and 75 days of age ($p < 0.01$) compared to controls. The peak rate of weight gain for all three treatment groups occurred between postpartum days 34 and 60. However, PMS treated rabbits continued to show accelerated weight gain (48.87 g/d) between days 60 and 75 compared to controls (40.60 g/d) and TAM (27.85 g/d) groups (Table V). Rate of weight gain for all three groups declined between postpartum days 75 and 92 and plateaued at approximately 100 days of age.

4.2.4.2 Ovarian Weights:

Treatment with TAM and PMS was found to have a significant effect ($P < 0.001$) on ovarian weights (Table VI). In the early age groups (25 and 40 days of age) no difference was found between the three treatment groups. In contrast, at 75 days of age the PMS treated ovaries were significantly ($P < 0.05$) heavier than those of the controls. At 108 days postpartum ovarian weights of controls were significantly ($p = 0.01$) heavier than ovaries from TAM treated rabbits.

Fig. 29. Developmental changes in the mean body weight of Tamoxifen (X) and Pregnant Mare Serum (•) treated rabbits vs controls (+). Comparisons between control and treatment means were made using Dunnett's test. $p < 0.05$, $p < 0.01$. Each data point is the mean +/- sem of between 4-6 animals. Simple linear regression of treatments vs age gave the following results:

	Tamoxifen	Control	Pregnant Mare Serum
r	0.9275	0.8631	0.9617
slope	28.77	33.21	42.36
y intercept	-323.25	-400.13	-635.19

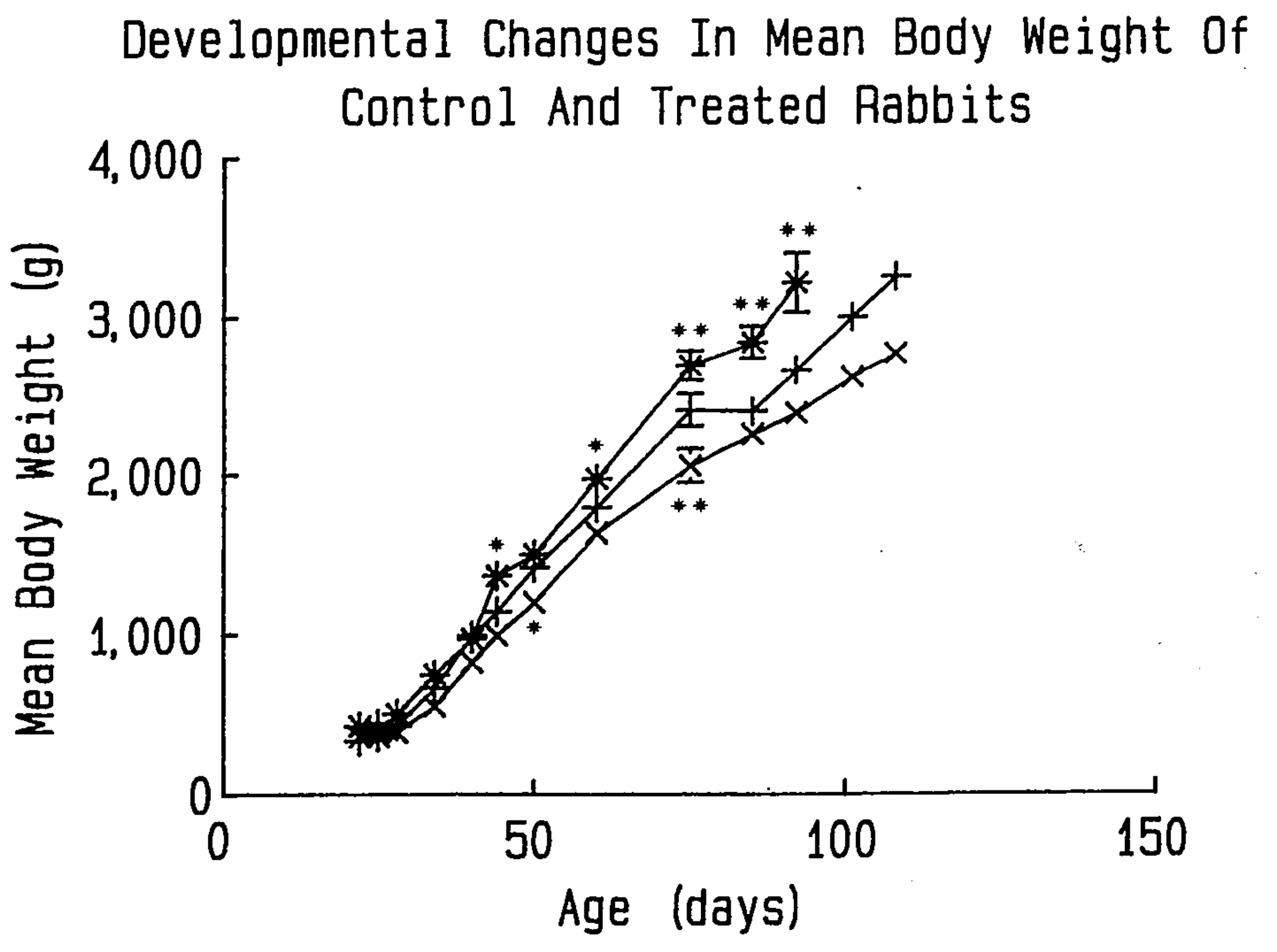

Developmental Changes In Mean Body Weight Of
Control And Treated Rabbits
Mean Body Weight (g)
4,000
3,000
2,000
1,000
0
0
50
100
150
Age (days)

Table V. Developmental changes in mean rate of weight gain (g/day) in Tamoxifen (TAM) and pregnant mare serum (PMS) treated rabbits vs controls (CON).

Age group	TAM	CON	PMS
22-28	4.25	15.73	12.93
34-50	41.19	47.63	47.56
50-60	43.82	37.80	47.15
60-75	27.85	40.60	48.87
75-92	16.39	14.85	25.20
92-101	31.03	38.02	36.22
101-108	21.43	36.83	-----
108-121	21.08	-----	-----

Table VI. Developmental changes in mean body, pituitary, adrenal, uterine, and ovarian weights of Tamoxifen (TAM) and Pregnant Mare Srum treated rabbits vs Controls.

Days Postpartum	Treatment	n	Body Weight (Kg)	Pituitary Weight (mg)	Adrenal Weight (mg)	Uterine Weight (g)	Ovarian Weight (mg)
	TAM	6	390.67 (24.51)	7.03 (0.29)	25.52 (0.29)	--------	13.67 (1.83)
25	Control	6	437.67 (29.47)	9.08 (0.88)	24.90 (2.03)	--------	13.06 (1.13)
	PMS	6	353.83 (35.67)	8.64 (2.23)	17.68 (1.65)	--------	14.48 (2.46)
	TAM	6	825.00 (18.77)	12.04 (1.09)	35.19 (2.88)	--------	9.94 (1.64)
40	Control	5	994.20 (65.61)	11.00 (1.73)	37.69 (4.18)	--------	18.59 (2.64)
	PMS	6	974.00 (49.89)	16.38 (1.45)	37.80 (3.36)	--------	15.36 (1.12)
	TAM	6	2060.00 (106.27)	29.90 (4.30)	88.89 (1.36)	--------	27.56 (1.45)
75	Control	4	2410.00 (104.78)[a]	38.42 (1.83)	107.78 (7.24)	--------	58.13 (9.45)[a]
	PMS	5	2695.60 (94.46)[a]	35.29 (2.37)	96.18 (5.74)	--------	65.81 (10.18)[a]
	Tam	5	2768.00 (60.03)	26.91 (2.62)	116.55 (15.26)	1.49 (0.22)	25.63 (4.63)
108	Control	4	3262.50 (29.05)[a]	36.54 (2.49)	149.45 (17.60)	10.08 (1.91)[a]	181.81 (21.10)[a]
	PMS	5	3673.60 (104.46)	38.41 (4.72)	146.08 (18.29)	--------	292.78 (39.01)

means with the same suprascript are not significantly different.
Numbers in parenthesis are the standard error of the mean. $P < 0.001$

4.2.4.3 Uterine and Pituitary Weights:

Mean uterine weights were significantly greater (p=0.001) at day 108 in controls (10.06 +/- 1.91) than in the TAM group (1.49 +/- 0.22). The uterine weights of the PMS group were not determined. Mean pituitary weights were not significantly different (p= 0.06) between the treatment groups at any age studied (Table VI).

4.2.5 Mating Response:

Rabbits of the PMS group weighed 3.0 kg by 85 days of age and all mated by day 92, in contrast to no successful matings in the control or TAM (P= 0.02) groups, neither of which had achieved a sexually mature body weight at this age. The control group weighed 3.0 kg at 102 days of age and mated at day 108 while the TAM group failed to mate (P= 0.02) in the time-frame of this study (Table VII).

At the time of mating the PMS treated rabbits had achieved a body weight of 3.1 +/- 0.1 kg (mean +/- sem) while the control and TAM group were 2.7 +/- 0.1 kg and 2.3 +/- 0.1 kg respectively. Control rabbits failed to successfully mate until a mean weight of 3.3 +/- 0.1 Kg was achieved at 108 days postpartum. Although TAM treated rabbits had attained a mean body weight of 3.2 +/- 0.2 Kg and were repeatedly exposed to two proven bucks around 131 days of age, no successful matings were observed.

Table VII. Mating Response Of PMS And Tamoxifen Treated Rabbits Versus Controls by Chi square analysis.

Treatment	Number receptive	Number unreceptive	P value
Day 92:			
PMS	5	0	0.0207
Control	0	4	------
Tamoxifen	0	5	NS
Day 108:			
Control	4	0	------
Tamoxifen	0	5	0.0207

NS = Not significant.
PMS = Pregnant mare serum.

4.2.6 Follicular Morphometry:

The mean number of follicles with 2 to 3, 4 to 6 and >6 layers of granulosa cells surrounding the oocyte were counted for each treatment group. Two-way analysis of variance revealed significant treatment effects for follicles with 2-3 ($P < 0.001$) and 4-6 ($P < 0.001$) granulosa cell layers. Only control rabbits at 40 days of age had a significantly ($P = 0.05$) greater number of follicles with 2-3 granulosa cell layers (Table VIII). At 75 days of age both controls and PMS treated rabbits had a greater number ($P = 0.01$) of follicles with 4-6 granulosa cell layers. However, treatments had no significant ($P = 0.14$) effect on follicles with more than 6 granulosa cells layers surrounding the oocyte or antral, atretic and anovulatory corpora lutea. Regardless, at 75 days of age PMS treated rabbits had a greater ($P = 0.05$) number of follicles with greater than 6 granulosa cell thickness than TAM treated rabbits. No difference was found between controls and TAM-treated rabbits.

A comparison of treatment means for antral follicles revealed a significant difference ($P = 0.05$) between both PMS and control rabbits versus TAM treated animals (Table VIII). No differences were found between treatment means for atretic follicles. Anovulatory corpora lutea were more prevalent ($P = 0.05$) in control rabbits at 75 days post partum relative to TAM treated rabbits.

T-tests of treatment means of corpora lutea showed

Table VIII. Developmental changes in follicle maturation in precocious and delayed puberty compared to age matched control rabbits.

Days postpartum	Treatment	2-3 GC	4-6 GC	>6 GC	antral	atretic	CL	anov CL
	TAM	106.25+/-43.72	5.00+/-3.00	0.25+/-0.25	0	1.50+/-0.65	0	0
25	Control	222.25+/-41.41	15.50+/-5.14	1.00+/-0.71	0	0	0	0
	PMS	198.00+/-61.24	15.75+/-8.58	0.25+/-0.25	0	0	0	0
	TAM	88.25+/-27.91	4.50+/-3.23	1.50+/-1.19	0	0	0	0
40	Control	402.00+/-102.44*	42.67+/-13.20	2.00+/-1.00	0	0	0	0
	PMS	275.75+/-47.23	18.75+/-6.32	4.50+/-2.33	0.50+/-0.50	0	0	0
	TAM	278.25+/-34.36	30.50+/-7.03*	2.75+/-1.89	1.00+/-0.71*	20.75+/-12.80	0	0*
75	Control	439.75+/-56.60	253.50+/-88.02	9.75+/-1.93	35.50+/-10.73	33.50+/-25.66	0	0.75+/-0.48
	PMS	437.25+/-75.55	176.25+/-39.06	10.75+/-5.36	35.50+/-17.93	4.25+/-1.93	0	0.25+/-0.25
	TAM	244.50+/-24.68	59.25+/-10.85	6.25+/-1.25	6.25+/-3.07	54.00+/-33.81	0*	16.50+/-2.40
108	Control	410.50+/-75.38	113.50+/-34.35	14.00+/-3.76	8.25+/-4.09	230.75+/-43.45	4.25+/-0.63	7.00+/-0.41
	PMS	288.00+/-99.79	104.75+/-22.16	24.00+/-9.04	51.00+/-18.83	93.50+/-53.68	7.25+/-1.25	7.50+/-4.41
130	TAM	189.00+/-21.82	47.00+/-2.74	4.50+/-0.96	6.25+/-2.36	91.25+/-21.92	0	0.25+/-0.25

TAM = Tamoxifen; PMS = Pregnant mare serum; GC = granulosa cell layers; CL = corpus luteum; anov CL = anovulatory corpus luteum. Numbers with the same superscript in an age group are not significantly different. Significance was tested at the 5% level.

that both PMS and control treated rabbits were significantly different (P= 0.05) from TAM treated rabbits. However, no difference was found between PMS and control rabbits.

4.2.7 Ovariectomy Experiment:

4.2.7.1 Plasma Gonadotropins:

In rabbits ovariectomized at 25 days of age plasma gonadotropins were markedly elevated ($p<0.001$) in comparison to sham-operated rabbits. Mean plasma FSH levels in ovariectomized rabbits (13.35 +/- 3.25) were significantly different from controls (1.89 +/- 0.60) as early as 37 days of age. On the other hand, mean plasma LH levels for ovariectomized rabbits (1.06 +/- 0.10) were not significantly different from control levels (0.90 +/- 0.06) until 58 days of age. Overall, the pattern of gonadotropin secretion in the two groups was similar, differing only in amplitude.

4.2.7.2 FSH/LH Ratio:

The ratio of circulating gonadotropins were markedly different between the two groups (Table IX). In the OVX group the FSH/LH ratio varied between 12:1 (day 51) and 25:1 (day 100) in contrast to the sham group in which the ratio varied between 1:1 (day 3) and 10:1 (day 72).

Table IX. Developmental Changes In The FSH/LH Ratio In Ovariectomized (OVX) And Sham Operated Female Rabbits.

Age (days)	OVX	Sham
37	16:1	2:1
44	18:1	9:1
51	12:1	6:1
58	19:1	9:1
65	19:1	8:1
72	24:1	10:1
79	20:1	6:1
86	19:1	3:1
93	23:1	1:1
100	25:1	1:1
103	19:1	1:1
117	13:1	1:1

4.2.7.3 Immunoreactive Cell Counts:

The number of immunoreactive GnRH neurons in half the hypothalamus of ovariectomized and sham-operated rabbits were counted in 30 um thick vibratombed serial sections. The mean number of sections counted was 220.00 +/- 29.72 (n=8). A correction factor of 0.70 was applied to raw cell counts to correct for duplicate counts of the same immunoreactive cell in adjacent sections. No difference in the total number of immunoreactive cells (1,100) were found in either of the two groups (Fig. 30). However, at sexual maturity (a body weight of 3.1 Kg) the number of rough immunoreactive cells in OVX rabbits was significantly (p<0.01) less than in the sham group. Differences between the number of immunoreactive cells with smooth processes did not approach significance. However, a trend for an inverse relationship between increasing rough cell numbers and decreasing smooth cell counts was observed.

4.3 Experiment III:

In 4 mature female rabbits daily s.c. injections of TAM (10 mg/kg/day) resulted in a significant (P=0.05) increase in mean plasma FSH following 8 days of treatment (Table X). Differences between the baseline and treatment levels of plasma LH were not significant. A small weight loss was found during the treatment period compared to pretreatment weights. However, the observed loss of weight did not become significant.

Fig. 30. The number of immunoreactive GnRH rough, smooth, and total cells counted in the right half of the hypothalamus of ovariectomized (OVX) and control rabbits both at a body weight of 3.1 Kg. A correction factor of 0.70 was applied to the raw cell counts. Each bar is the mean +/- sem of 4 animals. Comparisons between OVX and control means were performed by the method of least significant difference, p,0.05.

Rough Cells

Smooth Cells

Cell Total

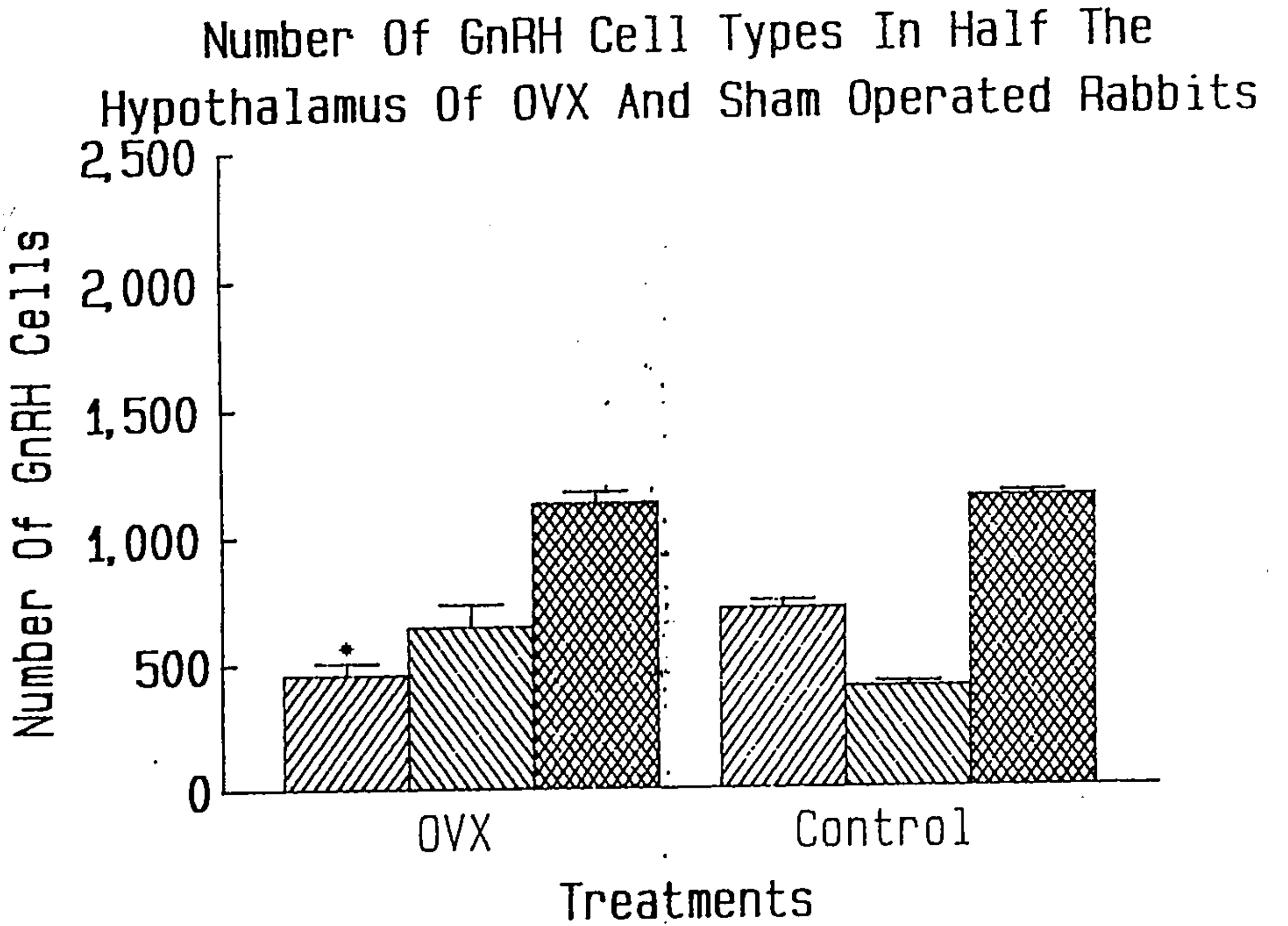

Number Of GnRH Cell Types In Half The
Hypothalamus Of OVX And Sham Operated Rabbits
2,500
2,000
1,500
1,000
500
0
Number Of GnRH Cells
OVX
Control
Treatments

Table X. Effect of Tamoxifen (10 mg/Kg/day) on body weight and peripheral gonadotropins levels (mean +/- s.e.m.) after two weeks of baseline sampling

Days Postpartum	Mean weight (Kg)	LH (ng/ml)	FSH (ng/ml)
Pretreatment Period:			
163	4.37 +/- 0.14	0.71 +/- 0.11	*
169	4.45 +/- 1.21	0.57 +/- 0.09	0.84 +/- 0.02
176	4.48 +/- 1.76	0.58 +/- 0.07	1.02 +/- 0.13
Treatment Period:			
180		0.61 +/- 0.11	2.63 +/- 1.21[a]
184	4.24 +/- 1.89	0.52 +/- 0.03	0.82 +/- 0.02
191	4.11 +/- 2.18	0.95 +/- 0.04	2.44 +/- 0.67[a]
200	4.13 +/- 2.15	0.69 +/- 0.11	1.38 +/- 0.24[a]

* = value below level of sensitivity of the assay.
a = significance at alpha = 0.01

CHAPTER V

DISCUSSION

5.1 Introduction

The hypothesis that sexual maturation is functionally related to developmental changes in the morphology of GnRH neurons has been proposed, but has not yet been tested empirically. Additionally, the rabbit is frequently used in studies examining the interrelationship of neurotransmitters and reproductive function. Consequently, a more detailed understanding than has been provided by preliminary studies (Barry, 1976; Flerko et al., 1978; Wendl and Sofroniew, 1980a) of the morphology and topography of the GnRH neuron in the brain of this species was necessary. The present study describes the morpholgy and topography of the GnRH neuron in the brain of the female rabbit by means of improved immunohistochemical techniques relative to prior studies involving this species. Immunoreactive neurons were quantified in half of the hypothalamus and two morphologically distinct GnRH cell types are described. Experimental manipulation showed that rough immunoreactive GnRH neurons increase in number while cells with relatively smooth contours decrease with advancing sexual maturation. These changes were prevented by estrogen receptor blockade and ovariectomy. In contrast, PMS treatment chronologically advanced these changes. The total number of immunoreactive cells remained constant between age groups and treatments. In PMS treated rabbits plasma estradiol was significantly elevated relative to controls between 25 and 34 days of age. Circulating levels

of estradiol in the PMS group remained comparatively high throughout the prepubertal period compared to baseline levels observed in controls between days 25 to 34. No differences were found for plasma estradiol levels in TAM-treated and control rabbits. Plasma gonadotropin levels were significantly suppressed in TAM-treated rabbits relative to controls. In contrast, plasma gonadotropins were significantly elevated in the PMS-treated rabbits at an earlier age than in controls. Rate of weight gain was greater in PMS rabbits than controls which in turn gained weight faster than TAM treated rabbits. While TAM rabbits attained a mature body weight of 3.1 Kg by day 128, they failed to become sexually receptive during the study, while the PMS group became mature earlier than controls (92 vs. 108). These results support the hypothesis that estradiol initiates developmental changes in GnRH neurons of the rabbit hypothalamus. Moreover, it is suggested that developmental changes seen in the GnRH neuron around the time of puberty onset are related to the development of inhibitory control of GnRH secretion.

5.2 Immunohistochemical Quantification, Morphology and Topography of GnRH Cells in the Brain of the Adult Rabbit:

5.2.1 Specificity Of GnRH Antisera

Since immunoreactivity was abolished by the various control measures employed, it is likely that the primary antiserum specifically interacts with GnRH-containing neural elements of the rabbit hypothalamus. The antiserum used recognizes amino acids 3,4,7,8,9 and 10 of the decapeptide (Silverman 1976, Silverman 1985; Schwanzel-Fukuda and Pfaff, 1989), and does not cross-react with any other known central nervous system peptide (Silverman 1976; Pelletier, 1987). The possibility of immunologically silent cells possessing a non-immunoreactive precursor or metabolite of GnRH cannot be excluded. However, such a possibility is considered to be remote for the following reasons. 1) Studies which have employed antisera generated against pro-GnRH and GnRH have not shown any difference in the distribution of these cells in the rat brain (Ronnekleiv et al., 1987; Culler et al., 1988). 2) In situ hybridization using the oligomer, 59mer, which is complimentary to mRNA coding for amino acids 5 to 15 of human GnRH preprohormone demonstrated the same distribution of GnRH cells as that reported by immunohistochemical techniques (Rothfeld et al., 1987). 3) Wray and Hoffman (1986c) found that colchicine pretreatment did not produce any

difference in the total number of immunoreactive cells counted in vibratome sectioned brains prepared similarly to those used in the present experiments. Consequently, it is concluded that the methods used provide a reliable means of quantifying GnRH cells in the rabbit hypothalamus.

5.2.2 Quantification of GnRH Cells:

Approximately 1,000 GnRH cells were counted in half the rabbit hypothalamus. This is in marked contrast to the variable number of cells (5 to 100/brain) reported by Barry (1976). The use of postembedding immunostaining of paraffin sections is the most probable reason for the significantly fewer numbers of GnRH cells reported by Barry (1976) since Goldsmith and Ganong (1975) have demonstrated that processing for paraffin embedding techniques deplete GnRH from the tissues. The number of GnRH cells counted in half the hypothalamus in the present study suggest a greater number than that reported for the rat (Wray and Hoffman, 1986a; 1986b; 1986c, Wray and Gainer, 1987) and hamster (Jennes and Stumpf, 1980), (1200 to 1300, and 800 respectively). In contrast, more GnRH cells (2400) have been reported for the baboon (Marshall and Goldsmith, 1980). GnRH cells were counted in the present study according to the method of Abercrombi (1946) and described in detail by Wray and Hoffman (1986b). In this method, raw cell counts are multiplied by a correction factor calculated by dividing the section

thickness by the sum of the section thickness and diameter of the cell. The method attempts to correct for duplicate counts of cells sectioned such that the same cell is present in two or more sections. With sections of a thickness greater than the diameter of the cell, this method will underestimate the actual number of cells. However, this method presently gives the best approximation of the total number of GnRH cells in the hypothalamus.

Two distinct cells types were observed and recorded in the present study. Cells with comparatively smooth contours accounted for approximately 34% of the 1,000 immunoreactive cells counted in the adult rabbit brain. In comparison, rough cells possessed irregular protuberances from the soma or their primary processes and represented about 64% of the total. Rough cells were further subdivided into two additional groups based on their morphology. One group of rough cells was characterized by numerous small bumps or blebs on the surface of their processes and soma and were clearly morphologically distinct from the smooth cells. The second group of rough cells were easily distinguished from smooth cells due to single or multiple spikes on the soma and processes. GnRH cells were not confined to specific hypothalamic nuclei but were distributed diffusely throughout the hypothalamus. Both cell types were distributed evenly throughout the hypothalamus. Multiple GnRH cell types have been described in prior studies in the rabbit (Weindl and Sofroniew, 1980a)

and rat brain (Krisch, 1980; Jennes et al., 1985; Wray and Hoffman, 1986a; 1986b; 1986c; Wray and Gainer, 1987; Merchenthaler et al., 1989). The percentage of cell subtypes found in the current study agree with those reported by Wray and Hoffman (1986a; 1986b) for the rat brain. Weindl and Sofroniew (1980a) describe bipolar and multipolar GnRH cells in the rabbit hypothalamus and show only a smooth contoured cell. The differences between this and the present study may be due to differences in processing of the tissue. Additionally, the results of this study (Weindl and Sofroniew, 1980a) are difficult to interpret since the age, sex of the rabbits, source of antisera and dilution used are not reported. Krisch (1980) demonstrated that in the adult rat brain two types of GnRH neurons exist, a bipolar smooth contoured cell and an irregular contoured unipolar cell. These findings have since been duplicated in the rat by Jennes et al., (1985), Wray and Hoffman (1986a; 1986b; 1986c), and Wray and Gainer (1987). Jennes and colleagues (1985) describe a "spiny" cell with thorn-like protrusions from the perikaryon and processes. Smooth cells were also described. Both cell types were reported to be similar in number and distribution in the rat brain. It was also reported that postsynaptic specializations were found at the level of the perikaryon and cell processes. However, synaptic contacts were found more frequently on spiny cells than on smooth cells. These authors suggest that these two populations of GnRH neurons differ on

the basis of their innervation and possess different integrative capacities. Wray and Hoffman (1986b) have shown that catecholaminergic innervation of irregular cells at the light microscopic level is increased in the rat brain as puberty is approached. Moreover, an increase in irregular cell numbers is proposed to be the result of transformation of aspinous cells into irregular cells with advancing sexual maturation. However, to date no ultrastructural studies have been performed to investigate whether catecholaminergic innervation of GnRH neurons is a necessary precursor to puberty onset. Wray and Hoffman (1986a) report that irregular GnRH cells possess both peduncular and sessile spines along the surface of processes. These findings are in contrast to those of the current study in which thick elevations or bumps along with sharp thin spikes were found on the GnRH cell soma and proximal processes. In the absence of ultrastructural studies it is difficult to interpret the significance of these observations. However, it is suggested that any change from a smooth contoured cell surface to rough increases the surface area for synaptic contacts and therefore integrative capacity of the cell. In the current study these changes are found on the cell soma and proximal segment of GnRH processes. These findings appear to agree with those of Wray and Hoffman (1986a; 1986b). Furthermore, Wray and Hoffman (1986b) showed that the number of catecholamine apposed smooth GnRH cells remained constant during sexual development while

catecholamine apposed LH-RH cells with "spine-like processes" increased. Apposition of GnRH cells by other neuropeptide and neurotransmitter-containing neurons during development has not been examined. Since in the rabbit irregular GnRH cells were characterized by small protrusions and spines on the soma and proximal part of the processes, the possibility of synaptic input by neurons possessing inhibitors of GnRH secretion can not be excluded.

A need exists for developmental studies examining the question of whether innervation of rough GnRH cells by neurons possessing neuropeptides and/or neurotransmitters other than catecholamines, and shown to regulate GnRH secretion, also increase as sexual maturity is achieved.

In the rabbit it is now suggested that two distinct GnRH cell types exist with different integrative capacities. It was not possible in this study to determine the neurochemical identity of the neurons which contact immunoreactive GnRH neurons. To date no double label immunohistochemical studies have been reported addressing this question in the rabbit. The neurochemical identity of the presynaptic contacts in the rabbit can be hypothesized to be of mixed type. Two presynaptic neurons, opioidergic and catecholaminergic are suggested as first priority for future double label immunohistochemical, ultrastructural studies in the rabbit.

5.2.3 Topography of GnRH Neural Elements:

GnRH cells were found to be distributed in a diffuse pattern throughout the hypothalamus of the female rabbit and are not confined to any of the classical hypothalamic nuclei. In the present study GnRH cells were found in the anterior hypothalamus, preoptic area, and medial basal hypothalamus. Barry (1976) reported immunoreactive perikarya to be scattered from the rostral mesencephalon to the paraolfactory area. The majority of GnRH cells are found in the preoptic-suprachiasmatic area (Flerko et al., 1978). However, in the present study the majority of GnRH neurons were found in the preoptic area and medial basal hypothalamus, which is consistent with earlier findings (Barry, 1976; Weindl and Sofroniew, 1980a) in the rabbit. This distribution is similar to the distribution of GnRH neurons in other mammals, including the guinea pig (Silverman, 1976; Silverman and Krey, 1978; Silverman, 1984), rat (Zimmerman, 1976), mouse (Hoffman et al., 1978) and rhesus monkey (Silverman et al., 1977).

The neural pathways observed in this study agree with earlier reports (Flerko et al., 1978) and expand those of Barry (1976) and Weindl and Sofroniew (1980a) in the rabbit. The preoptico-terminal and hypothalamo-infundibular tracts of Barry (1976) are viewed to be equivalent to the preoptico-infundibular and tubero-infundibular pathways of Flerko et al., (1978) and these pathways are confirmed in the present study. Weindl and Sofroniew (1980a) report that fibers of the

preoptic area travel dorsoventrally in the lamina terminalis and terminate on fenestrated capillaries of the OVLT. In the present study fibers from the preoptic area were followed to the external zone of the median eminence. Some fibers were also traced to the OVLT. Immunoreactive fibers, however, were not found in the infundibular stalk as reported by Weindl and Sofroniew (1980a). The present study does confirm the demonstration of subependymal and extrahypothalamic tracts as described earlier (Flerko et al., 1978). Weindl and Sofroniew (1980a) do not report finding a subependymal tract. Immunoreactive fibers of the subependymal tract were found in the present study at the basal surface and between ependymal cells lining the third ventricle. This was infrequently observed and is a novel finding in the rabbit but has been reported for the rat (Naik, 1975; Zimmerman, 1976; Kirsch and Leonhardt, 1980), sheep (Kozlowski and Zimmerman, 1974) and mouse brain (Kozlowski and Zimmerman, 1974; Naik, 1975; Burchanowski et al., 1979). GnRH has also been identified in the cytoplasm of ependymal cells in the rat (Pelletier et al., 1976). However, this later finding may be an artifact of the antisera used since other investigators have found contradictory evidence (Silverman and Desnoyers, 1976) at the ultrastructural level. Consequently, it is suggested that GnRH may affect ependymal cell function or be secreted directly into the third ventricle. In the monkey, GnRH pulses in the cerebrospinal fluid have been reported (Van Vugt

et al. 1985) to parallel LH pulses in the serum.

Several lines of additional evidence support the view that GnRH containing neural elements have a diffuse distribution in the rabbit hypothalamus and exit to other brain sites. Ramirez and colleagues (1986, 1987) investigated the in vivo functional characteristics of the GnRH pulse generator in response to locally applied NE in the adult rabbit brain. Their studies demonstrate that push pull perfusion of NE induced GnRH release in widely distributed regions of the rabbit hypothalamus. Immunoreactive processes in the area of the rostral commissure and medial preoptic area were observed to communicate with extrahypothalamic sites (Flerko et al., 1978). Electrophysiological studies in the rat have shown that exogenous application of GnRH by microiontophoresis produces changes in the spontaneous neuronal firing rate (Moss and Dudely, 1978). Additionally, immunoreactivity of fibres in the extrahypothalamic tract are reported to be lost by 48 h post copulation (Flerko et al., 1978). Consequently, it is concluded that GnRH neural elements have a broad and diffuse distribution throughout the rabbit hypothalamus. Additionally, as suggested by Goldsmith (1977), GnRH is proposed to act as a neurotransmitter or neuromodulator in addition to being a trophic hormone for the pituitary.

5.3 Developmental Changes in GnRH Neurons:

Developmental changes in GnRH neurons in the rat brain have been described in prior studies (Wray and Hoffman 1986a; 1986b; 1986c; Wray and Gainer, 1987). The current study extends these findings to another mammal, the rabbit. Moreover, the effect of experimentally advancing or preventing puberty onset on developmental changes in GnRH neurons are now described. Estradiol is shown to play a significant role in inducing developmental changes in GnRH neurons. However, an ovarian factor in addition to estradiol is suggested to be integral to initiating developmental changes in GnRH neurons in the female rabbit.

5.3.1 Immunoreactive Cell Counts:

The number of rough, smooth and total immunoreactive GnRH neurons were counted in models of advanced and impaired sexual maturation. The total number of immunoreactive cells (range 946 to 1042) were found to be constant between treatment groups throughout the study. In the present study, sexual maturation was impaired with the estrogen receptor blocker Tamoxifen (10mg/kg BW/day). In TAM treated rabbits developmental changes in GnRH neurons were found but progressed at a slower pace relative to changes found in control and PMS treated rabbits. In rabbits receiving TAM, plasma estradiol followed the same pattern as that for

controls but no significant differences in plasma concentration were observed at any age group studied. In every measure, other than plasma estradiol, TAM-treated rabbits remained morphologically and functionally immature. TAM is a triphenylethylene compound and possesses both estrogenic and antiestrogenic effects. The degree of antiestrogenic or estrogenic effects, however, varies depending on the species and tissue studied (Harper and Walpole, 1967; Skidmore et al., 1972; Forsberg, 1985). In the present study chronic treatment with TAM resulted in what appears to be both estrogenic and antiestrogenic behaviour. While uterine weights were not increased by TAM treatment (estrogenic), plasma gonadotropins were suppressed throughout the study, and developmental changes in GnRH neurons did not occur (suspected antiestrogenic effect).

The mechanism of TAM action is incompletely understood. Compounds of the antiestrogen group may interact with receptors other than the estrogen receptor or act by different molecular mechanisms (Black and Goode, 1981), thus accounting for their mixed effects. It has been shown that TAM competitively inhibits tritiated estradiol binding with the estradiol receptor from estrogen target tissues. TAM also binds with a second high affinity and saturable receptor (antiestrogen receptor) of unknown function (Fanidi et al., 1989). Regardless of the mechanism, Drouva and colleagues (1984) demonstrated that TAM reverses the estradiol

facilitation of K^+ evoked GnRH release in the rat. Therefore, it would appear that the hypothalamic neurons which excite GnRH release or GnRH neurons themselves in addition to pituitary gonadotropes are a potential site of TAM action. Direct evidence of TAM action on hypothalamic neurons is lacking at present.

Another approach to investigating estrogen induction of developmental changes in GnRH neurons would be to use aromatase inhibitors. These compounds inhibit aromatase to variable degrees (Brodie et al., 1986; Wing et al., 1988) and reduce circulating estradiol levels (Brodie et al., 1986). However, aromatase inhibitors have androgenic activities (Wing et al., 1988) and have not been very successful in inhibiting ovarian estrogen in the rabbit (Brodie, personal communication). Other approaches that could be pursued include immunization to GnRH or use of a GnRH agonist or antagonist. Selective removal of estrogens was not achievable with these alternatives and therefore these approaches were not pursued. Fraser (1984) has reviewed the benefits and drawbacks of the above alternatives.

The number of smooth cells were found to decrease while rough cells increased as control rabbits approached sexual maturity. These developmental changes were advanced in the PMS treated rabbits, and the change was significant at PND 75 compared to controls. The PMS treated rabbits were sexually mature by PND 92 in contrast to controls which were mature at

PND 108. Control rabbits became significantly different from the impaired sexual maturation group (Tamoxifen treated rabbits) at 108 days postpartum.

5.3.2 Developmental Changes in Plasma Estradiol and Gonadotropins:

In contrast to the findings of de Turckheim et al., (1983) plasma estradiol was detectable by radioimmunoassay in all three treatment groups studied. Plasma estradiol was significantly greater in PMS rabbits compared to controls between 25 and 34 days of age and then again at 75 days of age. The pattern of plasma estradiol concentration in the PMS group was triphasic, with peaks occurring on PND 34, 50, and 95. The initial increase in circulating estradiol was taken to be the result of PMS stimulation of ovarian aromatization. In the rat, PMS has been shown to cause superovulation by rescuing follicles from atresia (Brau and Tsafriri, 1980). PMS was also found to decrease atresia as well as to recruit "reserve" follicles in the hamster ovary (Chiras and Greenwald, 1978). Failure in the present study to find differences in follicular maturation between treatment groups was thought to be due to frequency of sampling. Measurement of follicular diameters may also have been a more sensitive indicator of follicle maturation. However, Jarrell et al., (1987) have shown that in the rat follicle diameters do not provide any information over that of estimates of follicle

populations. Regardless, a systematic comparison of the two methods is warranted since only a single measure of diameter has been used. Comparing both follicular maximum and minimum diameters may provide a more reliable measure for comparing treatment effects. It is hypothesized that in rabbits, PMS both supports follicles already growing and recruits sensitive follicles to begin growing.

In control rabbits, plasma estradiol was found to increase prior to successful matings and achievement of a mature body weight indicative of sexual maturity. These results appear to be consistent with the gonadostat hypothesis. Yet, circulating gonadotropins have already achieved adult levels by approximately 75 days of age and therefore appear to be uncoupled from the increased plasma estradiol concentration found just before sexual maturity is achieved. Increased plasma estradiol could be the consequence of decreasing synthesis and secretion of alpha fetoprotein as reported in the rat (Andrews et al., 1981). This is considered remote since PMS induced a significant increase in plasma estradiol levels measured between PND 25 and 34. Alternatively, ovarian sensitivity to plasma gonadotropins may be increased. Synergistic effects between LH, growth hormone (GH), and insulin-like growth factor-I (IGF-I) on ovarian steroidogenesis have been demonstrated in the rat (Cara and Rosenfeld, 1988).

The pattern of circulating estradiol concentration in

both control and TAM treated rabbits was biphasic with peaks on PND 50 and 95. However, no difference in plasma or ovarian estradiol concentration was found between the control and TAM treated rabbits. TAM may act independently of gonadotropins to stimulate ovarian estradiol release. In the follicular phase of pre-menopausal women TAM was shown to induce a 2 to 8 fold increase in estradiol secretion without producing significant changes in the secretion of FSH and LH (Groom and Griffiths, 1976). Alternatively, reduced prolactin secretion by TAM treatment may permit enhanced ovarian stimulation by normal concentrations of gonadotropins (Groom and Griffiths, 1976). In the current study measurements of plasma PRL were not made. Consequently, potential ovarian stimulation due to impaired PRL secretion in TAM treated rabbits cannot be determined. The latter alternative is unlikely since TAM markedly suppressed circulating gonadotropins. These results suggest that TAM induces estradiol secretion by the immature ovary in the rabbit. Results of the current study also suggest that PMS acts via stimulating the ovary to prematurely augment estradiol synthesis. It is suggested that estradiol plays a significant role in the process of sexual maturation in the rabbit. It is proposed that circulating estradiol is responsible for inducing developmental changes in GnRH cell morphology. By inducing increased circulating estradiol levels, PMS treatment advanced sexual maturity and developmental changes in GnRH neurons resulted. The time lag

between increased plasma estradiol concentration and GnRH morphological changes is difficult to understand since estradiol has a half-life of approximately 6 h (YoungLai, 1978; Bhavnani, 1988). It is possible that PMS activates the immature ovary prematurely and induces either maturation of the ovary or initiates maturation of the hypothalamus which then results in continued stimulation of the ovary. I propose that PMS in the present study activates the ovary which then continues to elaborate E_2 at elevated levels and induces maturational changes in the hypothalamus. In support of this hypothesis, Brawer et al. (1978) have found that plasma estradiol levels remained elevated 2 months after injection of immature rats with estradiol valerate. Recently, (Scully et al., 1989) a case of precocious puberty was described in a 6 1/2 year old girl in whom circulating levels of estradiol and testosterone were elevated abnormally but gonadotropins and adrenal steroids remained within normal limits. The patient had attained thelarche (normal 11 yr) and had an episode of vaginal bleeding. Ultrasonography revealed a large unilateral ovarian cyst. Following unilateral ovariectomy physical signs of puberty reverted to more immature stages but did not reach Tanner stages common for her age. The authors speculate that autonomous ovarian development may have resulted in induction of the hypothalamic pituitary axis. Conversely, the ovary may have been stimulated by premature activation of the hypothalamic pituitary axis which was then

suppressed by elevated levels of E_2. On the basis of the data (ie. rate of development, unilateral process, a single cyst and the size of the cyst) it is suggested that the precocious puberty was due to an autonomous ovarian process. A possible explanation is that estradiol effects are exerted through indirect mechanisms on nerve growth. Physiological doses of estradiol have been demonstrated to advance puberty in the rat (Ramirez and Sawyer, 1965). Moreover, estradiol induces synaptic remodelling of the rat brain (Nishizuka and Arai, 1981; Garcia-Segura et al., 1986) and these changes have been shown to be correlated with puberty onset (Clough and Rodriguez-Sierra, 1983). Garcia-Segura and Arai (1981) demonstrated that estradiol treatment in the adult rat induces increased neurite and dendritic growth as well as synaptogenesis in the brain. Withdrawal of estradiol results in a return to pretreatment levels by 32 weeks post treatment. Consequently, it is concluded that estradiol significantly affects the rate of sexual maturation through modification of hypothalamic circuitry. However, in the absence of direct neurochemical and neuroanatomical evidence it is not possible to determine whether puberty in the rabbit is the consequence of connection of excitatory inputs with GnRH cells or alternatively the consequence of resetting of the proposed "opiostat" (Wilkinson and Landymore, 1988).

5.3.3 Relationship Between Body Weight and Sexual Maturation in the Rabbit:

In the current study a body weight of 3.0 Kg was used as a means of determining sexual maturity. The relationship between body weight and sexual maturity has been established in prior studies (Hulot et al., 1982; YoungLai, 1986) in the rabbit. In the current study PMS treated rabbits were found to gain weight faster and achieve sexual maturity (PND 92) earlier than control rabbits (PND 108) which in turn were advanced relative to TAM (PND 130) treated rabbits. Weight gain in all three experimental groups was greatest between PND 40 and 75. Coincident with this period of accelerated weight gain was a maximal FSH/LH ratio. The rate of weight gain then slowed prior to achievement of sexual maturity. In contrast, in the rat the rate of weight gain was dramatically increased between PND 31 and 40, peaking on PND 35 (Wray and Hoffman, 1986c). The period of maximal rate of weight gain in the rat corresponds well with the beginning of vaginal opening. The significance of this finding is as yet unclear. It would appear that in the rabbit changes in weight precede developmental changes in GnRH neurons while in the rat both occur concomitantly. The results of the current study suggest that dramatic changes occurring in the hypothalamus affect GnRH secretion as evidenced by the FSH/LH ratio as well as growth hormone secretion (GH). It is interesting to note that the FSH/LH ratio in the PMS group at PND 22 and 25 was lower

than both the control and TAM groups. In the PMS group during this age period LH levels were also greater than in either the control or TAM groups while FSH levels were comparable thus accounting for a lower FSH/LH ratio. The LH level measured in the PMS group between PND 22 and 25 is proposed to have been elevated due to cross reaction of PMS with the primary antisera used in the radioimmunoassay for LH. Consequently, at this age the treatment groups were considered to have no differences other than treatments.

Correlation of body weight with sexual maturity in the female rabbit has been reported in prior studies (Hulot et al., 1982; YoungLai, 1986). The role of body weight in sexual maturation in the rabbit needs to be studied. A critical body weight (Frisch and Revell, 1970; 1971) and body composition (Frisch, 1980; 1984) have been proposed as triggers for the onset of menarche in girls. Body weight and the initiation of puberty has been reviewed by Baker (1985). However, this hypothesis has been severely criticized (Johnston et al., 1975; de Ridder et al., 1990). The weight range proposed by Frisch and Revell (1970; 1971) does not fit well with clinical observations (Johnston et al., 1975) and experimental findings (Wilen and Naftolin, 1977; Hansen et al., 1983; Glass et al., 1986). Nevertheless, results have been brought forward which inculpate diet in the process of sexual maturation. Glass et al., (1986) demonstrated that underfed male rats had lower body weights and lower body fat at puberty than ad libitum fed

males. In contrast, food restriction has been shown to be an effective means of inducing delayed puberty in the female rabbit (Hulot et al., 1982) and lamb (Foster and Olster, 1985; Foster et al., 1985). The role of diet on sexual development has also been studied by Smith et al., (1989) who demonstrated that a diet deficient in the essential fatty acids linoleic and linolenic acid delayed vaginal opening, first ovulation, and first diestrus in rats. The development of positive estrogen feedback was also delayed in this study. Smith and colleagues (1989) speculate that the puberty delay is due in part to decreased synthesis of the prostaglandin E_2 (PGE_2) or impaired ability of NE to stimulate PGE_2 release. It is reasonable from these observations to speculate that graded underfeeding delays puberty onset through mechanisms which alter the regulation of releasing factors or pituitary responsiveness. Recently, (Thomas et al., 1990) found that adult ovariectomized ewes fed a restricted diet had increased plasma GH levels while plasma gonadotropins were decreased. On the basis of the present studies, the effect of diet on the regulation of hypothalamic releasing or inhibiting factors and anterior pituitary function can not be deduced. It can be hypothesized from these studies that nourishment and body weight modifies the rate of sexual development by delaying the development of the mechanisms which regulate GnRH secretion. However, there does not appear to be a critical body weight or composition which triggers puberty onset.

Rosenfield (1990) draws attention to a number of disorders with diffuse etiologies such as childhood illness, undernutrition, GH deficiency and chronic anaemia, all of which are associated with pubertal delay. Illness, chronic anaemia and undernutrition affect a broad range of physiological processes. Under such conditions the stressed organism may not have the reserves necessary to support the developing reproductive system, resulting in pubertal delay. In contrast, GH has been implicated as a factor involved in the onset of puberty by several lines of evidence. GH deficiency with consequent deficiency of IGF-I is frequently associated with sexual infantilism (Laron and Sarel, 1970,; Ramaley and Phares, 1980; Cara and Rosenfield, 1988; Nogami et al., 1989; Rosenfield, 1990). Similarly, in rats passively immunized to growth hormone releasing hormone (GHRH), sexual maturation is delayed (Arsenijevic et al., 1989). Laron and Sarel (1970) showed that pubertal development could be resumed in patients with GH deficiency and arrested pubertal development by GH replacement therapy. Similarly, in GH deficient mice, GH administration restores fertility (Bartke, 1964). GH effects on pubertal development are postulated to be through induction of IGF-I productions. Cara and Rosenfield (1988) demonstrated that IGF-I and insulin synergize with LH to stimulate androgen production by normal thecal interstitial cells in culture.

While the evidence to date strongly supports the

involvement of GH in sexual maturation the nature of the GH role is unclear. GH may have direct effects on pituitary gonadotropes or the developing gonad itself. Alternatively, GH effects may be mediated entirely by IGF-I. Given that GH plays a permissive role in the process of sexual maturation it would be beneficial to investigate developmental changes in hypothalamic GHRH containing cells.

5.3.4 Ovariectomy Impairs Developmental Changes in GnRH Neurons:

The role of the immature ovary in the postnatal development of GnRH cell subtypes needs further clarification. In the present study ovariectomy was used to investigate ovarian effects on the total number of detectable GnRH cells and postnatal developmental changes in these cells. The total number of immunoreactive GnRH cells (1,100) were not altered by ovariectomy. Additionally, the distribution of GnRH cells in the rabbit hypothalamus was unaffected. However, development of GnRH cell subtypes was prevented by ovariectomy. This contrasts with a recent report (Wray and Gainer, 1987), in which ovariectomy did not alter the developmental characteristics, overall cell number, or distribution of GnRH neurons in the rat hypothalamus. In this study rats were sacrificed on postnatal days 50 to 71 (puberty at approximately 35 days post partum). The possibility that developmental changes in GnRH morphology progress at a slower

pace in the absence of the ovary, and therefore gonadal steroids, cannot be excluded. Additionally, developmental changes which occur postpubertally must also be considered. Witkin (1987) has demonstrated that synaptic input to GnRH cells increases threefold by middle age and tenfold by old age in the rat. It would be desirable to examine the brains of ovariectomized rats at the time of normal puberty onset. In the current study, circulating levels of gonadotropins remained elevated well beyond the normal time for puberty onset in the rabbit. The FSH/LH ratio throughout the study remained equivalent to sham operated rabbits between PND 40 and 72. These results suggest that GnRH neural inputs, in the rabbit, are prevented from developing the adult pattern of organization by removal of the ovary immediately after weaning (around PND 22).

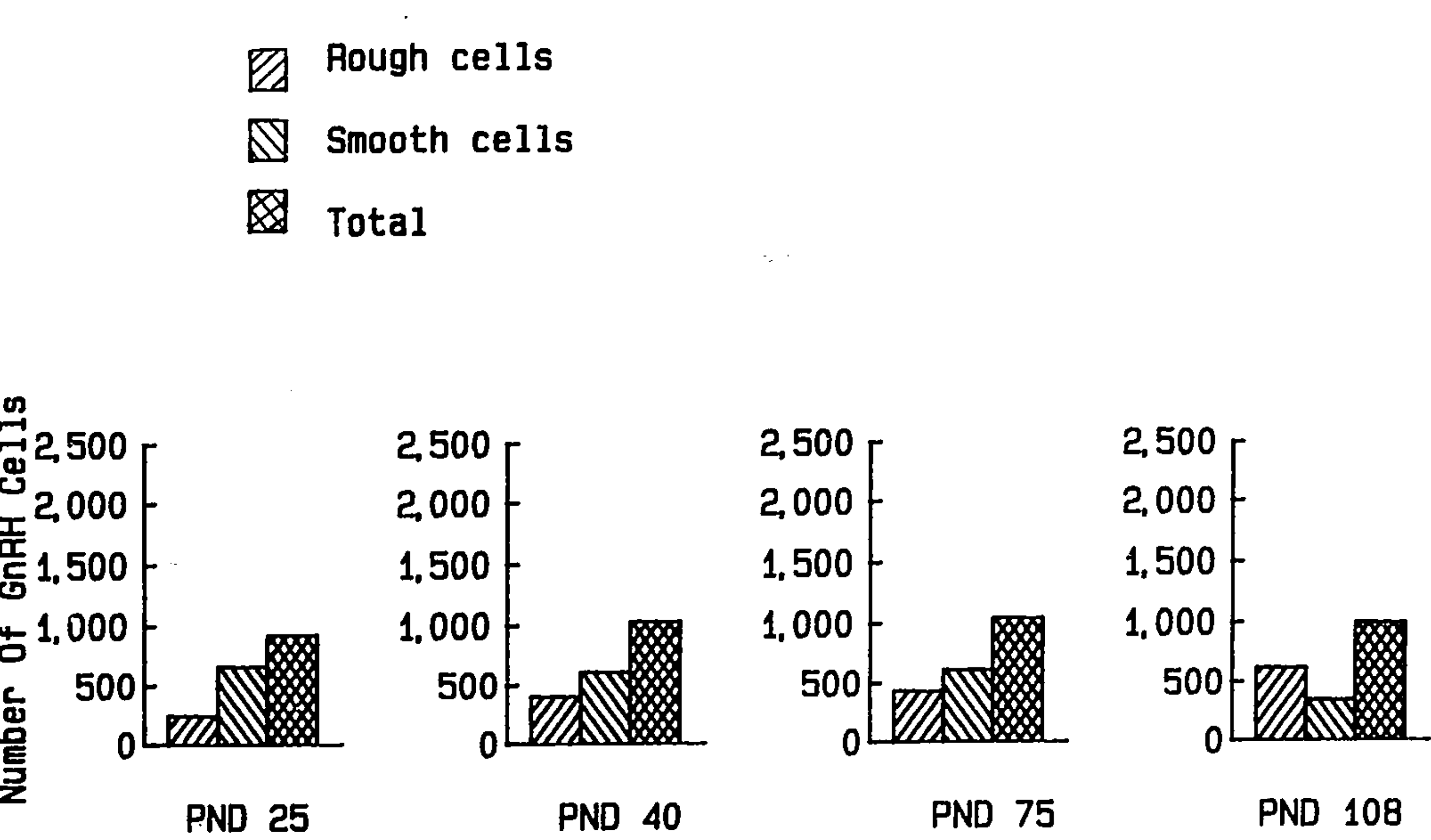
It is proposed that negative feedback of gonadal steroids on GnRH release is mediated by opioidergic neurons. In the ovariectomized rat, estrogen or testosterone propionate were reported to reduce the ovariectomy-induced increase in circulating LH levels (Van Vugt et al., 1983). A single injection of NAL was able to reverse the effect of the gonadal steroids. Similarly, NAL injection in push pull perfusion experiments of metestrous rats 4 to 8 days post ovariectomy produced a significant increase in GnRH release (Karahalios and Levine, 1988). These results suggest that steroid inhibition of GnRH release is mediated by the opioid system.

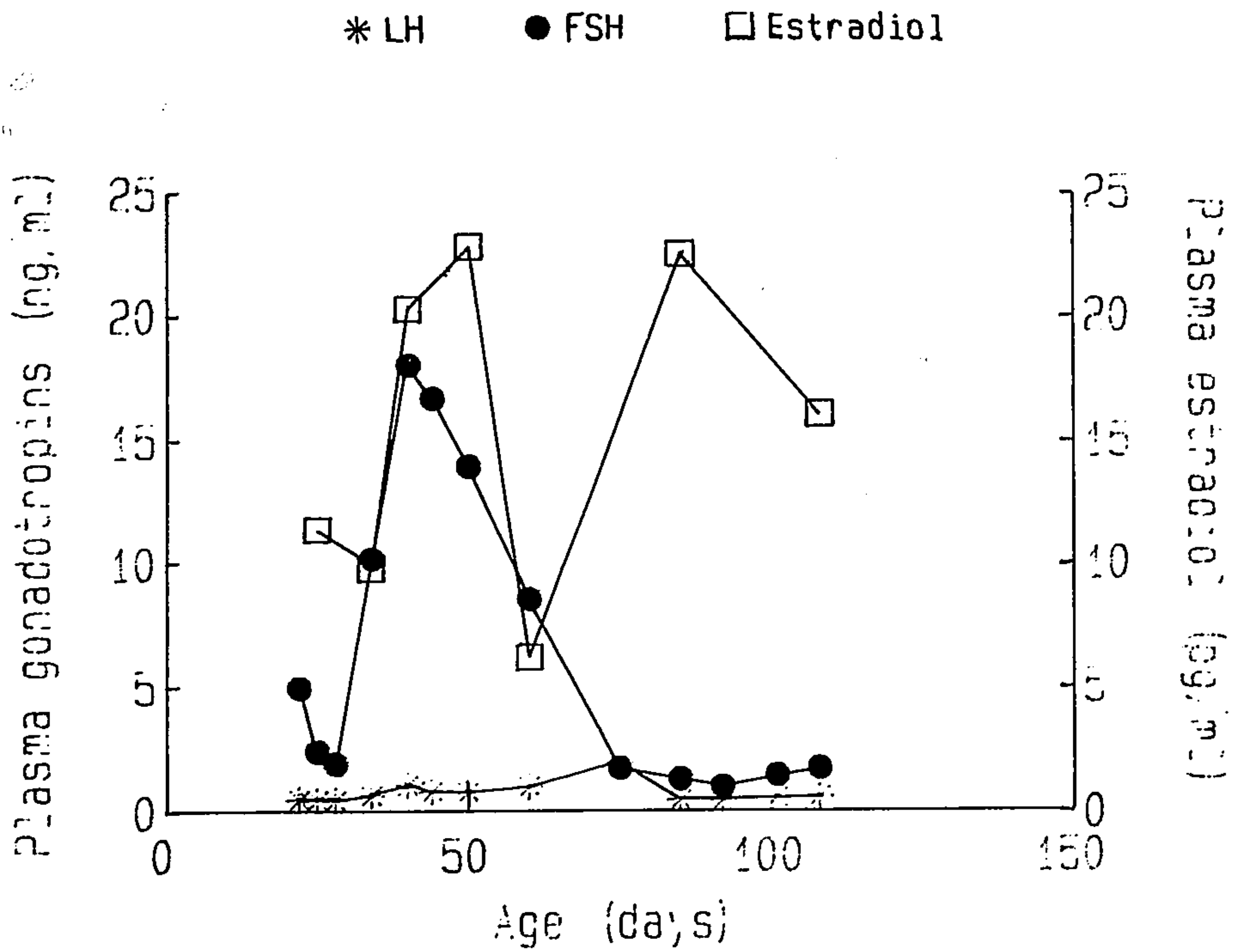
However, the possibility that gonadal steroids are capable of inducing both inhibitory and excitatory effects on GnRH secretion via catecholaminergic neurons cannot be excluded.

5.3.5 Summary of Developmental Changes in the GnRH Neuron:

The developmental changes in GnRH cell populations, plasma gonadotropins and estradiol of control rabbits are summarized in figure 31. The results of the present studies suggest that estradiol induces developmental changes in GnRH cell morphology which are speculated to be related to the development of the neural circuits which are both inhibitory and stimulatory for GnRH secretion. Several lines of evidence in the rat show that GnRH neurons receive multiple inputs. Functionally, opioids (Blank et al., 1979; Leadem et al., 1985; Wilkinson and YoungLai, 1985; Orstead and Spies, 1987; Karahlios and Levine, 1988; YoungLai et al., 1988; Wiemann et al., 1989) and catecholamines (Sawyer et al., 1974; Fuxe et al., 1978; Pau and Spies, 1986; Ramirez et al., 1986; Clough et al., 1988; Terasawa et al., 1988) have been shown to regulate GnRH and gonadotropin secretion in the rabbit. Additionally, immunohistochemical evidence in the rat showed that GnRH neurons are innervated by opioidergic neurons (Leranth et al., 1988; Lashin et al., 1988; Chen et al., 1990). Catecholaminergic innervation of GnRH neurons has also been demonstrated in the rat brain (McNeill and Sladek, 1978; Abata et al., 1979; Ajika, 1979; Jennes et al., 1982; Kuljis

Fig. 31. Developmental changes in GnRH cell population, plasma gonadotropins, and estradiol of control rabbits.





LH
FSH
Estradiol
Plasma gonadotropins (ng/ml)
Plasma estradiol (pg/ml)
Age (days)
0
5
10
15
20
25
0
50
100
150

and Advis, 1989; Chen et al., 1989). Moreover, in the rat (Wray and Hoffman, 1986b) and mouse (Hoffman et al., 1982) it has been shown that GnRH neuron subtypes (spiny vs smooth) are differentially innervated by catecholaminergic neurons. The developmental course for opioid and catecholaminergic innervation of GnRH neurons needs further study and may vary for different mammalian species. It is difficult to infer changes in GnRH input based on circulating hormone levels without direct evidence of innervation.

In the rabbit, estradiol may act on either catecholaminergic, opioidergic neurons, or both to augment growth of these neurons directly. Morphological studies have shown that both catecholaminergic and opioidergic neurons concentrate tritiated estradiol (Shivers et al., 1983; Jerikowski et al., 1986), while less than 0.2% of GnRH cells were found to concentrate estradiol. Brown and colleagues (1989) have demonstrated that radiolabeled estradiol is localized to nerve cells of the developing rat brain and is excluded from glial cells. An alternative explanation could have estradiol acting synergistically with an ovarian factor to promote morphological changes in the hypothalamus consistent with sexual maturation. In the rabbit, a protein produced by ovarian interstitial cells has been temporally associated with sexual maturation (Washenik and Dunbar, 1988). However, this protein "maturin" does not appear until 18 weeks or about 126 days of age. Since sexual maturation occurred

between PND 100 and 108 in control rabbits in this study it is suggested that maturin is likely a consequence of sexual maturation rather than a factor inducing puberty onset.

5.4 Chronic Treatment With Tamoxifen Appears To Prevent The Development Of Estrogen Negative Feedback:

It was unexpected that TAM treatment would fail to produce a rebound increase in circulating gonadotropins. This finding suggests that estrogen inhibition of gonadotropins is weak or absent in the immature rabbit. In order to evaluate the effect of acute TAM treatment on gonadotropin secretion in the adult rabbit, a final experiment investigated the effect that TAM treatment had on circulating gonadotropin levels in the adult female rabbit. Circulating FSH but not LH was found to be significantly elevated 2 weeks after TAM treatment compared with pretreatment levels. Consequently, these results suggest that negative estrogen feedback is absent in the immature female rabbit but is functional in the adult.

5.5 Proposed Stages of Sexual Development in the Rabbit:

Based on morphological changes in the GnRH neuron, measurements of circulating gonadotropins and estradiol, and physical measurements, 4 stages of sexual development are

proposed for the female rabbit. The first stage or neonatal period commences with birth and concludes around PND 25. This period was not studied in the experiments described here but is proposed on the basis of evidence from prior studies (de Turckheim et al., 1983; YoungLai, 1986). It is characterized by elevated circulating gonadotropins which peak around 9 to 10 days postpartum (de Turckheim et al., 1983; YoungLai, 1986). The second developmental period was suggested to begin at about 25 days of age and concludes at 75 days of age. This stage is designated the critical period because it is characterized by peak gonadotropin secretion, maximal FSH/LH ratio, elevated plasma estradiol concentrations and accelerated weight gain. Circulating levels of estradiol fall in the later part of this stage coincident with falling FSH levels. Pituitary responsiveness to GnRH in the rabbit only becomes diminished at 72 days of age (YoungLai et al., 1989). These data suggest that pituitary response to GnRH is functionally inhibited after 72 days of age. In the rat, the pituitary response to GnRH is also diminished just before vaginal opening (Debeljuk et al., 1972). These changes may be mediated by changes in pituitary GnRH receptor number. It is feasible that rising circulating estradiol levels seen in this study beginning around PND 60 may inhibit pituitary response to GnRH. Increased plasma estradiol in the rat may also account for the increased content of GnRH in the hypothalamus seen just before vaginal opening (Araki et al.,

1975).

It is suggested that gonadotropin secretion during this period is due to altered hypothalamic sensitivity to inhibitory factors. Elevated gonadotropin levels have been correlated with a 40 % reduction in [^{3}H] NAL binding of the rabbit medial basal hypothalamus at 51 days of age (YoungLai et al, 1988). Two possible explanations can be offered. On one hand, opioid receptors are reduced as a result of desensitization. In contrast, if opioid receptors autoregulate their numbers then decreased binding conceivably would suggest diminished activity in this cell group and reduced availability of endogenous opioids.

Elevated plasma estradiol concentrations found in this study are taken to be the result of increased gonadotropin secretion by the pituitary. Plasma estradiol levels measured in this thesis agree well with peak ovarian estradiol content (50-60 days, and 90 days) measured previously in the rabbit (de Turckheim et al., 1983). Follicular maturation was found to begin during this age period in this and other studies (de Turckheim et al., 1983; Deanesley, 1972), however, significant differences between the treatment groups could not be found in the present study. It is interesting to note that this stage of development is associated with the maximal period of weight gain as discussed earlier. It is proposed that this age period is not only typified by pituitary ovarian activation but that important organizational changes are

occurring in the hypothalamus relevant to reproduction and other physiology processes as well.

The third or peripubertal stage of development occurs between 75 and 110 days of age. In this age group plasma gonadotropins attain the adult concentration coincident with increased circulating estradiol. It is during this period that synaptic changes are proposed to reach their peak with hypothalamic GnRH cells. Rough GnRH cells were, for the first time, found to comprise a larger percentage than smooth cells in this age group. Since, in this age group plasma gonadotropins are at adult levels while plasma estradiol increases to a peak at about PND 85, it is proposed that significant numbers of GnRH inputs become opposed to GnRH cells at this time. However, it is difficult to explain the cause and function of increased circulating estradiol in the presence of adult levels of gonadotropins. It is possible that ovarian sensitivity to gonadotropins is enhanced during this period while pituitary responsiveness to GnRH has been demonstrated to be suppressed (YoungLai et al., 1989). The possibility that the sensitivity of the rabbit hypothalamus to gonadal steroids has been diminished cannot be excluded. In the rat evidence has been brought forward (Cicero et al., 1980; Van Vugt and Meites, 1980; Wilkinson and Bhanot, 1982) that suggests that one of the processes by which the maturing hypothalamus changes its sensitivity to steroid feedback is via a change in opioid sensitivity. Indeed, Wilkinson and

colleagues (Wilkinson and Bhanot, 1983; MacDonald and Wilkinson, 1990) have shown that the timing of puberty in the rat is the consequence of a gradual decrease in opioid feedback, together with the emergence of an excitatory drive. Based on the findings in this study the timing of puberty in the rabbit could be the consequence of a similar decrease in opioid feedback and augmented ovarian sensitivity to pituitary gonadotropins.

The fourth and final stage of development is adulthood which begins around 110 days of age. In the adult female rabbit plasma gonadotropin levels are stable and spike only after mating has been performed. Suppression of gonadotropins in the adult rabbit is suggested to be the consequence of opioidergic inhibition of GnRH secretion to prevent inappropriate ovarian stimulation and to maximize the potential of maximum litter size with each mating.

5.6 Summary:

The topography and morphology of the GnRH neuron in the adult rabbit hypothalamus has been described in this thesis. The number of immunoreactive GnRH neurons is now reported and GnRH cell subtypes described. Additionally, postnatal developmental changes in GnRH cell subtypes are reported. Moreover, it was shown that premature ovarian activation accelerates developmental changes in GnRH cells

while competitively inhibiting estradiol at its receptor or removal of the ovary prevents these developmental changes from occurring. The mechanism triggering developmental changes in GnRH neurons is unclear. It is proposed that the critical period (PND 25 to 75) is a stage of reproductive development during which significant changes in the hypothalamus are initiated. In order to better address some of the issues raised above ultrastructural studies of the rabbit hypothalamus with more frequent sampling during this period would be informative. Also, the use of TAM or ovariectomy provides only a very crude means of examining the influence of estradiol and the ovary on the developing brain. The use of aromatase inhibitors or passive immunization to GnRH may provide better means to dissect the actual mechanism triggering the observed developmental changes in GnRH neurons.

Based on previous reports and the present studies four stages of sexual development are described for the rabbit. Of these, the critical period (PND 25 to 75) raises the most questions regarding developmental changes in the rabbit hypothalamus. It is proposed that during this stage of development major changes in hypothalamic circuitry are taking place and affect numerous physiological systems.

5.7 Conclusions:

1. Two morphologically distinct GnRH cell types are present in the hypothalamus of the female rabbit.
2. GnRH neural elements are widely distributed throughout the rabbit hypothalamus and cells are not confined to classical hypothalamic nuclei.
3. Since the total number of GnRH cells counted was constant it is concluded that GnRH cells with smooth contours are transformed to rough cells, and these developmental changes are functionally relevant to puberty onset.
4. Estradiol induces developmental changes in GnRH neurons which are related to sexual development.
5. Tamoxifen citrate, an antiestrogen, blocks estradiol induced developmental changes in GnRH neurons.
6. Ovariectomy also prevents developmental changes in the GnRH neurons in the rabbit.
7. Stages of reproductive development can be suggested for the female rabbit.

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