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THE ROLE OF ANDROGENS IN THE REGULATION OF THE HYPOTHALAMO-PITUITARY-TESTICULAR AXIS

by

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ANDROGEN REGULATION OF THE HYPOTHALAMO-PITUITARY-TESTICULAR AXIS

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ABSTRACT

The importance of discontinuous hormonal signals in endocrine communications is suggested by the ability of sustained hormonal signals to shut down, as opposed to stimulate, target cells, and by the observation that a change in pulse pattern can alter the response elicited. Serial sampling of the serum in catheterized, conscious, freely-moving rats was employed to investigate two aspects of pulsatility in the hypothalamo-pituitary-testicular feedback loop regulating reproduction in the adult male. First, in response to treatment with moderate to high doses of the testosterone (T) metabolite, dihydrotestosterone (DHT), pituitary responsiveness to exogenous LHRH was suppressed, and elevated post-castration mean LH levels declined due to a dose-dependent suppression of LH pulse frequency and amplitude. LH pulse amplitude was not suppressed by low dose DHT treatment, in spite of a dramatic suppression of pituitary responsiveness as measured, implying that a stimulatory androgenic influence on amplitude might be exerted upon hypothalamic LHRH release; blocking the endogenous opioid peptide system did not abolish this phenomenon. A stimulation of LH pulse amplitude was observed in orchidectomized rats given a low (sub-physiological) dose of T, and again, this effect was apparently mediated at a supra-pituitary site as pituitary responsiveness appeared unaltered at this T dose. These results suggest that the feedback regulatory actions of exogenous DHT, like those of T, are mediated at both the hypothalamus and the pituitary; this is consistent with the hypothesis that endogenous DHT mediates some of the negative and postive feedback effects of T in intact or T-treated orchidectomized animals. A second aspect of pulsatility was investigated in testes-intact rats. Fluctuations in serum T concentration are of a dual nature, reflecting both acute pulsatile bursts and prolonged episodes of testicular secretory activity, the precise pattern of which varies between and within individuals. While there is a correspondence in the general level of secretory activity between T and its secretagogue, LH, the correlation between pulses is imperfect, suggesting the influence of modulatory factors; light may play a role in the regulation of LH and T secretion. Thus, pulsatile LH release from the hypothalamo-pituitary complex in male rats is regulated by DHT in a manner similar to T, and subject to an irregularly pulsatile androgen signal from the testes.

CONDENSE

L'importance des signaux hormonaux discontinus dans la communication endocrinienne est suggérée par l'habilité des signaux hormonaux constants a inhiber, au lieu de stimuler, les cellules cibles, aussi bien que par l'observation qu'un changement de la sécrétion pulsatile des signaux hormonaux peux altérer la réponse suscitée. L'échantillonnage en série du sérum chez les rats mâles cathétérisés, conscients, et libre de mouvement, a été employé afin d'étudier deux aspects de la sécrétion pulsatile dans la rétroaction hypothalamique-hypophyse-testiculaire. En premier lieu, les effets des doses modérées et élevées du métabolite de la testostérone (T), la dihydrotestostérone (DHT), ont été étudié. Les niveaux de LH qui sont fortement élevé âpres castration, ont été diminué par la DHT. Cette diminution, qui dépendait de la dos de DHT utilisée, a été achevé par une réduction de la vitesse de fréquence de la sécrétion pulsatile et de amplitude des pics de LH; une diminution de la sensibilité de l'hypophyse au LHRH a aussi été noté. Cependant, avec la dose la plu petite de DHT utilisée, l'amplitude des pics de LH ne fut pas réduite, en dépit d'une suppression dramatique dans la sensibilité de l'hypophyse au DHT. Ceci insinuerait que l'influence androgénique sur l'amplitude est modulé a un niveau plus élevé que l'hypophyse. Les peptides opioidiques endogènes ne semblent pas moduler cet effet. Une petite dose, équivalente a un cinquième du niveau moyen physiologique, de T chez des rats castrés mène à une augmentation de la taille des pics de LH. Il est également conclus que cet effet était modulée a un site supra-hypophysaire car la sensibilité de l'hypophyse ne fut pas changée par cette dose de T. Ces résultats suggèrent que, semblablement à la T, la DHT module ses effets sur la rétroaction au niveau de l'hypothalamus et de l'hypophyse. Ceci est consistant avec l'hypothèse qu'au moins certains des effets de la T sur les mécanismes de rétroaction positif et négatif chez le rat intacte ou castré et traité avec le la T ont comme médiateur la DHT. En deuxième lieu, les liens entre la sécrétion pulsatile du LH et de la T chez des rats intactes ont été

étudié. Les fluctuations dans les concentrations sériques de T furent de nature double, reflètent les pics aigus et des épisodes prolongés d'activité sécrétoire du testicule; des changements assez marqués furent notés non seulement entre individus mais aussi chez le même rat échantillonné plusieurs fois. Même lorsque une relation de l'activité sécrétoire entre la T et sont sécrétagogue, le LH, pouvait être établi, elle était imparfaite, suggèrent l'influence d'autres facteurs modulateurs. La lumière semblerait jouer un rôle dans la sécrétion de la T et du LH. On peut donc conclure que, chez le rat mâle, la sécrétion pulsatile du LH provenant du complexe hypothalamo-hypophysaire est réglés par la DHT de façon semblable à la T, et qu'elle est sujette a un signale irrégulier d'androgène provenant du testicule.

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ACTH	adrenocorticotropic hormone, adrenocorticotropin, corticotropin
$B\backslash B_0$,	ratio of tracer bound in the presence (B) and absence (B_0) of competing ligand
cAMP	cyclic adenosine 3',5'-monophosphate
cDNA	complementary deoxyribonucleic acid
CNS	central nervous system
DBB	diagonal band of Broca
DHT	dihydrotestosterone = 5α-androstan-17ß-ol-3-one
DA	dopamine
EB	estradiol benzoate
GAP	GnRH-associated peptide
ID	inner diameter
IU	international units
i.v.	intravenous
LH	luteinizing hormone, lutropin = ICTH = interstitial cell-stimulating hormone,
LHRH	luteinizing hormone-releasing hormone = GnRH = gonadotropin releasing hormone
MBH	medial basal hypothalamus
ME	median eminence
MPOA	medial preoptic area
MUA	multi-unit activity
NA	noradrenaline
NPY	neuropeptide Y
OVLT	organum vasculosum of the lamina terminalis
OD	outer diameter
P450c17	17α-hydroxylase/17,20 lyase
P450scc	20-hydroxylation/22-hydroxylation/20,22-lyase = 20,22 desmolase
PEG	polyethylene glycol 8000
RIA	radioimmunoassay
SCN	suprachiasmatic nucleus
SE	standard error
T	testosterone = 4-androstene-17ß-ol-3-one
Tp	testosterone proprionate
3α-diol	5α-androstan-3α,17ß-diol
3ß-diol	5α-androstan-3ß,17ß-diol
3ß-HSD	3ß-hydroxysteroid dehydrogenase/isomerase
17ß-HSD	17ß-hydroxysteroid dehydrogenase = 17 ketosteroid reductase = 17-oxidoreductase

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INTRODUCTION

The contribution of the adult male mammal toward the goal of successful reproduction is to deliver fertile sperm into the reproductive tract of the female; this requires the coordination of several functionally distinct systems in the male. The key processes of spermatozoal development and maturation occur in the testes and epididymides, and the accessory sexual organs make significant contributions to the seminal medium that carries the sperm into the female tract. Male behavioural strategies requisite to successful interaction with the female arise in the central nervous system, as do hormonal signals important for spermatogenesis and the production of the male steroid hormone, testosterone (T). Common to each of these tissues is a dependence upon T for crucial supportive and/or regulatory functions; thus, it is the paracrine and endocrine actions of this androgen that orchestrate the diverse events underlying successful male reproduction.

Testosterone is responsible for the feedback regulation of its own production in the testes. Intermittently, hypothalamic neurons secrete luteinizing hormone-releasing hormone (LHRH) into the pituitary portal blood, which carries the neuroendocrine signal directly to the pituitary to elicit the pulsatile release of luteinizing hormone (LH) into the peripheral circulation. These LH pulses augment T release by increasing the steroidogenic activity of the Leydig cells; as higher serum T levels reach the CNS and pituitary, they reduce LH output, thus completing the feedback loop. The research reported in this thesis investigates the stimulatory and inhibitory effects of the potent T metabolite, dihydrotestosterone (DHT), upon the hypothalamo-pituitary axis in the orchidectomized rat, and characterizes the intermittent nature of testosterone secretion in the intact male animal. As background to this work, this introductory section will review relevant information on the three components of the hypothalamo-pitutiary-testicular loop, and the endocrine interactions between them, with an emphasis on rats - the animal model used in experiments reported in this thesis.

1 LUTEINIZING HORMONE-RELEASING HORMONE

1.1 HISTORICAL CONTEXT

The notion that endocrine interactions between the hypothalamus, pituitary and gonads are important for the successful functioning of the male and female reproductive systems evolved in the first half of this century. The reciprocal influence between the testes and pituitary was noted by Moore and Price who determined that the maintenance of testicular and accessory tissue weights required activities found in hypophysial extracts, and that sex hormones secreted from the testes exerted some control over the endocrine function of the hypophysis {Moore & Price, 1932}.

The first studies to implicate hypothalamic involvement in reproduction demonstrated that electrical stimulation of the sub-thalamic region could elicit a gonadal response in the rabbit {Harris, 1937; Haterius & Derbyshire, 1937}, and this was supported by the clinical observation that hypothalamic pathologies were often associated with hypogonadism or sexual precocity {Hetherington & Ransom, 1942; Bauer, 1954}. Anatomical studies detected the presence of a specialized portal vasculature linking the hypothalamus and hypophysis, suggesting that neurovascular communications between these tissues might be important for the generation of the endocrine message to the gonads {Green & Harris, 1947}. This was confirmed in experiments that disrupted the communication pathway: pituitary stalk section in female rats, monkeys, and rabbits led to a temporary loss of reproductive function which only lasted until the regeneration of the portal vessels {Harris, 1950a, 1950b; Jacobsohn, 1954}; and similarily estrous cyclicity was disrupted in hypophysectomized female rats, but recovered if a donor pituitary was implanted under the median eminence (but not if placed under the

frontal lobe) {Harris & Jacobsohn, 1952}. The role of the median eminence as the point of converging neural systems for the endocrine regulation of anterior pituitary function was confirmed when hypothalamic tissue or extract was found capable of regulating the in vitro release of numerous pituitary trophic hormones from anterior lobe cells {Guillemin, 1955; McCann et al, 1960; Garcia & Geschwind, 1966}. It subsequently became evident that a hypothalamic signal, luteinizing hormone releasing hormone (LHRH), was communicated via the portal circulation to the anterior pituitary, and was essential for stimulating the pulsatile secretion of the gonadotropin, luteinizing hormone (LH) .

1.2 LHRH - THE MOLECULE

LHRH was isolated from portal blood and characterized in 1971, and has the following amino acid sequence:

(pyro)Glu-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly(NH_2)

{Schally et al, 1971; Amoss et al, 1971}. Two years later this peptide was localized in cell bodies in the hypothalamus {Barry et al, 1973} and subsequently within granular (secretory) vesicles in axon terminals in the perivascular space of the median eminence {Styne et al, 1977}. It is now known that LHRH is synthesized in the soma of hypothalamic neurons as a prohormone {Seeburg & Adelman, 1984}, processed and transported in vesicles to the nerve terminals {Rubin et al, 1987; Liposits et al, 1991}, and secreted periodically into the capillary plexus of the median eminence {Carmel et al, 1976}. Because the pulsatile peptide signal is a stimulus for the release of follicle-stimulating hormone (FSH), as well as LH, from the anterior pituitary gonadotropes, LHRH is also known as gonadotropin-releasing hormone (GnRH).

While portal blood sampling {Carmel et al, 1976}, push-pull cannula {Dluzen & Ramirez, 1987}, and microdialysis {Meredith & Levine, 1992} investigations detect LHRH pulses arriving at the anterior pituitary, venous blood leaving the pituitary contains, at best, very low concentrations of the decapeptide, probably due to dilution in the circulation and its rapid degradation in the pituitary {Handelsman & Swerdloff, 1986}. LHRH which does reach the peripheral circulation is subject to further enzymatic degradation in the serum and other tissues {Bauer & Horsthemke, 1984}. As the decapeptide is not glycosylated and doesn't bind plasma proteins {Tharandt et al, 1979; Chan & Chaplin, 1985}, its half-life in serum is short (< 10 min in humans) {Griffiths & Kelly, 1979; Bennett & McMartin, 1978}.

1.2.1 *The LHRH/GAP gene*

The DNA sequence for the gene encoding LHRH {Seeburg & Adelman, 1984} reveals that it is initially part of a larger 92 amino acid precursor. LHRH processing involves the removal of the 23 amino acid signal peptide, successive cleavage of the Gly-Lys-Arg amino acids on the N-terminal end of the decapeptide, amidation, and cyclization of the carboxy-terminal of LHRH. While in some species, such as the human, processed LHRH appears to be stored in the perikarya {King & Anthony, 1984}, several lines of evidence suggest that in the rat the cleavage of LHRH from the 56 amino acid C-terminal moiety, "GnRH-associated peptide" (GAP), occurs in the vesicles within axonal processes and nerve terminals. Unlike polyclonal antibodies raised against unprocessed LHRH, antisera directed against the (processed) decapeptide do not react with soma in the hypothalamus unless the rat has been treated with colchicine to prevent axonal transport {Setalo et al, 1975}. In immunocytochemical studies anti-GAP and anti-LHRH appear to co-localize over the same (secretory) vesicles {Phillips et al, 1985; Liposits et al, 1991}. The coincidence of GAP

pulses with portal blood LHRH episodes also suggests that this processing takes place only subsequent to precursor sequestration into vesicles. The function of GAP has not yet been determined, though a role in the regulation of pituitary hormones has been investigated; in vitro studies on the rat adenohypophyseal cells indicate that GAP is a potent stimulator of gonadotropin release and strongly inhibits prolactin release, and at doses of 10^{-5}M a 13 amino acid fragment of the peptide also shows the same activites in monkey pituitary {Millar et al, 1986; Nikolics et al, 1985}.

1.2.2 *The pulsatile nature of LHRH secretion*

Initial attempts to define daily serum LH rhythms yielded inconsistent results, and the resulting confusion cleared once it was realized that LH secretion is intermittent {e.g. Gay & Sheth, 1972}. Shortly thereafter, serial sampling of the pituitary stalk blood in monkeys revealed acute fluctuations in the LHRH signal en route to the pituitary, providing evidence for the hypothalamic mediation of episodic LH discharge {Carmel et al, 1976}. Since then, temporal associations between LHRH pulses and serum LH pulses have been demonstrated in monkeys {Van Vugt et al, 1985; Levine et al, 1985; Pau et al, 1989}, sheep {Clarke & Cummins, 1982; Levine et al, 1982; Karsch et al, 1987; Caraty & Locatelli, 1988}, goats {Mori et al, 1991}, rabbits {Pau et al, 1986}, and rats {Urbanski et al, 1988; Levine & Duffy, 1988; Phelps et al, 1992}. In addition to regulating gonadotropin release, LHRH stimulates gonadotropin peptide biosynthesis {Starzec et al, 1986} and glycosylation in cell cultures {Liu & Jackson, 1978; Vogel et al, 1989}, and regulates pituitary responsiveness to LHRH as will be discussed in the following paragraphs.

The pulsatile pattern of LHRH release is important for its effects on gonadotropes, as continuous infusion of the decapeptide renders the gonadotrope refractory to further LHRH

stimulation (homologous desensitization), while intermittent LHRH administration does not {Belchetz et al, 1978; Ferin et al, 1978; Nakai et al, 1978; Valk et al, 1980; Smith & Vale, 1981; Barkan et al, 1985}. The reduced pituitary responsiveness that follows apulsatile LHRH treatment is associated with internalization of the receptor molecule, which decreases the number of LHRH receptors on the cell surface (down-regulation) {e.g. Hazum et al, 1980; Clayton, 1982; Loumaye & Catt, 1983; Conn et al, 1984; Zilberstein et al, 1983}. This is not the only mechanism of desensitization, however, since measurable desensitization occurs even when internalization is blocked {Gorospe & Conn, 1987}, and persists even after LHRH receptor numbers have returned to control levels {Conn et al, 1984}.

The pattern (amplitude and frequency) of LHRH pulses changes throughout the female reproductive cycle, and also varies, albeit more subtly, throughout the day in the intact male animal and in castrated rats. There are several lines of evidence to suggest that variations in the pulse pattern are of functional significance. In women with primary hypogonadotropic amenorrhea, pulsatile LHRH therapy induces ovulation with a greater success rate if the frequency is hourly rather than every 2 hours; changes in the dose administered per pulse will be reflected in altered hormone levels {Filicori et al, 1989}. Changes in the frequency of the pulsatile LHRH stimulus alter the relative potency of the signal on α- and ß- subunit content, and on LH and FSH secretion {Haisenleder et al, 1991; Dalkin et al, 1989; Papavasiliou et al, 1986; Leung et al, 1987; Wildt et al, 1981}. Thus, the pulsatile delivery of the LHRH signal is not only essential for the maintenance of normal gonadotrope responsiveness, but also encodes a message that varies with the frequency and amplitude of the signal.

1.3 THE NEUROANATOMY OF HYPOTHALAMIC LHRH NEURONS

1.3.1 *The organization of the hypothalamus*

The nuclei, or neuron clusters, in the medial region of the hypothalamus may be subdivided according to their rostral-caudal position relative to the tubero-infundibulum (pituitary stalk). The anterior region includes the medial preoptic area, the anterior hypothalamus, suprachiasmatic nucleus, supraoptic nucleus and paraventricular nucleus. The tuberal group, found between the optic chiasm and mammillary bodies, comprises the dorso- and ventro- medial nuclei, and the arcuate nucleus, which is positioned above the median eminence (ME) adjacent to the third ventricle. Of least significance to hypothalamic regulation of male reproductive function is the posterior group: the mammillary complex, the posterior hypothalamic nucleus, and the supra- and tubero- mammillary nuclei.

1.3.2 *Anatomy of the portal vasculature*

It was Green & Harris {1947} who initially described the close apposition of nerve terminals in the median eminence with capillaries that drain into portal blood vessels, and proposed a neurovascular link between the hypothalamus and anterior pituitary. More detailed microscopic studies have recently shown that LHRH-immunoreactive neurons in the median eminence terminate on a pervasive vasculature, known as the primary capillary plexus. Anatomically, this network of capillaries has been compared to that of the lung and gastric mucosa where the exchange of materials between the blood and tissue is rapid {Sobin & Tremer, 1977}.

Blood from the superior hypophyseal artery (arising from the supraclinoid internal carotid arteries and the vessels of the circle of Willis) is directed through the numerous small fenestrated vessels of the primary plexus to acquire secretory contributions from innervating neurons, then reunites to flow, via portal vessels superficial to the pituitary stalk, directly to

the anterior pituitary {Page, 1988}. This portion of the adenohypophysis is thoroughly perfused with the neural secretions of the median eminence, as the portal blood is distributed by a second capillary plexus before it drains away via the lateral hypophyseal veins into the adjacent cavernous sinuses. This perfusion route is distinct from that for the neural lobe, which receives its blood supply from the inferior hypophyseal arteries and drains via the inferior hypophyseal veins {Page, 1988}.

It is noteworthy that in some mammalian species (e.g. the bat and ferret, but not the rat) a significant proportion of LHRH fibers project beyond the median eminence into the pituitary stalk to terminate close to the anterior pituitary, or even in the neural lobe {Anthony et al, 1984}; in these species the importance of the portal circulation in debatable.

1.3.3 *LH releasing activity in the hypothalamus*

The presence of LHRH in median eminence extracts was first detected by bioassay {McCann et al, 1960}. A combination of Palkovits' "punch" microdissection technique and radioimmunoassay indicated the presence of LHRH in many hypothalamic nuclei and some extrahypothalamic CNS regions of the rat brain: the highest LHRH concentrations were found in the median eminence (ME), with the next highest levels in the arcuate nucleus and organum vasculosum of the lateral terminalus (OVLT) totaling less than 5% of the amount (concentration) localized to the ME {Selmanoff et al, 1980; Palkovits et al, 1974}.

1.3.4 *LHRH neurons terminating in the ME*

The isolation of the medial basal hypothalamus {Brownstein et al, 1976; Weiner et al, 1975} or anterior hypothalamus {Kalra et al, 1977} from the rest of the brain depletes the ME of most of its LHRH content, suggesting that a majority of the ME LHRH originates outside

the hypothalamus. The use of anti-LHRH antibodies has permitted a more precise localization and morphological characterization of LHRH-containing neurons. In both males and female mammals LHRH cell bodies are small, fusiform in shape, relatively few in number (e.g. 1300-1500 in rat brain {Merchenthaler et al, 1984}) and sparsely distributed, singly or in small clusters {e.g. King et al, 1982; Witkin et al, 1982}. In the rat basal forebrain, polyclonal antibodies against various portions of the LHRH peptide in its unprocessed form detect numerous scattered immunoreactive soma along the midline between the diagonal band of Brocca rostrally and the mammillary bodies caudally, i.e. in the diagonal band of Brocca, organum vasculosum of the lamina terminalis, the preoptic area, and anterior hypothalamus {Setalo et al 1976; Witkin et al 1982; Liposits et al, 1984; Shivers et al 1983; Hiatt et al, 1992}.

On the basis of more recent immunohistological studies, King has proposed an "onion skin model" to describe several distinct populations of LHRH neurons that are differentially activated depending on the endocrine milieu of the rat {see Hiatt et al, 1992}. Along the midline adjacent to the third ventricle, distributed over a roughly prism-shaped area in the rostral preoptic area around the OVLT, is a relatively dense core of LHRH neurons that are consistently detectable. As detectability reflects the presence of supra-threshold quantities of LHRH antigen, changes in detectability are thought to represent physiological differences in the functional state of LHRH neurons. Surrounding this core population of LHRH cells are successive "layers" of more scattered peripheral neuronal subgroups (extending from the diagonal band of Broca to the anterior hypothalamus, and laterally into the diagonal band of Broca, OVLT and preoptic area) which are variably detectable depending on the sex, time after gonadectomy, and stage of the estrous cycle {Hiatt et al, 1992}. In the male rat, there is a depletion of the more "superficial" population(s) of LHRH cells within one day of

castration, while in the longer term (3 week) gonadectomized rats the LHRH cell population expands to exceed that of the intact animals {King & Rubin, 1992}. King's group have interpreted these studies to suggest a functional subgrouping of LHRH neurons, with the more peripheral populations modulating the coordinated release of LHRH from the core subgroup, in response to different endocrine contexts {Hiatt et al, 1992}.

Those LHRH neurons that project to the ME travel along periventricular and lateral pathways that pass via the anterior hypothalamus and retrochiasmatic area. Thirty to fifty percent of the LHRH-immunopositive neurons in and caudal to the diagonal band of Broca do not label for a retrograde tracer injected at the median eminence, and thus probably represent a subset of LHRH-containing cells which project to other regions of the hypothalamus or extrahypothalamic regions of the CNS {Silverman et al, 1987; Merchenthaler et al, 1989}. Interestingly, the single-labelled neurons are neither geographically nor morphologically distinct from those which do project to the median eminence. These double-labelling studies suggest that the LHRH content of the ME originates in part from a variety of rostrally-situated extrahypothalamic nuclei, and are thus consistent with studies in which the deafferentation of the anterior hypothalamus, lesion of the OVLT (but not the diagonal band of Broca-septal region), or the complete isolation of the medial basal hypothalamus caused a decrease in ME LHRH content; {Kalra et al, 1977; Samson & McCann, 1979a; Ibata et al, 1979; Brownstein et al, 1976; Weiner et al, 1975}. Lesions of the medial preoptic area decreased LHRH in the ME only if the midline and ventral structures were destroyed {Ibata et al, 1979; Samson & McCann, 1979a}.

1.4 A PULSE GENERATOR DRIVES LHRH SECRETION

While episodic LHRH release explains the pulsatile nature of serum LH levels, the source of the intermittent circhoral pattern of LHRH release remains an enigma. It has long been postulated that there exists a pulse generator that determines the pattern of episodic release of LHRH and perhaps of other neuronal events as well. In particular it is presumed to control the amplitude and frequency of LHRH pulses and perhaps other parameters such as the slope of the rising edge of LHRH pulses. The pulse generator is thought to be a group of neurons, capable of regular firing in patterns that are determined intrinsically but subject to modulation by external influences. To effect the pulsatile release of LHRH at the level of the median eminence, this body of cells would have to directly activate all contributing LHRH neurons simultaneously, or trigger a spread of excitation through a network of intervening neurons that would ultimately elicit the coordinated release of the decapeptide as a pulse. This latter indirect means of activation could involve a third (non-LHRH) type of neuron to spread the release signal, or may work through LHRH neurons synapsed amongst themselves to effect coordinated release.

The anatomical location and nature of synchronization of the putative pulse generator LH secretion is not yet fully understood. LH secretion is not impaired by radiofrequency or electolytic lesions of the septal region, medial preoptic area, OVLT, or suprachiasmatic nucleus {Samson & McCann, 1979a; Samson & McCann, 1979b; Bishop et al, 1972}. Several lines of evidence suggest that the MBH is necessary for LHRH pulse generation: deafferentation to isolate the mediobasal hypothalamus (MBH; which includes the arcuate and periventricular nuclei) from the rest of the CNS does not disrupt the pulsatile pattern of LH release, whereas separation of the MBH from the retrochiasmatic area, or a lesion that encompasses the arcuate nucleus region, abruptly stop LH secretion {Halasz & Gorski, 1967; Blake & Sawyer, 1974; Krey et al, 1975; Plant et al, 1978; Soper & Weick, 1980}.

However, there are differences in rodent and primate neuroanatomy which should be appreciated before extrapolating anatomical data across species {King & Anthony, 1984; Summerlee, 1986}; e.g. the presence of LHRH-containing neurons in the arcuate nucleus of the rat is not generally accepted {King et al, 1982}, though lesion of this region may nonetheless halt LH release, by disrupting the release of LHRH from fibers passing through this nucleus {Page, 1988}.

Knobil has explored the MBH with multi-tipped electrodes in the rhesus monkey: each volley of "multi-unit activity" (MUA) detected in this region, is faithfully followed by a pulse of LH in the peripheral serum {Wilson et al, 1984}, and the one-to-one correlation of MUA volleys with serum LH pulses is maintained in the presence of agents that decrease pulse frequency such as anesthesia {Wilson et al, 1984}, morphine {Kesner et al, 1986} and α-adrenergic blockers {Kaufman et al, 1985}. These data suggest that the synchrony with which the LHRH neurons release their peptide into the median eminence vasculature is established at the level of the MBH where many LHRH perikarya are found. It does not, however, lead to the identification of the cell(s) that drive this synchronous release. These MUA volleys may represent the release effort of a single LHRH neuron, the firing of a (the?) pacemaker cell of the pulse generator, or the electrical activity of a (non-LHRH?) neuronal system which coordinates the activation of widespread LHRH neurons. As 10% of all terminals on LHRH dendrites and perikarya are LHRH-immunopositive, LHRH neurons themselves may coordinate release of the decapeptide {Leranth et al, 1985; Chen et al, 1989}.

There is some controversy, however, regarding the interpretation of MUA volleys as a faithful representation of the activity of LHRH neurons (or their afferents). This interpretation has been questioned since the midcycle LH surge was thought to be the direct

result of a very high frequency of LHRH portal pulses, and yet a decrease in the frequency of MUA volleys is recorded coincident with the initiation of the natural or estradiol-induced preovulatory LH surge in female monkeys {O'Byrne et al, 1991; Kesner et al, 1987}. However, studies in the ewe show that LHRH concentrations in the portal blood increase to very high levels during at least the initial phase of the LH surge in ewes, and while fluctuations are evident at this time, the concentration profile is more suggestive of continuous high release than of rhythmic pulsatile secretion {Moenter et al, 1990; Moenter et al, 1991}. It seems possible that the pulse-for-pulse relationship in the female is dominant throughout the estrous cycle except during the pre-ovulatory stage when an outpouring of LHRH drives the LH surge {Clarke, 1992}. Thus, it may be hypothesized that the MUA volleys detected in the MBH do indeed represent the activity of the putative pulse generator that determines the pulse frequency of LHRH (and LH) continuously in the male, and during most stages of the cycle in the female, and LHRH neurons rostral to the MBH may provide afferent input that augments the release of the decapeptide when an increase in pulse amplitude or an apulsatile outpouring of LHRH is appropriate. However, in the human female LH pulses occur during the mid-cycle LH surge {Djahanbakhch et al, 1984; Crowley et al, 1985}, generating some confusion due to species differences.

1.5 THE REGULATION OF PULSATILE LHRH RELEASE

The neural circuitry involved in the control of LHRH and LH secretion is complex and not fully understood. However, it is clear on the basis of neuroanatomy alone, that there is potential for a great many afferents to influence the preoptico-tuberal tract of LHRH neurons, and thus the release of LH. Indeed, there are many neuromodulators and hormones

which alter LH release experimentally: e.g. the classical neurotransmitters such as noradrenaline, adrenaline, dopamine, serotonin, histamine, gamma-aminobutyric acid, and acetylcholine; an extensive array of peptide neuromediators including somatostatin, neurotensin, neuropeptide-Y, peptide YY, galanin, thyrotropin-releasing hormone, corticotropin-releasing factor, angiotensin II, vasoactive intestinal polypeptide, and gastrin; as well as the endogenous opioid peptides {see review by Weiner et al, 1988}. Important progress will have been made when the relative importance of endogenous input from each of these modulators has been determined. The role of selected neuromediators thought to be of major physiological significance will be discussed in the subsequent sections.

Many of the neuroendocrine modulators of LH release are thought to work by altering LHRH release from the hypothalamus, though relatively few in vivo studies have assessed the effects of putative regulators on the LHRH release pattern per se, since accurate measurements of portal LHRH concentrations are technically difficult to obtain (especially from small animals). The quantity and variety of neuronal interconnections in the hypothalamus, and the rarity and sparse distribution of LHRH neurons make it difficult to physically identify, access, isolate and culture LHRH neurons, and thus in vitro studies on LHRH cells have been limited mainly to the perfusion of hypothalamic fragments. Wetsal's group {Mellon et al, 1990} recently circumvented this obstacle by obtaining an immortalized LHRH-producing hypothalamic cell line from transgenic mice genetically targeted for tumourigenesis.

1.5.1 *Noradrenergic regulation of LHRH release*

There is anatomical evidence of interactions between noradrenaline (NA) and LHRH neuronal systems: two major NA systems innervate the septal-preoptic-tuberal pathway,

emanating from the midbrain (A1, A2, A5, and A7) and the locus coerulus (A6) and projecting to the medial basal hypothalamus and more rostral regions respectively {Palkovits, 1981; Ungerstedt, 1971; Swanson & Hartman, 1975; Sawyer & Clifton, 1980; for review see Moore & Bloom, 1979}. While LHRH and dopamine ß-hydroxylase staining show an anatomical proximity suggestive of direct synaptic interactions in the diagonal band of Broca, septal region, and medial preoptic area, this was less common in the arcuate nucleus and ME regions suggesting that the most direct action of the noradrenergic afferents upon LHRH release occurs rostral to the ME {Jennes et al, 1982; Hoffman et al, 1982}. In the ME, the majority of NA fibers end in the internal layer, rather than the external layer where LHRH terminals abut the portal vessels, and histofluorescence studies placed monoaminergic terminals near but not immediately adjacent to LHRH terminals, and thus do not support a direct influence (e.g. via axo-axonic contacts) of NA over LHRH release at the ME {McNeill & Sladek, 1978; Ajika, 1980; Füxe & Hökfelt, 1969}.

The role of NA in regulating LH release has been investigated mainly in the ovariectomized rhesus monkey and rat. In early studies Knobil proposed that NA stimulates LH release since α-adrenergic receptor blockers could suppress pulsatile LH release {Bhattacharya et al, 1972}. The same investigators have subsequently observed a reduction in the frequency of MUA volleys in the medial basal hypothalamus in association with the inhibition of LH release by α-adrenergic antagonists {Kaufman et al, 1985} suggesting that NA stimulation is effected at the level of the hypothalamus, i.e. upon LHRH secretion. This hypothesis is supported by recent work with hypothalamic push-pull perfusion cannulae in the monkey and rabbit, which reveal an increase in pulsatile LHRH release following the administration of adrenergic agonists {Terasawa et al, 1988; Ramirez et al, 1986}, or its suppression by α-antagonists {Terasawa et al, 1988; Pau et al, 1989; Gearing & Terasawa,

1991; Pau et al, 1991}. In the rat, pharmacological interventions such as α-receptor blockade {Weick, 1978}, inhibition of NA synthesis {Negro-Vilar et al, 1982; Drouva & Gallo, 1976; Gnodde & Schuiling, 1976}, or chemical lesioning of NA-producing cells {Hancke et al, 1977} resulted in the disruption or suppression of pulsatile LH release.

In the male rat, there is evidence of a positive influence of NA upon LH release, though not all studies support this finding. Pharmacological data suggests that NA may mediate the post-castration rise in LH levels {Ojeda & McCann 1973}, and in vitro LHRH release from ME tissue is stimulated by NA, except in the presence of an α-adrenergic blocker {Negro-Vilar et al, 1979; Ojeda et al, 1982}. On the other hand, while some investigators report that intraventricular NA increases serum LH concentrations, others observe no change {Kamberi et al, 1970; Schneider & McCann 1970}. Nor was LH release affected by noradrenergic denervation which greatly reduced the hypothalamic NA content of the intact and castrated male rats studied by Nicholson and colleagues {Nicholson et al, 1978}, though others noted a temporary decline in LH levels following 6-hydroxydopamine injections into the medial forebrain bundle, or the preoptic area, or the lateral ventricle {Kitchen, 1974; Fenske & Wuttke, 1976}.

Thus, it remains to be determined which positive and negative modulatory roles of NA afferents are physiologically relevant to the portal secretion of LHRH, and whether these actions are exerted directly upon the neurosecretory cells projecting to the median eminence, or are secondary to effects on intermediary NA-responsive neurons.

1.5.2 *Opioid regulation of LH release*

The three major groups of endogenous opioid peptides, endorphins, enkephalins and dynorphins, are produced in separate neurons and have distinct patterns of distribution in the

rat brain. Proopiomelanocortin, the 241 amino acid precursor of ß-endorphin, is produced in large amounts in neurons of the arcuate nucleus, which project to a variety of brain structures including the periventricular nucleus, medial preoptic area, suprachiasmatic nucleus, and lateral ME where ß-endorphin is secreted into the portal blood {Finley et al, 1981a; Emson et al, 1984; Wardlaw et al, 1980}. Proenkephalin is widely distributed throughout the brain with immunoreactive fibers in the hypothalamus including the external layer of the ME {Sar et al, 1978; Wamsley et al, 1980; Finley et al, 1981b}, and is cleaved to yield met-enkephalin and leu-enkephalin. Prodynorphin (or proenkephalin B) is synthesized mainly in the neurons of the paraventricular and arcuate nucleus {Watson et al, 1982} and is found in processes innervating the median eminence and MPOA {Khachaturian et al, 1982; Code & Fallon, 1986}. Thus the anatomical background exists for interactions between the opioid-containing neurons and the LHRH neurosecretory system.

A substantial amount of evidence has accumulated to implicate endogenous opioid peptides in the role of tonic inhibitory modulators of LH release. Though not all studies are in agreement {Takahara et al, 1978; Cicero et al, 1980; Piva et al, 1986}, the majority of experiments in man and in rats have demonstrated that serum LH levels are suppressed by exogenous opiate agonists and elevated following antagonist administration {e.g. Cicero et al, 1976; Bruni et al, 1977; Meites et al, 1979; Morley et al, 1980; Kinoshita et al, 1980}. Intrahypothalamic injections of antisera to the endogenous opioids, ß-endorphin and dynorphin (but not met-enkephalin), will also stimulate LH release, suggesting that one or both of these opioids may be the endogenous agonist(s) for this inhibitory input, though ß-endorphin is the more potent of the two {Schulz et al, 1981}, and has been the focus of most studies to date. Also consistent with an inhibitory effect of opioids on LH secretion is the clinical observation that narcotic addicts with decreased sexual function and hypogonadism also have depressed

gonadotropin levels {Morley, 1981; Delitalia et al, 1983; Grossman et al, 1981; Reid et al, 1981}. While the hormonal effects produced by the administration of morphine or endogenous peptides could represent pharmacological effects, those observed following the administration of specific antagonists such as naloxone or naltrexone, may more closely approximate physiologic effects secondary to alterations in endogenous opiate levels.

The stimulation of LH release by naloxone can be blocked by LHRH antagonists, suggesting that the endogenous opioid peptides exert their inhibitory effects at the level of LHRH release from the hypothalamus {Blank & Roberts, 1982}. Support for this comes from the demonstration that naloxone induces LHRH release from hypothalami in vitro {Rasmussen et al, 1983; Drouva et al, 1981; Rasmussen, 1991}, but does not alter LHRH-induced LH release from pituitary fragments in vivo or in vitro {Cicero et al, 1979; Grossman et al, 1981; Wiesner et al, 1984}, though there is some evidence to the contrary in studies using cultured pituitary cells {Blank et al, 1986; Cacicedo & Franco, 1986}. Endogenous opioids appear to interact with other hypothalamic influences on LH (LHRH) secretion, such as the stimulatory noradrenergic system; in the rat, the opioid-induced attenuation of LH release can be prevented by prior depletion {Kalra, 1981} or blockade {Kalra & Simpkins, 1981} of hypothalamic α-adrenergic systems. The ability of α-adrenergic agonists (clonidine {Kalra & Simpkins, 1981} and noradrenaline and adrenaline {Kalra & Gallo, 1983}) to elicit LH release in a morphine-blocked rat, suggests that endogenous opioid peptides may suppress LH release by limiting stimulatory adrenergic input to LHRH neurons, e.g. via axo-axonal connections in the hypothalamus {Kalra & Crowley, 1982; Van Vugt et al, 1981; Veldhuis et al, 1983a}. However, an interaction with the NA system is apparently not required for endogenous opioid peptides to influence LH secretion, as a secretory response was elicited by naloxone in rats with lesions of the ascending NA tract (Miller et al,

1985}, and co-administration of an opioid antagonist and an adrenergic agonist had a synergistic rather than merely an additive effect on LHRH release {Clough et al, 1990}.

The opioid-induced suppression of LH release appears to be steroid-dependent as endogenous and exogenous opiate modulation of LH release disappears with time following orchidectomy in the immature and mature rat {Cicero et al, 1982; Bhanot & Wilkinson, 1983; Bhanot & Wilkinson, 1984}, and returns with the administration of sufficient amounts of replacement steroids (Tp, DHT, or EB) {Bhanot & Wilkinson, 1984; Gabriel et al, 1986}. The dose-response curve for morphine's acute inhibition of serum LH (but not its antinociceptive effect) is shifted to the right in animals orchidectomized for 31 versus 3-days, increasing the ED50 by 9-fold; thus exogenous opiates are still effective, but are much less potent in "chronically" castrated rats due to a tolerance acquired with time following the gonadectomy {Cicero et al, 1982}.

In the female rhesus monkey, Ferin and colleagues {Ferin, 1989} have correlated high levels of ovarian steroids with maximal ß-endorphin concentrations in the hypophyseal portal blood, and a low frequency of LH pulse secretion {Wehrenberg et al, 1982; Wardlaw et al, 1982}. Gallo observed a naloxone-induced increase in both the frequency and the amplitude of LH pulses at several stages of the rat estrous cycle, and concluded that these effects must stem from a hypothalamic effect of the opioid antagonist, since this treatment did not alter pituitary responsiveness {Babu et al, 1987; Babu et al, 1988a; Babu et al, 1988b}. Naloxone increased the frequency and duration of MUA volleys in the long-term ovariectomized rat {Kimura et al, 1991}, and in ovariectomized monkeys, Knobil and colleagues have directly observed a morphine-induced (and naloxone-reversible) reduction in the frequency of hypothalamic MUA volleys {Kesner et al, 1986}. These observations suggest that high levels of ovarian steroids augment hypothalamic ß-endorphin release, and that this in turn causes a

reduction in LH pulse frequency by slowing the LHRH pulse generator.

Work in the female of various other species indicates that they too are subject to opioid inhibition of LH pulse frequency and amplitude. Naloxone increases both parameters in the adult human {Grossman et al, 1981; Moult et al, 1981; Quigley & Yen, 1980}, sheep {Whisnant et al, 1991}, and rabbits {Younglai et al, 1988; Younglai & Byrne, 1989}. Opioids appear to play the same role(s) in the human male {Grossman et al, 1981; Moult et al, 1981; Ellingboe et al, 1982; Veldhuis et al, 1983b; Delitala et al, 1983}. In contrast to the female rat (see above) there is no data in the literature on the effects of opioids on LH pulse parameters in the male rat, which is the subject of this thesis.

1.5.3 *Neuropeptide Y (NPY) regulation of LHRH release*

In the last 5 years, numerous studies have investigated the regulation of LH and LHRH by NPY, a 36 amino acid peptide, first isolated from porcine brain {Tatemoto et al, 1982}. This neuropeptide is found throughout the rat and primate brain, with the highest concentration of NPY-immunopositive cells in the arcuate nucleus; a few cells are also in proximity to the hypophysial portal veins in the ME {Allen et al, 1983; Chronwall et al, 1985; Khorram et al, 1987a; Sar et al, 1990b}. In both the male and the female rat the administration of NPY elicits an increase in LH secretion {Rodriguez-Sierra et al, 1987; Kalra & Crowley, 1984}, and in the female monkey and rabbit NPY infusion into the MBH increased LHRH release, as measured by push-pull-perfusion {Woller & Terasawa, 1991; Khorram et al, 1987b}. Endogenous NPY and LHRH levels in the hypophysial-portal circulation change in concert throughout the estrous cycle in the rat {Sutton et al, 1988}, and on a pulse-per-pulse basis in the stalk/ME of male and female primates {Woller et al, 1992}. Further evidence of a stimulatory role for the endogenous neuropeptide comes from

experiments in which anti-NPY antisera suppressed the pulse frequency and amplitude of LHRH {Woller et al, 1992} and LH {Kaynard & Spies, 1991} and prevented the steroid-induced LH surge in ovariectomized rats {Sutton et al, 1988}.

The hypothalamic LHRH neuronal system is not the only site of NPY action; studies in the female rat indicate that NPY can also augment LH release induced by exogenous LHRH in vivo {McDonald et al, 1989; Bauer-Dantoin et al, 1991} and in vitro {Crowley et al, 1987}. A stimulatory effect of NPY on basal LH release from pituitary cells in vitro has also been shown by some investigators {McDonald et al, 1985; Crowley & Kalra 1988; Chabot et al, 1988}, but not by others {Kerkerian et al, 1985; Rodriguez-Sierra et al, 1987; Parker et al, 1991}. NPY receptors have been found in both the hypothalamus and the anterior pituitary {Unden et al, 1984}.

Central NPY administration can also inhibit the secretion of LHRH {Khorram et al, 1987b} and LH {McDonald et al, 1985}, and serial sampling studies show that a decline in pulse frequency and amplitude underlie the NPY-induced fall in mean LH levels {McDonald et al, 1989; Kaynard et al, 1990}. Typically, this suppressive effect of NPY has been observed in ovariectomized animals that were not given steroid replacement, whereas the stimulation of LH (LHRH) by NPY occurs in intact or steroid-replaced castrate animal models. Thus it seems that the effect(s) of NPY on LH secretion, like those of noradrenaline and the opioid peptides, are modulated by the gonadal steroid milieu; indeed, the steroid modulation of at least a subpopulation of hypothalamic NPY cells is also suggested by data showing the concentration of [^{3}H]-estradiol by 10-20% of the NPY-immunopositive neurons in the arcuate nucleus {Sar et al, 1990b}. Also, in male rats, testosterone replacement restored castration-depleted NPY concentrations in the arcuate nucleus, ME and ventromedial hypothalamus, consistent with the hypothesis that steroid-responsive NPY neurons in these

regions may constitute a local excitatory circuit that augments LHRH (and NPY) release into the portal system {Kalra et al, 1988}.

1.5.4 *Dopaminergic regulation of LHRH release*

Dopaminergic perikarya in the rostral periventricular nuclei (A14), the mesencephalic dopamine (DA) groups (A8, A9, A10), and the periventricular-arcuate nucleus (A12) project to the septal-preoptic-tuberal LHRH tract {Füxe & Hökfelt, 1969; Day et al, 1980; for review see Palkovits, 1981}. Unlike the noradrenergic afferents, many DA axons terminate in the external layer of the ME, some in close apposition to LHRH neurons and to the portal capillary system {Kizer et al, 1976; McNeil & Sladek, 1978; Palkovits, 1981}. Intraventricular DA administration augmented LH secretion in vivo {Schneider & McCann, 1970; Kamberi et al, 1969}, as well as from hypothalamic tissues of testes-intact male rats in vitro, and the latter effect could be blocked by treatment with a DA antagonist {Schneider & McCann, 1969; Bennett et al, 1975; Rotsztejn et al, 1977; Negro-Vilar et al, 1979}. In conflict with these studies implying that DA can acutely stimulate LHRH release from the ME, hypothalamic/ME tissue from the castrated male rat failed to release LHRH in response to DA in vitro {Rotsztejn et al, 1977; Negro-Vilar et al, 1979}, and the post-castration hypersecretion of LH lagged several hours behind an increase in DA turnover in the arcuate nucleus and ME {DePaolo et al, 1982}. A dissociation between DA turnover and LH (LHRH) secretion was also noted in the MPOA of castrated rats (the terminal bed of periventricular DA cells) where a decrease in DA turnover can be elicited by intracranial androgen treatment, but is not associated with altered LH secretion {Simpkins et al, 1980a; Simpkins et al, 1980b; Simpkins et al, 1983}. On the other hand, DA turnover increased in terminals originating in the tubero-infundibular tract, in association with the testosterone-

induced suppression of high post-castration serum LH levels and this is suggestive of an inhibitory effect of DA {Simpkins et al, 1980b; Simpkins et al, 1983}. Thus, the data indicate that DA is involved at least as a modulator of LH (LHRH) release, though further studies are required to characterize the positive and negative influences of the dopaminergic afferents.

2 LUTEINIZING HORMONE

2.1 HISTORICAL CONTEXT

Physically, the pituitary gland, or hypophysis, is divided into an anterior (pars distalis) and posterior lobe (comprising the pars nervosa and the pars intermedia), both adjoined to the base of the brain at the median eminence by the pituitary stalk. While both lobes secrete a number of hormones into the peripheral circulation, each accomplishes this in a different manner.

The concept of the pituitary as a hormone-secreting tissue awaited technological and conceptual developments of the late 19th and early 20th century, when improved microscopes and staining procedures revealed the structural specialization of the glandular anterior pituitary for "internal" secretions, and the discovery of secretin established that molecules released into the circulation could act as blood-borne messengers for specific distal tissues. Anatomical studies had also revealed the highly vascularized nature of the anterior lobe, as well as the neuronal link between the hypothalamus and the posterior lobe of the pituitary, leading Cushing to propose that pituitary substances were carried to the brain via the circulatory or neuronal linkages, and had a role in the regulation of brain function. This hypothesis was abandoned after Wislocki and King reported that intravascular dyes that stained the pituitary heavily did not penetrate beyond the median eminence, and correctly surmised that the direction of blood flow was "downward" from the brain toward the pituitary. This was subsequently confirmed by direct observation in a number of species {for review see Everett, 1988}.

The initial association of pituitary hormones with reproductive processes followed

from studies in which the administration of bovine anterior pituitary tissue or extract produced ovarian enlargement and estrous cycle suppression in intact female rats, and effected gonadal repair and maintainance in rats that were hypophysectomized or treated with suppressive doses of sex steroids {Moore & Price, 1932}. Meanwhile ablation and replacement studies had indicated that in addition to hormonal influences on the gonads, the anterior pituitary contained several other activities controlling body growth, mammary glands, thyroids, and the adrenal cortex. Eventually, as six peptide hormones were isolated from the anterior pituitary and characterized chemically, and LH and FSH were identified as the activities acting on the gonads, i.e. the gonadotropins {see Page, 1988 for review}. The major effect of LH on the testis is to stimulate testosterone production by the Leydig cells.

2.2 ORGANIZATION OF THE PITUITARY

Embryologically, the neurohypophysis originates from a downward-growing process that develops on the floor of the diencephalon, and this tissue retains its neuronal identity into adulthood. Nerves arising in the hypothalamic supraoptic and paraventricular nuclei course through the median eminence and down the pituitary stalk to the pars nervosa, forming the hypothalamo-hypophyseal tract. The infundibular stem is that portion of the stalk that carries these axons to the infundibular process (pars nervosa, neural lobe) of the posterior lobe. Together, the median eminence, infundibular stem, and pars nervosa comprise the neurohypophysis.

Hormones synthesized in the hypothalamic soma of these nerves are secreted from nerve terminals in the pars nervosa, and reach the circulation after passing through the

extracellular and perivascular space into fenestrated capillaries. These secreted hormones (e.g. neurophysins, oxytocin, vasopressin (also known as arginine vasopressin AVP, and antidiuretic hormone ADH)) regulate lactation, uterine contractions, and urine volume.

The pars tuberalis is that portion of the pituitary stalk which connects the pars distalis (anterior lobe) to the median eminence; together the pars tuberalis, pars distalis, and pars intermedia are known as the adenohypophysis (glandular lobe). With the exception of the pars intermedia, the adenohypophysis is embryologically derived from Rathke's pouch, a dorsal outpocketing of the roof of the mouth.

The cells of the anterior pituitary are clustered in close proximity to the thin-walled sinusoids of the vascular system. Most of the cells in the anterior lobe are chromophobic (50%), and are thought to be precursors of the chromophilic cells, which may be subdivided into acidophils (40%) and basophils (10%) according to their dye staining properties {for review see Baker, 1974}. The acidophils of the pars distalis are somatotropes and lactotropes which secrete growth hormone and prolactin, respectively, into the circulation, whereas the basophilic cells include the thyrotropes and corticotropes which secrete thyroid-stimulating hormone and proopiomelanocortin-derived hormones, respectively, and the gonadotropes which secrete both LH and FSH. Light and electron microscope studies indicate that LH- and FSH- immunoreactivities are co-localized in most gonadotropes, though some do appear to be monohormonal {Baker et al, 1972; Herbert, 1975; Moriarty, 1976}.

2.3 THE GONADOTROPE

The effects of LHRH upon LH synthesis and release from the gonadotropes are mediated through a membrane bound receptor, and are subject to modulation by the testicular

steroids. Exogenous androgens and estradiol alter the ability of the pituitary to secrete LH in response to an LHRH pulse, but it is not clear whether the steroids affect these actions via the LHRH receptor or post-receptor mechanisms of signal transduction. In the adult male rat there are both estrogen and androgen receptors in the anterior pituitary {Kato, 1975; Jouan et al, 1973; Korach & Muldoon, 1974; Sar et al, 1990b}, though the aromatase system that converts testosterone to estradiol is apparently not present {Naftolin et al, 1972; Kato, 1975; Naftolin & Ryan, 1975}, and circulating estradiol levels are so low in the intact male as to suggest that their impact is small compared to that of testosterone {deJong et al, 1973}, notwithstanding the higher relative activity of estradiol (affinity 10^{-10}M) which augments the physiological significance of small amounts of the estrogen {Weichman & Notides, 1977}.

2.3.1 *The LHRH receptor*

The LHRH receptor is a plasma membrane bound {Marian & Conn, 1983} 60 kD glycoprotein {Hazum, 1982} found on the surface of 90-100% of gonadotropes, but not on other cell types in the anterior pituitary {Childs et al, 1983a; Childs et al, 1983b}. Until high-affinity agonist binding triggers receptor cross-linking, clustering, and endocytosis, these receptors are evenly distributed on the gonadotrope cell surface {Hopkins & Gregory, 1977; Hazum et al, 1980}. While receptor cross-linking is correlated with ligand potency (with respect to LH release) {Conn et al, 1982a; Conn et al, 1982b; Gregory et al, 1982}, the processes of clustering and endocytosis are apparently neither sufficient nor necessary for LHRH-induced LH secretion, as both events also occur following exposure to antagonists {Suarez-Quian et al, 1986; Jennes et al, 1984}, and may be prevented (e.g. by linking the agonist to uninternalizable beads) without inhibiting LHRH action {Conn et al, 1981; Conn & Hazum, 1981}. Endocytosed photo-affinity labelled ligand-receptor complexes have been

found in lysosome-like organelles, the Golgi complex, and in secretory granule membranes, where they are presumably subject to degradation, or recycled back to the cell surface {Schvartz & Hazum, 1987}. Interestingly, the fate of the internalized ligand-receptor complex seems to depend upon the nature of the ligand, as antagonist conjugates have not been detected in the Golgi apparatus but do appear in lysosomes {Jennes et al, 1986}.

Homologous up-regulation of the LHRH receptor occurs within 8-24 h after exposure to low concentrations of LHRH, and appears to be a calcium stimulated and protein-synthesis-requiring process {Conn et al, 1984; Loumaye & Catt, 1983}. In the model of LHRH action proposed by Huckle and Conn {Huckle & Conn, 1988}, the calmodulin and protein kinase C pathways are also involved in receptor up-regulation, and mediate the effects of LHRH on gonadotropin release and biosynthesis respectively. The sensitization, or "self-priming" of gonadotropes {Pickering & Fink, 1976; Waring & Turgeon, 1980} occurs within a time period insufficient for measurable receptor up-regulation, suggesting that it is post-receptor factors that govern the self-priming action of LHRH.

2.3.2 *Luteinizing hormone - the molecule*

LH was originally named luteinizing hormone in recognition of its role in female mammals in eliciting ovulation and luteolysis. In the male the same glycoprotein stimulates androgen production by interstitial (Leydig) cells of the testes, and hence its other name interstitial cell-stimulating hormone (ICSH). In both sexes of a given species the primary sequence of LH is the same. In the rat, distinct genes encode two peptides, α and ß peptides of 96 and 121 amino acids respectively, which are glycosylated and combine to form a heterodimer {Burnside et al, 1988; Chin et al, 1983; for review see Gharib et al, 1990}.

The dimer construction of LH

The α/ß dimer construction of LH closely resembles that of two other adenohypophyseal hormones, FSH and TSH, as well as of the placental hormone, chorionic gonadotropin (CG). The non-covalently linked α and ß subunits impart species and biological specificity respectively. The coding region of the α-subunit shares a high degree of similarity (70-90%) among several species (e.g. human, cow, rat and mouse) though the variable length of the first intron (range 5.4 - 13 kb) introduces considerable size differences between the full-length α-subunit genes {Gharib et al, 1990}. Within a species, the primary amino acid and corresponding nucleotide sequences of both the placental and pituitary α-subunits are identical, though the presence of different upstream regulatory elements indicates a tissue-dependent manner of α-subunit gene expression {Delegeane et al, 1987; Ocran et al, 1990; Kendall et al, 1991}. The distinct target tissue responses elicited by LH, FSH, and TSH are attributed to the receptor specificity imparted by their dissimilar ß-subunits (homologies of 30-40%), while the structural similarity of LH and CG ß-subunits (80% homology at the amino acid level) permits these α/ß complexes to interact with the same receptor, and thus underlies their functional similarity {Fiddes & Goodman, 1980; for review see Pierce & Parsons, 1981}. Though the dimer conformation that imparts receptor specificity is determined by the ß-subunit, the importance of the α-subunit for binding is indicated by the fact that as monomers neither peptide subunit alone shows more than weak receptor binding activity {Mougdal & Li, 1982; Reichert et al, 1973}. Recently, the transcription and translation of LH α- and ß-subunits have been the focus of many studies on the modulation of LH release at the pituitary level. As the α-subunit is generally present in much greater abundance than LH-ß, control of LH synthesis is thought to be achieved by regulating the amount of ß-subunit available for dimerization {Blackman et al, 1978; Kourides et al, 1980a; Hoshina & Boime,

1982; Vogel et al, 1989}. While LHRH stimulation may induce the release of the free (undimerized) α-subunit in addition to the assembled LH hormone, LH-ß is not found outside the pituitary except in dimer form {Hoshina & Boime, 1982}.

Glycosylation

Each LH subunit contains five or six intrachain disulfide bonds and is glycosylated before (non-covalent) dimer assembly in the endoplasmic reticulum. Subsequently, extensive oligosaccharide processing in the endoplasmic reticulum and Golgi apparatus yields the secreted form of the dimer {Ryan et al, 1987; Sairam, 1989}. The two N-linked oligosaccharide groups on the α-subunit and the single carbohydrate moiety on LH-ß constitute approximately 16% of the 28 kD weight of LH; additional carbohydrate moieties on FSH-ß give the hormone a higher weight (approximately 33 kD) {Parsons & Pierce, 1981}. There is evidence that both the nature and the degree of dimer glycosylation have functional implications. A high sialic acid content in glycoproteins slows their clearance from the circulation, and this is thought to account for the progressively longer half-lifes of LH (2% sialic acid), FSH (5% sialic acid) and human CG (10% sialic acid) as desialylation results in rapid in vivo clearance {Tsuruhara et al, 1972; Morell et al, 1971; Moyle et al, 1975}. O-linked sugars found on the free α-subunit in the pituitary and placenta, alter subunit stability and prevent dimerization and thus function {Kourides et al, 1980b; Parsons et al, 1983; Cole et al, 1984}; it has been hypothesized that the regulation of O-glycosylation provides an indirect means of regulating hormone function {Corless & Boime, 1985}. In addition it has been shown in both in vitro and in vivo studies that while a sugar-free hormone can still bind its receptor, even with enhanced affinity, it cannot elicit a cellular response, and thus it acts as a competitive antagonist in the presence of the functional glycosylated form of the hormone

{Moyle et al, 1975; Channing et al, 1978; Sairam & Bhargavi, 1985}. Physiological variations in the degree of glycosylation might thus yield dimers of lesser potency (partial agonists) and this could constitute a means of modulating hormone activity. Indeed, there are multiple forms of differentially glycosylated gonadotropins in the pituitary and serum {e.g. Bogdanove et al, 1974; Graesslin et al, 1976; Robertson et al, 1977}, and the presence and relative quantity of these species of varying biological and immunological activity (at least in the pituitary) appears to depend on the endocrine milieu in vivo {e.g. Peckham & Knobil, 1976; Robertson et al, 1982}.

2.3.3 *LH pools*

The releasable pool of LH, defined as that portion of the pituitary LH content which is released upon acute challenge with a supramaximal dose of LHRH, represents 2 - 8% of total pituitary LH content in the intact female rat, except during the LH surge of the female when it is as high as 40%; changes in the size of the releasable pool do not correlate with pituitary LH content {Pickering & Fink, 1979}. Continuous incubation of hemi-pituitaries from male rats with LHRH releases LH that is of greater biopotency than that remaining in the pituitary, and it has been suggested that this may be attributed to differences in the carbohydrate content between the two pools {Mukhopadhyay et al, 1979; Sharpe et al, 1975}. It has been shown that LHRH can increase the rate and degree of LH glycosylation {Liu & Jackson, 1978; Vogel et al, 1986; Starzec et al, 1986}, as well as shorten the delay between LH subunit synthesis and release {Vogel et al, 1989}, but it is not known whether these biochemical changes are associated with the movement of LH into the releasable pool, or simply with a change in the degree of glycosylation (and thus biopotency) within the releasable pool. Processes leading up to the transfer and differential glycosylation of LH

molecules may be important sites of regulation.

The gonadotrope population can also be divided by centrifugal elutriation into subpopulations with different size/density characteristics. Childs and colleagues {Childs et al, 1992a, 1992b} have hypothesized that the smaller LH-immunopositive cells are relatively inactive with respect to LH secretion, but mature into medium or large LH-releasing cells in preparation for the LH surge in the female rat. They have shown that, at different stages of the estrous cycle of the female rat, there are indeed shifts in the number of anterior pituitary cells in each size/density group, and in the number of cells per group that stained for LH mRNA {Childs et al, 1992a, 1992b}. While these data loosely support their proposal that shifts occur between the gonadotrope pools as the amount of LH secreted changes with cycle stage, and indeed are not inconsistent with a conversion of small LH-containing cells to large gonadotropes in preparation for the midcycle LH surge, the hypothesis needs to be adjusted to account for observations such as the maximally intense LH staining apparent during estrous, i.e. just after maximal LH release {Childs et al, 1992a}.

2.4 PULSATILE LH RELEASE

2.4.1 *LH secretory patterns*

It is clear from the literature that adult male mammals secrete LH in a pulsatile manner. This has been documented by serial sampling studies in ferrets {Sisk & Desjardins, 1986}, monkeys {Steiner et al, 1980; Plant, 1982}, sheep {Sanford et al, 1974; D'Occhio et al, 1982}, bulls {Katongole et al, 1971}, mice {Coquelin & Desjardins, 1982}, rabbits {Rowe et al, 1975}, rats {Ellis & Desjardins, 1982; Steiner et al, 1982} and man {Naftolin et al, 1973; Santen & Bardin, 1973}. The normal (testes-intact) adult male rat has basal LH

concentrations near the limit of detectability, interrupted by low amplitude LH pulses occurring irregularly, with interpulse intervals as brief as 30 minutes and sometimes more than 12 h (roughly 2.5 h is the average among those animals having at least 2 pulses per 8-12 hour sampling session) {Ellis & Desjardins, 1982; Steiner et al, 1982}. The rising edge of an LH pulse is relatively steep, typically achieving peak levels in 5-10 minutes, while 50-70 minutes may be required for the complete decline of LH levels after a secretory burst {Desjardins, 1981; Ellis & Desjardins, 1982; Steiner et al, 1982}. This rise and decay pattern can be seen clearly in the male of many mammalian species {Santen & Bardin, 1973; Schanbacher, 1984; Sisk, 1987}.

The majority of data available suggests that the intermittent bursts of LHRH, released from the hypothalamus into the hypophysial portal blood, drive the release of LH pulses. The most convincing support for this hypothesis comes from the many reports of close temporal associations between LHRH and LH pulses (see section 1.2.1), and experiments in which the intermittent administration of exogenous LHRH restores pulsatile LH secretion to animal models in which endogenous LHRH release has been neutralized {e.g. Nansel & Trent, 1979; Wildt et al, 1981; Clarke & Cummins, 1984}.

Though a pulsatile pattern of LH release from unstimulated fetal anterior pituitary cells in vitro has been reported {Gambacciani et al, 1987}, in most in vitro studies using anterior pituitary cells, LH is below or barely above the assay detection limit (in the absence of secretagogues), suggesting that the unstimulated pituitary secretes minimal amounts of LH {Schally et al, 1973; Kamel et al, 1987a; McIntosh & McIntosh, 1983}. It is possible, however, that in vitro experiments overestimate endogenous basal LH release due to non-physiological release induced by sample preparation and in vitro conditions. Between LH pulses in intact animals, there is a basal level of LH secretion which persists near or drops

below the assay detection limit following the loss of pulsatile LH secretion due to hypothalamic lesion {Soper & Weick, 1980}, or to the disruption of the neurosecretory link in hypothalamo-pituitary disconnected sheep {Clarke et al, 1983; Tilbrook et al, 1991}, in rats with their pituitaries grafted to a distal position {Strobl et al, 1989}, or in animals treated with antiserum to LHRH {Ellis et al, 1983; Koch et al, 1973; Lincoln & Fraser, 1990}. Thus it seems that the pituitary is intrinsically able to release low levels of LH, but owes the pulsatile aspect of the LH secretory pattern to episodic LHRH release.

2.4.2 *Physiological determinants of LH pulse frequency and amplitude*

The frequency of LH pulses is determined by the frequency of hypothalamic MUA volleys which drive the pulsatile release of LHRH (see section 1.2.2), and by pituitary sensitivity. Typically there is a one-to-one relationship between LHRH and LH pulses, though LHRH pulses may be "silent" (not associated with LH pulses) if they are of such low amplitude that their peak concentrations do not exceed the detection threshold of the gonadotropes {Levine & Duffy, 1988; Urbanski et al, 1988; Ramirez et al, 1991}. Generally, however, caution is advised in the interpretation of silent LHRH pulses, as their number may be overestimated due to false-negative errors in the identification of LH pulses, i.e. if the peaks in LH secretion evoked by the putatively silent LHRH pulses, are too short to achieve statistical significance in the pulse-detection protocol.

In general, it may be said that the magnitude of an LHRH pulse is reflected in the amplitude of the LH pulse it elicits {Rebar et al, 1973; McIntosh & McIntosh, 1983; Kamel et al, 1987b}, but this amplitude relationship can be modified by influences such as the shape of the LHRH pulse {McIntosh & McIntosh, 1983; Handelsman et al, 1988}, the frequency of LHRH pulses, and the endocrine milieu which may alter pituitary responsiveness. Changes in

pituitary responsiveness are recognized experimentally, when there is a difference between treatment groups in the magnitude of the acute LH increment elicited by an exogenous LHRH bolus of constant size; the physiological basis for such differences in responsiveness includes changes in LHRH receptor number (but not affinity) as well as post-receptor mechanisms of LH secretion {Garcia et al, 1984; Clayton & Catt, 1981b; Frager et al, 1981}. Studies in vitro and in LHRH-deficient animal models have established that changes in pituitary responsiveness can be effected directly at the level of the pituitary {e.g. Kao & Weisz, 1975; Cheung & Davidson, 1977; Strobl et al, 1989}, or indirectly, by modulating LHRH pulse frequency, which is inversely related to LH pulse amplitude {Wildt et al, 1981; Clarke et al, 1984; Katt et al, 1985; Finklestein et al, 1988; McIntosh & McIntosh, 1983; Kamel et al, 1987b; Lambalk et al, 1987}. Thus, regulators of LH pulse amplitude can exert their effect at the level of the hypothalamus, by altering LHRH pulse amplitude or frequency, or at the level of the pituitary itself.

TESTICULAR ANDROGENS

Early investigators of the gonadal influence over pituitary function noted that castration caused pituitary enlargement due to the formation of vacuolated "castration cells," which were characterized as basophils and later as gonadotropin-secreting cells. The first clue to the steroidal nature of the gonadal substance(s) was in the reversal of this phenomenon and inhibition of other pituitary-mediated reproductive events by the administration of lipid extracts of gonadal tissue. In the 1930's it was shown that testosterone was the testicular factor that caused growth of sex accessory tissues in the male, and soon after it was identified as the gonadal steroid capable of inhibiting pituitary function in the male {Moore & Price, 1932; see Eik-Nes, 1975}.

3.1 THE ORGANIZATION OF THE TESTIS

The adult mammalian testis has two main functions: spermatogenesis and androgen production, which take place within the avascular seminiferous tubules and the vascularized interstitium, respectively. The closely packed, looped seminiferous tubules are comprised of germ cells and Sertoli cells, enclosed by a basement membrane and layers of myoid cells. Sertoli-Sertoli cell tight junctions separate the spermatogonia and early spermatocytes from spermatozoa and the luminal environment; also outside this "blood-testis barrier" are Leydig cells, blood and lymphatic vessels, macrophages and loose connective tissue which, altogether, comprise 10-60% of testicular volume depending on the species; in the male rat, the clusters of Leydig cells occupy roughly 5% of the testicular volume and are surrounded by peritubular lymphatic sinusoids {Fawcett et al, 1973}. Steroids produced in the Leydig

cells are carried away in the blood stream, the lymph, or the luminal fluid of the seminiferous tubules.

Throughout adulthood, waves of primordial germ cells, initially poised at the circumference of the seminiferous tubules, develop into spermatozoa as they advance through a layer of Sertoli "nurse" cells toward the fluid-filled lumen of the tubule. The luminal sperm then flow out the end of the tubule loop and are channeled by the rete testis and efferent ducts into the convoluted epididymal tubule where they mature while in transit and then remain stored in the cauda epididymidis until ejaculation. Accessory organs, such as the seminal vesicles, Cowper's gland (absent from the rat), and prostate gland contribute to the volume and content of the semen before it exits via the urethra. In the adult male human, Leydig cells play an important role locally, providing androgen support for sperm development in the adjacent seminiferous tubules and maturation in the epididymides, and more distally, in maintaining the sex accessory tissues and effecting male sexual behaviour at the CNS level.

3.2 TESTOSTERONE SYNTHESIS IN THE TESTIS

While a variety of steroids are synthesized and released by the Leydig cells their major secretory product is testosterone (T) (serum production of 7 ng/day), and it contributes >95% of serum T in the human male {Eik-Nes & Hall, 1965; Lipsett et al, 1966}. The other site of steroidogenesis in the male is the adrenal gland cortex, which produces mainly glucocorticoids and mineralocorticoids, though a small percentage of its basal steroid production is of androgens (mainly androstenedione in rats, mainly dehydroepiandrosterone in humans) which may be converted to T in the periphery {Coffey, 1988}. Where the details of testicular testosterone production have been investigated, they have been found to be similar

to the equivalent steroidogenic processes in the adrenal cortex, though steroidogenesis in the latter tissue has been more thoroughly characterized.

The steroid precursor of the androgens, cholesterol, is from one of four sources: 1) plasma lipoproteins, 2) cholesterol esters stored within the Leydig cell, 3) cholesterol present in the inner and outer mitochondrial membranes, and 4) de novo synthesis in situ from two-carbon acetate precursor molecules. The cholesterol available in the inner mitochondrial membrane is significantly depleted (to $<40\%$) within 2.5 min of the the application of steroidogenic stimuli, at least in the bovine adrenal gland, and thus it has been suggested that this source of substrate is used in the most immediate response to steroidogenic stimuli {Cheng & Kimura, 1985}.

Whereas the first (cholesterol - pregnenolone) and last (androstenedione - testosterone) reactions are common to two pathways, the intermediates between pregnenolone and androstenedione may initially retain the double bond in the original Δ^5 position (between C5 and C6 in the B-ring) until the penultimate step (Δ^5 pathway), or undergo isomerization of the double bond to the Δ^4 position (between C4 and C5 in the A-ring) directly after pregnenolone formation (Δ^4 pathway) {for review see Hall, 1988}.

The 27-carbon cholesterol molecule has an 8-carbon aliphatic sidechain in the C17 position of the perhydrocyclopentanophenanthrene steroid nucleus. The first step in androgen synthesis, the removal of all but two carbons of the sidechain from cholesterol to yield pregnenolone, is catalyzed by the cytochrome "P450scc" enzyme in the inner mitochondrial membrane (scc=side chain cleavage). In the testes, the remaining sidechain carbons are cleaved in the second of a two-step process catalyzed by the 17α-hydroxylation and 17,20-lyase activities of the "P450c17" enzyme in the endoplasmic reticulum, to yield (17-keto) dehydroepiandrosterone (DHEA), via the Δ^5 path; this is unlike adrenal steroidogenesis in

which virtually all of the 17α-hydroxylated intermediates of P450c17 retain their 2-carbon sidechain and are converted to 21-carbon corticosteroids. The 3ß-hydroxysteroid dehydrogenase and Δ^5-Δ^4 isomerase activities are both found in one microsomal enzyme, known simply as the 3ß-hydroxysteroid dehydrogenase enzyme (3ß-HSD), which can act on the substrate (pregnenolone), intermediate (17α-hydroxypregnenolone), or product (DHEA) of the P450c17 reaction to yield progesterone, 17α-hydroxyprogesterone, or androstenedione respectively. The 17ß-hydroxysteroid dehydrogenase enzyme (17ß-HSD) that converts the 17-keto group of androstenedione to the hydroxyl function of testosterone, is also a microsomal enzyme {Hall, 1988}. Thus, most of the reactions converting cholesterol to testosterone occur at the smooth endoplasmic reticulum; indeed the testosterone-producing capacity of the Leydig cell is proportional to the surface area of the smooth endoplasmic reticulum {Zirkin et al, 1980}. A notable exception to this localization of steroidogenic proteins is in the first step in cholesterol metabolism, i.e. sidechain cleavage, which occurs in the mitochondrial inner membrane {Christensen, 1975}.

3.2.1 *Other steroids produced in the testes*

The human and rat male gonads contain a number of other steroid-metabolizing enzymes, such as the 11ß- and 16α- and 21- hydroxylase activities, and 20α-hydroxysteroid dehydrogenase. No physiological role has been assigned for the steroid products of these various enzymatic activities, though it has been suggested that the reduction of testicular 17α-hydroxyprogesterone at carbon 20 yields an inhibitor of 17,20-lyase activity, and thus 20α-hydroxysteroid dehydrogenase may regulate testosterone production {Eik-Nes, 1975; Shikita & Tamaoki, 1965}. Some of the intermediates in T synthesis are secreted from the testes, as are those steroids produced by further testicular metabolism, though only in minor quantities

relative to T {Christensen, 1975}. The aromatase and 5α-reductase activities of the testes contribute to the serum levels of the two major active T metabolites, estradiol and dihydrotestosterone (DHT) (2 and 350 pg/ml respectively {de Jong et al, 1973; Coyotupa et al, 1973}), thougl. these steroids never amount to more than one-tenth the circulating T levels. Dehydroepiandrosterone and androstenedione of adrenal origin may also be converted to DHT, and peripheral conversion of minor amounts of serum androstendione (<2%) and T (<1%) together contribute a total of 75-90% of the total peripheral estrogens present in the young human male, catalyzed mainly by the aromatase present in adipose tissue. The low levels of circulating estrogens present in the male are of undetermined importance.

3.3 TESTOSTERONE SYNTHESIS DRIVES SECRETION

It is generally assumed that free (unconjugated) steroids present in the Leydig cells are not stored, but diffuse into the circulation in amounts that roughly parallel their unbound intracellular concentrations. This is in contrast to the strategy of vesicular storage employed to regulate the post-synthesis release of protein and peptide hormones and neurotransmitters; it is generally believed that such membrane-dependent compartmentalization could not similarly restrict the movement of lipophilic compounds such as unconjugated steroids. Thus steroid release rate is a reflection of synthesis rate, concentration gradient, (which is influenced by the presence of binding proteins and testicular blood and lymph flow), and the cytoplasm/membrane distribution for each steroid. Acute changes in serum T concentration are effected primarily by the LH-mediated regulation of its synthesis , though the contribution of temporal and spatial variations in local blood flow and in the permeability of the

microvasculature may be an important mechanism by which local cellular products can influence regional T concentrations {Eik-Nes, 1975; Desjardins, 1989}. The distribution of newly synthesized T between venous serum and reproductive tract fluid may also be affected by the concentrations of steroid-binding proteins in the serum (e.g. albumin in all mammals, and sex-hormone binding globulin in mammals other than the rat), and in adjacent tissues (e.g. the androgen-binding protein produced in the Sertoli cells) {Eik-Nes, 1975}.

3.4 THE LH RECEPTOR

Pulses of LH released from the pituitary bind specifically and with high affinity to membrane bound receptors on the Leydig cells, and the testes responds within 3 to 6 minutes with an increased rate of testosterone release {Eik-Nes, 1967}. Cloning of the LH receptor has shown it to be a single 674 amino acid (93 kD) polypeptide composed of two domains of roughly equal size; the carboxy-terminal domain contains 7 hydrophobic regions which are thought to span the cell surface membrane, and cytoplasmic regions with phosphorylation sites implicated in regulatory roles, and thus suggests that this protein is a member of the G-protein coupled family of receptors {McFarland et al, 1989; Loosfelt et al, 1989; Minegish et al, 1990; for review see Segaloff et al, 1990}. The glycosylated extracellular amino terminal is unusually large (341 amino acids; 50-60 kDa) {McFarland et al, 1989; Rodriguez & Segaloff, 1990}.

LH (or hCG) binding triggers receptor aggregation and the internalization of the receptor complex {Podesta et al, 1986; Conn et al, 1978; Ascoli, 1982}; ligand-receptor binding is followed by activation of adenylate cyclase and increased formation of cAMP and

T. Under normal conditions, only a small fraction of LH receptors need be occupied to evoke maximal testosterone synthesis. This indicates that Leydig cells contain "spare" receptors {Catt & Dufau, 1973}, and implies that, following LH-induced desensitization, LH responsiveness could recover before the full complement of receptors does. cAMP-dependent protein kinases are thought to regulate the activity of proteins in the steroidogenic pathway, and thus the production of testosterone {Dufau et al, 1977}. Though cAMP appears to be the primary second messenger mediating the steroidogenic response to LH stimulation, and is certainly the best studied second messenger system to date, there is also evidence that mediaters such as arachidonic acid, calcium and inositol phosphates may be involved {Lowitt et al, 1982; Gudermann et al, 1992; see Cooke et al, 1992}.

While a number of different hormones, growth factors and second messengers modulate the responsiveness of the steroidogenic cell to an LH stimulus, the most important regulator appears to be LH itself. Leydig cell stimulation by elevated LH levels induces a time- and dose- dependent desensitization of steroidogenesis to further hormone exposure, due to a loss of cell surface LH receptors (down-regulation; cAMP-reversible) {e.g. Tsuruhara et al, 1977; Saez et al, 1978; Freeman & Ascoli, 1981} and/or an uncoupling or altered function of the G-proteins that mediate the stimulation of adenylate cyclase (also cAMP reversible) {Freeman & Ascoli, 1981; Dix et al, 1982; Rebois & Fishman, 1986; Ekstrom & Hunzicker-Dunn, 1989}. Additional mechanisms must also be involved, since Leydig cells desensitized to LH/hCG can also become resistant to the steroidogenic effects of exogenous cAMP {Tsuruhara et al, 1977; Rebois & Patel, 1985; Pereira et al, 1988}, and since T synthesis may remain depressed even after the ability of Leydig cells to respond to LH with a rise of cAMP has recovered, indicating that large doses of LH down-regulate Leydig cell activity beyond the generation of this cyclic nucleotide {Davies et al, 1978; Dufau & Catt, 1978}. Receptor

down-regulation can be distinguished from post-receptor mechanisms of desensitization by its time-course, and its requirement for protein synthesis {Dix & Cooke, 1981}. While the majority of these studies utilize protocols involving the prolonged exposure of steroidogenic cells to high (pharmacological) levels of LH or hCG in vitro, Leydig cell desensitzation (with respect to both cAMP and T production) and LH receptor down-regulation have also been observed in in vivo and in vitro studies using physiological and sub-physiological concentrations of LH, suggesting that these phenomema may indeed be physiologically relevant, and possibly necessary to maintain the normal response of Leydig cells to LH in vivo {Hsueh et al, 1977; Habberfield et al, 1987; see also Catt et al, 1979}. Ligand-induced decreases in LH receptor binding are preceded by a decline in the abundance of receptor mRNA transcripts, suggesting that down-regulation is achieved in part by reducing receptor synthesis {LaPolt et al, 1991}.

3.5 TESTOSTERONE SECRETORY PATTERNS

The importance of the discontinuous hormonal signals in communication is suggested not only by the abundance of hormones for which pulsatile serum patterns have been reported {Tannenbaum & Martin, 1976; Willoughby et al, 1977; Culler & Negro-Vilar, 1986}, but also by the observation that sustained hormonal signals can shut down, as opposed to stimulate, target cells {Wildt et al, 1981}. The physiological importance of fluctuations in testosterone levels has been implied by studies in rats and rabbits, in which intact males showed suppressed spermatogenesis and LH levels in response to a quantitatively physiological but non-fluctuating serum testosterone concentration {Stratton et al, 1973; Berndtson et al, 1974; Verjans et al, 1975; Damassa et al, 1976; Robaire et al, 1979; Steiner et al, 1982}. A one-to-one relationship has been demonstrated for serum pulses of LH and

testosterone in several species, e.g. man {Naftolin et al, 1973; Santen & Bardin, 1973}, monkey {Steiner et al, 1980; Plant, 1981}, ram {Sanford et al, 1974; Lincoln, 1976; D'Occhio et al, 1982}, rabbit {Rowe et al, 1975}, ferret {Sisk & Desjardins, 1986}, mouse {Coquelin & Desjardins, 1982}, and bull {Katongole et al, 1971; Smith et al, 1973}, however, testosterone fluctuations have not been well-characterized in the rat. Most studies investigate daily variations in testosterone using infrequent sampling protocols (samples every 30-480 min.) {Kalra & Kalra, 1977; Sodersten et al, 1983; Steiner et al, 1984} and conclude that there is a circadian rhythm in testosterone secretion, but there is no consensus on the number and timing of testosterone peaks in a day. Wong et al {1983} suggest that such inconsistencies may be due in part to differences in strain, age and season.

Only one study has systematically investigated the relationship between LH and testosterone in the male rat using a frequent sampling protocol; Ellis and Desjardins {1982} sampled adult Sprague Dawley rats for 12 h at 5 min intervals and reported only testosterone episodes that were irregular and sustained (3-6 h long). While these episodes were sometimes preceded by 1-2 h with a train of closely coupled LH pulses, this was clearly not the one to one correspondance commonly observed in other species. Though it was not discussed, their data also exhibited an occassional testosterone spike of a duration and amplitude suggestive of a pulse {Ellis & Desjardins, 1982}.

3.6 DIHYDROTESTOSTERONE

While DHT is released from the testes, serum levels of this androgen are so low that the impact of circulating DHT is minor compared to that of T unless the target tissue contains the enzyme 4-ene steroid 5α-reductase (5α-reductase; EC 1.3.1.22) which irreversibly converts T to DHT. The activity of 5α-reductase is quite high in the accessory reproductive

tissues relative to other peripheral androgen-dependent tissues. For example, just one hour after a subcutaneous injection of [^{3}H]-testosterone, 70% of the total radioactivity in the ventral prostate and in the seminal vesicles was isolated as DHT (and 15% as unmetabolized T); in contrast the liver and rectus abdominous muscle contained 37% of the radioactivity in its unmetabolized form, and less than one percent as DHT {Tveter & Aakvaag, 1970}. Thus, in some target tissues the role of circulating T may be that of pro-hormone for DHT, which effectively amplifies the androgen signal, while in others T itself mediates the androgen effects.

In the adult male rat 5α-reductase is present and active in both the neuroendocrine sites involved in the regulation of LH release. The high levels of 5α-reduced metabolites found in the anterior pituitary are second only to those of the prostate gland and seminal vesicles, and the hypothalamus also contains relatively high reductase activity {Jaffe, 1969; Massa et al, 1972; Martini, 1982}. This is true for a number of species, such as the human {Jenkins & Hall, 1977}, monkey {Sholiton et al, 1974}, dog {Perez-Palacios et al, 1970}, mouse {Attardi & Ohno, 1976} and guinea pig {Sholl et al, 1975}. Thus, the potential exists for DHT to mediate some or all of the feedback actions that T exerts on the hypothalamus and pituitary in adult male mammals.

3.7 ANDROGEN EFFECTS

In 1786 it was noted that castration led to the regression of certain accessory male reproductive tissues {Hunter, 1786}. Testicular extracts were able to prevent and reverse the castration-induced atrophy, and this activity was later associated with the testosterone content of the testis. In adults, the prostate, seminal vesicle and bulbourethral (Cowper's) glands as well as the epididymides are dependent upon androgen support for maintenance of their size

and function.

Testosterone's actions are also important at earlier stages of life, and are widespread beyond the reproductive organs. Testicular androgens affect the development of the male sex accessory tissues and external genitalia in the embryo, and cause neonatal imprinting in tissues such as the brain, kidney and liver. Androgen effects are also evident in the pubertal development of secondary sex characteristics and their maintenance in adulthood; T acts upon the musculature, stimulates skin and hair follicles, and hematopoeisis, and also influences male aggression, libido, and sexual behaviour (pre-copulatory behaviour, mounting, intromission, ejaculation, and post-ejaculatory behaviour) {Coffey, 1988}.

To regulate its own production, testosterone acts upon the hypothalamic release of LHRH, and LH secretion from the pitutary.

3.7.1 *Negative feedback by testicular androgens*

It is well-established that the frequency and magnitude of LH secretory bursts depend on the steroid environment of the animal. In the testes-intact rat, small, infrequent LH pulses are superimposed irregularly on a baseline of LH that hovers only slightly above the assay detection limit {Ellis & Desjardins, 1982; Steiner et al, 1982; Grosser, 1987}. Following gonad removal there is a dramatic and prolonged rise in the mean serum LH levels: the steepest (e.g. 20-fold) increase occurs within the first 24 hours post-surgery, and subsequent more gradual increases plateau 2-4 weeks later, a further 2 to 4 fold above intact levels {Gay & Midgley, 1969; Yamamoto et al, 1970; Grosser, 1987}. These augmented LH concentrations are due to an increase in secretion rather than a delayed removal of the hormone from the circulation {Gay & Bogdanove, 1968; Gay & Midgley, 1969}, and are associated with hypertrophy and ultimately hyperplasia of the pituitary gonadotropes {see

Tougard & Tixier-Vidal, 1988}.

Serial sampling in castrated male rats reveals a progressive post-orchidectomy rise in LH pulse frequency, amplitude and nadir, initiated within 1 day of castration {Ellis & Desjardins, 1984a; Grosser, 1987}. The rates of change of these parameters parallel that of mean serum LH levels: those that immediately follow castration are the most dramatic, and more gradual changes in each of these three pulse parameters contribute to the continued elevation of mean serum LH {Steiner et al, 1982; Ellis & Desjardins, 1984a; Grosser, 1987; Culler, 1990}. After 21 days without gonadal feedback, LH pulse frequency, amplitude, nadir and mean are respectively about 10-, 10-, 30-, and 40- fold what they were in the intact adult male rat {Steiner et al, 1982; Grosser, 1987}. If an orchidectomized rat is treated with sufficient amounts of T the frequency and amplitude of LH pulses can be brought back to intact levels {Steiner et al, 1982; Grosser, 1987}.

The means by which endogenous T effects its regulation of LH in the intact male are incompletely understood, but clearly there are two levels at which control might be exerted: 1) over the hypothalamic release of LHRH pulses into the portal vasculature, and 2) over the secretory response of LHRH-stimulated gonadotropes. Androgen receptors have been localized to the anterior pituitary and hypothalamic areas containing LHRH neurons {McGinnis et al, 1983; Handa et al, 1987; Sar et al, 1990a}, thus hypothalamic effects on LHRH pulse frequency or amplitude may result from T modulation of input to the LHRH pulse generator system, and/or on neurons which regulate the LHRH neurons in parallel with the pulse generator. Effects upon the gonadotropes could result from androgen binding to receptors within the gonadotropes ("direct" effects), or from T-induced modulations of hypothalamic secretions which in turn effect changes at the level of the pituitary ("indirect" effects upon the gonadotropes).

That the orchidectomy-induced elevation in LH levels is mediated at least partly by LHRH is evident from the striking drop in serum LH that immediately (within 30 min) follows the infusion of anti-LHRH antiserum, or an LHRH antagonist into the gonadectomized rat {Clayton et al, 1982a; Ellis et al, 1983; Almeida et al, 1989}. Direct evidence of testicular regulation of LHRH secretion comes from studies monitoring LHRH released from the median eminence in orchidectomized versus intact rats, however there are numerous discrepancies in the results of such studies. While four studies showed a castration-induced increase in mean LHRH levels in the portal blood {Eskay et al, 1977}, the pituitary perfusate {Dluzen & Ramirez, 1987; Ramirez et al, 1991}, and the peripheral blood {Wheaton & McCann, 1976}, four others showed no change {Levine & Duffy, 1988; Phelps et al, 1992; Meredith & Levine, 1992} or a decrease {Dluzen & Ramirez, 1985}. However, two of the latter studies {Dluzen & Ramirez, 1985; Levine & Duffy, 1988} employed a technical approach that has been criticized; it has been suggested that LHRH signals en route to the pituitary cannot be accurately characterized from push-pull perfusate samples of the hypothalamus/median eminance, because the perfusate contains LHRH from cells proximal to the cannula, and the neurons at a particular implantation site may or may not be actively contributing to the ongoing pulsatile LHRH secretion in a given endocrine condition {Dluzen & Ramirez, 1987; Hiatt et al, 1992}.

However, push-pull cannulae placed in the pituitary have also yielded inconsistent results regarding orchidectomy-induced changes in the frequency and amplitude of the LHRH signal. In two different experiments on rats castrated up to 7 days previously, Phelps et al {1992} detected no change in either LHRH frequency or amplitude (n=8-12/group), or an increase in both parameters (n=5-6/group), though the amplitude increase did not achieve significance. They considered the data showing no effect of T on LHRH release more

reliable by virtue of the larger number of animals in each treatment group. Meredith & Levine {1992} recorded a 150% increase in LHRH frequency, but unaltered amplitude in acutely (4 day) orchidectomized rats, while two other reports indicate that amplitude does increase to 250% in 14 day {Ramirez et al, 1991} and chronically (≥28 day) {Dluzen & Ramirez, 1987} castrated male rats, though a quickened pace of pulses was detected only in the chronically castrate group. Only one study has directly tested the ability of testicular androgens to alter LHRH in the male rat. A testosterone-releasing capsule, implanted between 14 and 21 post-orchidectomy, brought LH back down to intact levels yet mean LHRH concentration in the pituitary perfusate was not different from that in untreated 21-day castrates {Ramirez et al, 1991}. This variation in results suggests a need for further studies involving larger numbers of animals, as well as more sensitive hormone assays that permit a decrease in sample volume, and thus an increase in sampling frequency, with the associated improvement in the accuracy of pulse amplitude measurements (see Appendix 3). Nonetheless, while it would be premature to derive definite conclusions from the present studies on the steroid regulation of LHRH pulse parameters, clearly the detectable orchidectomy-induced changes are consistent with a disinhibition of LHRH release following the removal of testicular hormones.

Both T and DHT inhibit pituitary responsiveness, decreasing LHRH-induced LH secretion in vivo {Debeljuk et al, 1972; Verjans & Eik-Nes, 1976; Cheung & Davidson, 1977} and in vitro {Schally et al, 1973; Kao & Weisz, 1975}. As the interactions between the components of the hypothalamo-pituitary-testicular loop are ongoing in the in vivo animal model, it is difficult to determine whether androgen suppression of pituitary responsiveness is achieved indirectly, e.g. by eliciting a change in LHRH secretion that in turn alters pituitary

responsiveness, or more simply by a direct action of the androgen upon the pituitary gonadotropes. Levine's group isolated the pituitary from orchidectomy-induced changes in endogenous LHRH secretion by grafting a pituitary under the kidney capsule of hypophysectomized rats receiving exogenous pulsatile LHRH stimulation. By 18 hours post-orchidectomy the mean plasma LH concentration had risen 3-fold above that in normal intact rats, but in contrast to castrated animals without hypophysectomy or a pituitary transplant, LH did not continue to rise over the next 6 hours. These data suggest that direct effects of endogenous T on the gonadotropes are at least partially responsible for the acute (< 18 h) castration-induced increases in pituitary responsiveness and LH pulse amplitude, though subsequent changes in LH pulse amplitude may be the result of incoming LHRH signals of greater amplitude {Strobl et al, 1989}.

That DHT can also directly affect pituitary responsiveness is suggested by experiments in the acutely orchidectomized adult rat in which endogenous LHRH secretion is abolished by phenobarbital administration and replaced with exogenous LHRH pulses {Nansel et al, 1979}. In this hypothalamic clamp model those orchidectomized animals that received a DHT implant at orchidectomy show a decrease in pituitary responsiveness to LHRH pulsed at 30 min intervals. Interestingly, this difference was not apparent if the LHRH was given at hourly intervals {Nansel et al, 1979} or as an infusion of constant dose {Nansel & Trent, 1979}. Thus while DHT can have a direct effect upon pituitary responsiveness, it may depend in some manner on a permissive hypothalamic influence.

In association with changes in pituitary responsiveness, an increase in pituitary LHRH receptor number follows castration and is prevented by T and DHT administration {Clayton & Catt, 1981a; Frager et al, 1981; see review by Clayton & Catt, 1981b}. LHRH positively autoregulates its own receptor (as well as post-receptor LH secretory mechanisms) {Frager et

al, 1981; Clayton, 1982; Katt et al, 1985}, so it is possible that gonadal steroids alter receptor number indirectly by their effects on LHRH secretion, but in vitro studies suggest that androgens can also exert a direct effect upon gonadotrope LHRH receptor levels {Giguere et al, 1981}. Direct or indirect androgen regulation of pituitary responsiveness may also be effected at post receptor sites, though the details of such modulatory actions have yet to be elucidated {Pieper et al, 1984; Katt et al, 1985}.

3.7.2 *Positive feedback by testicular androgens*

The majority of studies investigating the role of T in the feedback loop use an orchidectomized model in which T is replaced in a controlled fashion. In a few of these studies, where relatively low doses have been used, T treatment has resulted in an increase in LH levels {Swerdloff & Walsh, 1973; Bloch et al, 1974; Verjans et al, 1975; Gay & Dever, 1971; Negro-Vilar et al, 1973a}. This effect is also observed in vitro {Mittler, 1974; Schally et al, 1973; Tang & Spies, 1975}, and in other species including the pig {Ford & Schanbacher, 1977}, primate {Resko et al, 1977; Resko et al, 1981; Resko & Horton, 1983}, and man {Dorner et al, 1975b; Kulin & Reiter, 1976; Capell et al, 1973}. In the female, estradiol-induced postive feedback generates the midcycle pre-ovulatory LH surge, a phenomenon which can be mimicked by testosterone administration to an ovariectomized estrogen-primed female rat, or by estradiol given to castrated and primed male rats, men and monkeys {Dorner et al, 1975a, 1975b; Karsch et al, 1973}. Common to all these observations of positive feedback on LH release is the presence of a low background level of gonadal steroid.

The pattern of LH pulses underlying the increased mean LH levels in the orchidectomized male rat indicates that the positive feedback effect is selectively upon LH

pulse amplitude; dose-response studies show that while increasing doses of T have either a neutral or negative effect on LH pulse frequency, LH pulse amplitude is suppressed by higher doses of T but stimulated by low doses (e.g. doses that yielded serum T concentrations just 20 - 30% of intact levels) {Steiner et al, 1982; Grosser, 1987}. There is no evidence that T can increase pituitary responsiveness at the doses eliciting positive feedback, thus it appears that the androgen-induced stimulation of LH pulse amplitude is mediated at the level of the hypothalamus {Grosser, 1987}.

3.8 STEROID RECEPTORS

Steroid receptors are characterized by a modular structure comprised of three domains, with a centrally located, highly conserved, 66-68 amino acid DNA-binding domain that mediates the interaction of the steroid-receptor complex with specific acceptor sites on the chromatin. The carboxy-terminal end is the ligand-binding domain, and the poorly conserved amino-terminal domain is primarily involved in transactivation function of the receptor. Probes based on the highly conserved DNA-binding region have been used to isolate homologous proteins from the cDNA libraries of various steroid-responsive tissues; this approach yielded the sequence of numerous hormonally-regulated transcriptional activators, as well as receptors for which no ligand is known ("orphan receptors"), and proteins lacking the hormone-binding domain which are presumed to be transcription factors regulated by some other means. Together these proteins form the steroid receptor superfamily.

3.8.1 *The androgen receptor*

The last of the known steroid receptors to be cloned was the androgen receptor (AR).

Using a consensus oligonucleotide probe for the steroid receptor DNA-binding domain, the human AR was cloned from a ventral prostate cDNA library in early 1988 {Chang et al, 1988; Lubahn et al, 1988a}. DNA sequence analysis indicates that the organization of functional domains of the AR clones conforms to that of other proteins in the steroid receptor superfamily, and that the steroid- and DNA-binding domains are identical in the human and rat AR {Lubahn et al, 1988b}. It is a 98 kD MW protein with 902 amino acids encoded in a 10 kb mRNA species {Tan et al, 1988}.

Mice carrying a mutated nonfunctional androgen receptor gene are equally resistant to the actions of T and DHT {Goldstein & Wilson, 1972}, providing evidence consistent with earlier biocemical data that both hormones act via a single receptor protein. Nonetheless, the conversion of T to DHT serves to amplify the androgen signal, as DHT possesses twice the affinity of T for the receptor, and dissociates at one-fifth the rate {Kovacs et al, 1984; Grino et al, 1990}. In vitro, when both hormones are supplied in equal concentration, the binding of DHT is favoured over that of T by a ratio of 4 to 1; high concentrations of T offset this relatively weak binding activity, at least with respect to androgen-induced receptor upregulation {Grino et al, 1990}. DHT also amplifies the androgen effects of T by a second mechanism, as it cannot be aromatized to estrogen and thus commits more of the available T to act via the androgen receptor rather than through an estrogenic metabolite {Thompson et al, 1971}.

3.8.2 *The mechanisms of steroid receptor mediated actions*

Intense research efforts over the last decade have outlined the numerous and complex events that translate steroid receptor binding into a modification of cellular function. Ligand-modulated transcriptional activity of the receptor is the best-studied outcome of steroid-

receptor interaction, though evidence is accumulating to suggest that the steroid receptor can influence the protein population of the cell, and thus the cell's function, by mechanisms that do not involve gene transcription, {e.g. Brock & Shapiro, 1983; Puia et al, 1990; McEwen, 1991}. Ligand-induced changes in steroid receptor conformation trigger a cascade of events that include the dissociation of receptor-associated proteins, the induction of kinase activity, receptor phosphorylation, nuclear localization, receptor dimerization, binding to chromatin "acceptor" sites, and association with other nuclear transcription factors {Landers & Spelberg, 1991}. It has become apparent that the receptor itself is but a part of an oligomeric complex of proteins which direct, regulate and fine-tune steroid-induced events, while the role of the steroid is to induce (and maintain?) the conformational shift(s) in the receptor that determine the membership and activity of the other proteins in this oligomeric complex.

While experimental approaches tend to measure steroid-induced receptor events in terms of a single testable endpoint (e.g. gene transcription), or perhaps several endpoints representing different stages in the cascade of post-binding events (e.g. nuclear translocation, DNA binding, and gene transcription), it is important to realize that a given steroid-receptor interaction might elicit numerous distinct cellular responses, each mediated by a parallel and/or serially connected branch of the cascade of biochemical processes triggered by the initial binding event. A hypothetical example would be that testosterone binding to the androgen receptor in the ventral prostate might lead to the stabilization of protein A, the transcription of two distinct genes for proteins B and C, and in addition the stabilization of the mRNA species of proteins B and D. There might be many such distinct "outcomes" which together would drive the cell to change in a manner appropriate to the particular steroid environment.

Several recent studies suggest that the inhibitory effects of different antagonists upon

steroid action may be manifested at different stages of ligand-mediated receptor events, such as nuclear translocation {Kemppainen et al, 1992; Ylikomi et al, 1992}, DNA-binding {Schauer et al, 1989; Reese & Katzenellenbogan, 1991}, or processes subsequent to DNA-binding {Berry et al, 1990; Brown & Sharp, 1990; Lees et al, 1989}. It is presumably the molecular structure of each anti-hormone that determines the extent and nature of binding-induced conformational changes in the steroid receptor, and thus at what stage it will interrupt the post-binding cascade of events; a given ligand-specific receptor conformation might thus confer a degree of selectivity on the outcome(s) of binding, by permitting some branches in the cascade to go to completion, while blocking others at an intermediate step. Thus, the structural details of a ligand could determine the selection of genes regulated, if the associated ligand-receptor conformation is permissive to the formation of certain receptor-DNA complexes, but not others.

STATEMENT OF THE PROBLEM

Regulation of testicular function by the gonadotropins depends upon the operation of a hormonally mediated feedback loop between the testes and the brain-pituitary axis. Testosterone, the major circulating steroid of testicular origin and the best characterized feedback regulator of LH secretion, has a well-known negative feedback effect upon serum LH concentration, which reduces mean serum LH concentrations by inhibiting hypothalamic LHRH secretion, and diminishing pituitary responsiveness to the incoming LHRH signal. Clinical and experimental studies also show that low quantities of testosterone can augment LH pulse amplitude and mean serum concentration to magnitudes greater than those associated with orchidectomy. The stimulation of LH mean and LH pulse amplitude occurs in the absence of a change in pituitary responsiveness, suggesting that the positive feedback effect(s) of T are mediated at a hypothalamic locus. It is unknown by what means the different doses of testosterone elicit these opposite effects on LH pulse amplitude (positive feedback with low doses, negative feedback with higher doses).

In some peripheral reproductive tissues the 5α-reduction of T to DHT is integral to the androgen-dependent regulation of tissue growth and function, and this may also be true for those tissues important in the regulation of LH secretion. Consistent with the possibility that T is a pro-hormone for DHT-mediated negative feedback effects on the brain-pituitary axis, mean serum LH concentration is suppressed by DHT even more potently than by T. As the 5α-reductase enzyme that converts T to DHT is present in both the pituitary and hypothalamus, DHT is potentially responsible for any of the T-induced effects exerted at these sites in the feedback loop. One might postulate that the opposing effects of T upon LH pulse amplitude are due to distinct actions of T and its 5α-reduced metabolite on the hypothalamo-pituitary complex.

While the positive feedback effects of low (sub-physiological) androgen doses on LH

pulse amplitude and mean serum concentration have been demonstrated in the orchidectomized rat, it is unknown if this phenomenon is important for homeostasis within the hypothalamo-pituitary-testicular loop, or even if such low levels of testosterone occur in the normal (testes-intact) rat. In many species other than the rat, acute fluctuations have been observed in serum testosterone concentrations in the male, and typically, these T pulses are tightly coupled to pulses of LH, (though this is not always the case, a notable exception being man). In the adult male rat, investigators have studied circadian rhythms in T, typically finding one, two or three daily T maximums based on protocols with a serum sampling interval (every 30-480 min) not suited to the detection of moment-to-moment hormone fluctuations. The inconsistency of these results, and the high degree of variability associated with mean T levels are suggestive of underlying pulsatility in serum testosterone concentrations.

In this thesis, the following questions were addressed to investigate the role of DHT in the hypothalamo-pituitary-testicular feedback loop, and characterize testicular secretory activity:

- Which parameters of plasma LH patterns are altered by suppressive DHT concentrations?
- Is DHT (low doses) capable of exerting a positive feedback effect on plasma LH?
- Are the feedback effects of DHT on LH release sufficiently similar to those of T, to suggest that DHT mediates T effects on the neuroendocrine reproductive axis?
- What is the nature of plasma T and LH patterns in intact male rats and what is the relationship between them? What are the acute effects of daily changes in lighting on these hormone concentrations?
- Are the changes in T concentration in the intact animal of a magnitude that suggests that physiological variation in T levels might support both positive and negative feedback effects?

MATERIALS and METHODS

4 ANIMALS AND HOUSING

The use of rats in these studies was approved by the McGill University Animal Care Committee. Adult male Sprague-Dawley rats (Charles River Canada Ltd., St. Constant, Quebec) weighing between 375-475 g, (88-95 days old) were given Purina rat chow and water ad libitum. The animals were housed in rooms of controlled temperature (22-25°C) and humidity (50-70%), with lights on between 07:00 and 19:00.

During the first part of the DHT studies, a minor endemic viral infection in the Animal Centre required that the experimental animals were brought in a full month before use. This permitted the rats to contract the highly contagious sialodacryloadenitis (SDA) virus, undergo the course of the infection (a mild and transitory inflammation of salivary and lacrimal glands), and then to recover before the experiment began. No differences were observed between these animals and those acquired shortly before use, other than a slight increase in body weight range.

4.1 STEROID TREATMENT

To experimentally control circulating androgen levels in the adult male rat, the testes were removed and replacement steroids were administered in subdermally implanted constant-release capsules. As the testes produce 95% of the circulating androgen in adult male rats {Eik-Nes & Hall, 1965; Lipsett et al, 1966}, their removal leads to the depletion of endogenous serum testosterone within 12 hours {Ellis & Desjardins, 1984a; Sodersten et al, 1983}. The capsules were implanted immediately following orchidectomy (day 0) and left in

place until after serial blood sampling (usually day 21). Previous studies have shown that following castration, with or without replacement steroids, changes in the LH pattern are quite dramatic within the first few days, then the rate of change decreases and LH pulse parameters become relatively stable between 2 and 4 weeks {Ellis & Desjardins, 1984; Grosser, 1987}. Thus, in the experiments reported herein, serial LH data from steroid-treated rats were based on samples collected between 3 and 4 weeks post-orchidectomy and steroid treatment.

4.1.1 *Orchidectomy*

With the rat under light ether anesthesia, the lower abdominal region was shaved and swabbed with 70% alcohol, and the peritoneum accessed through a 1.5 cm midline incision through the skin and muscle. One at a time the testes were exposed through this incision, and cut away after first clamping and tying off the efferent ducts as close to the testes as possible. The epididymides and associated fat pad were replaced in the peritoneum, and the abdominal muscle and external wound closed with sutures (5-0 Dermalon; Cyanamid Canada Inc., Montreal, Quebec). Steroid capsules were then implanted (see below) before the rat recovered from the anesthesia.

Rats having testes weighing more or less than 1.59 $\pm$ 0.32 g (mean $\pm$ 2*S.D.), or that differed from each other by more than 10% in weight, were excluded from these studies.

4.1.2 *Steroid implants*

Steroid implants were constructed from washed polydimethylsiloxane (PDS) tubing (Silastic, Dow-Corning Corp., Midland, MI, Cat. #602-305, ID 1.98 mm, OD 3.17 mm)

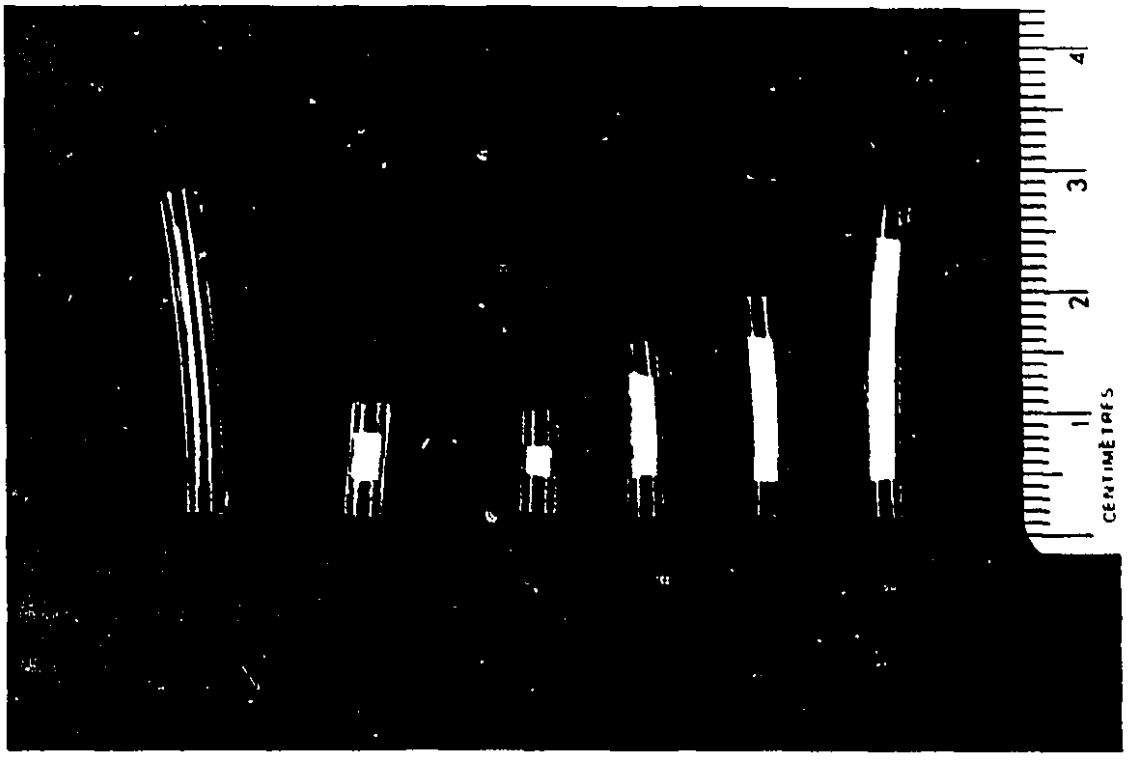

Figure 1. Sustained release polydimethylsiloxane implants. Shown left to right are an empty 20 mm implant, a 3 mm testosterone-filled implant, and 2 mm, 8 mm, 12 mm, and 20 mm dihydrotestosterone-filled implants.

STEROID DOSAGES FROM CONTINUOUS RELEASE IMPLANTS	Steroid release rate (in vitro)	Implant length	Steroid released per implant
Testosterone	3.0 μg/mm/day	3 mm	9,0 μg/day
Dihydrotestosterone	2.4 μg/mm/day	2 mm	4,8 μg/day
		8 mm	19 μg/day
		12 mm	29 μg/day
		20 mm	48 μg/day

Table 1. Daily steroid doses released by the testosterone and dihydrotestosterone implants used in these studies, as inferred from in vitro release rates {Robaire et al, 1979; Brawer et al, 1983}.

packed with the desired crystalline steroid (Steraloids, Wilton, NH) and plugged at either end with silicone adhesive (Silastic Medical Adhesive Type A, Dow-Corning Corp., Midland, MI, Cat. #891). Placed in a fluid environment, the steroid diffuses out of the PDS implant at a rate that is proportional to the surface area of the tubing, the thickness of the tubing wall, and the molecular properties of the steroid {Kincl et al, 1968; Stratton et al, 1973}. In vitro, T is released from these PDS implants at a rate of 3.0 μg/mm/day {Robaire et al, 1979} and DHT at a rate of 2.4 μg/mm/day {Brawer et al, 1983}. Thus, the 3 mm testosterone implant released approximately 9 μg T/ day, and the 2, 8, 12, and 20 mm dihydrotestosterone implants released approximately 5, 19, 29, and 48 μg DHT/day respectively (see Figure 1, and Table 1). The control treatment for steroids administered as implants was an empty, plugged, 20 mm length of PDS tubing.

Prior to use in the experimental animals, steroid release from the capsules was initiated and allowed to stabilize over three days by implanting them in carrier rats not otherwise used in these experiments. On experimental day 0, the steroid-filled capsules were removed from the carriers, rinsed briefly in 70% ethanol, and dried. Through a 10-20 mm incision in the lower back of the experimental rat (castrated 5 minutes previously), the capsule was guided subcutaneously along a thin grooved spatula to a resting position just below the shoulders, deposited there, and the incision closed with sutures or wound clips.

In the largest experiment, the large number of animals involved (n=145 rats) required that they be ordered, treated and sampled in numerous small batches (12 batches of 7-15 animals each). To minimize the possibility that inter-treatment differences in the experimental parameters might be due to inter-batch variability, each treatment was administered to one or more rats in each batch.

4.1.3 *Tissue collection*

The weights of androgen-dependent accessory sex tissues were determined from androgen-treated animals to provide a biological index of androgenicity. Following decapitation of the animal, paired seminal vesicles and the ventral prostate were dissected from orchidectomized steroid-implanted adult male rats on post-castration day 27 $\pm$ 3. The contents of the seminal vesicles were expelled gently, and all tissues blotted dry and their weights noted. Orchidectomized rats with empty implants and an additional group of weight-matched normal (intact, untreated) male rats yielded control values for these tissues. The body weights of the rats did not differ significantly between the treatment groups, so tissue weights were expressed as absolute values (mg).

4.2 SERIAL BLOOD SAMPLING

4.2.1 *Catheter Surgery*

To determine the serum pattern of a hormone secreted in pulses the blood must be sampled serially for an extended period at intervals suited to the half-life of the hormone and the frequency of pulsatile hormone release. To minimize the stress of repeated blood withdrawal from conscious freely-moving rats, each animal was equipped with a chronic indwelling atrial cannula using a modification of the technique described by Grosser et al {1987}.

The rat was anesthetized with sodium pentobarbital (Somnotol, MTC Pharmaceuticals, Hamilton, Ontario) at a dose of 65 mg/kg body weight, i.p., and given a prophylactic injection of penicillin G procaine, 100,000 IU in 0.33 ml i.m. (Ayercillin, Ayerst Laboratories, Montreal, Quebec). The surgical sites were shaved and swabbed with 70%

ethanol, and a 1 cm chest incision was made rostral and medial to the rat's right shoulder. The right external jugular vein was exposed by blunt dissection, and the end of an 11 cm length of ethanol-washed Silastic medical grade tubing (Dow Corning Corp., Midland, Michigan; Cat. #602-155; 0.64 mm ID, 1.19 mm OD) inserted into the jugular vein in the direction of the heart and secured in place with ligatures. The catheter was positioned such that the beveled tip lay within the right atrium (roughly 4.0 cm from the insertion site) and the other end was tunneled subcutaneously to the top of the head where it was exteriorized through a 1.5 cm midline incision rostral to the ears. This end of the catheter was attached to an elbow (a 3 cm portion of 20 gauge needle bent in the middle at a 90° angle) which was fixed to the skull with screws. Slightly caudal to the elbow, another screw anchored a 1 cm metal loop (modified paper clip) in place, perpendicular to the skull surface, for later use as an attachment point. Dental cement further secured these fixtures and closed the head incision; the chest wound was closed with wound clips or nylon sutures (5-0 Dermalon; Cyanamid Canada Inc., Montreal, Quebec). The protruding portion of the catheter elbow was sealed with crimped Tygon tubing when the catheter was not in use.

Post-operatively, the rat was examined 2-3 times in the first week, and at least weekly thereafter; on these occasions the catheter was flushed with 200 IU of heparin sodium per ml (Heparin Sodium Injection USP, Allen and Hanburys, Montreal, Quebec) diluted in bacteriostatic (1.5% benzyl alcohol) 0.9% saline (Bacteriostatic Sodium Chloride Injection USP, Squibb Canada Inc., Montreal, Quebec). The animals were allowed to recover from surgery for at least 7 days prior to undergoing serial blood sampling; previous studies have shown that it takes roughly one week following atrial cannulation for rats to return to their normal rate of body weight gain and food intake {Tannenbaum & Martin, 1976}, and for disrupted estrous cyclicity to normalize in female rats {Blake et al, 1973}.

Numerous refinements of this catheterization procedure were introduced when many of the animals developed a "one-way" block of the cannula which rendered them unsampleable (see Appendix 1). As none of these modifications consistently reduced the rate of one-way catheter blockage, experiments were continued using the original procedure except that the minimum recovery interval between surgery and sampling was reduced to 5 days.

4.2.2 *Preparation for sampling*

On the day before sampling the rats were moved to wire mesh cages suspended within isolation boxes equipped with food, water, individual timed fluorescent lighting (lights on between 07:00 and 19:00), and a one-way mirror for observation (Fig. 2). This system provides a controlled, reduced-stress environment for the rats during periods when serial blood samples are obtained.

As shown in figures 2 and 3, a 70 cm polyethylene sampling line (PE-100; Clay-Adams #7420; 0.86 mm ID, 1.52 mm OD) containing heparinized saline (200 IU/ml) ran from a syringe outside the isolation box through a small hole in the top of the box, to the catheter elbow on the rat's head. This line was protected from the rat within the cage by a suspended flexible wire coil and the attached alligator clip which anchored the coil to the metal (modified paper clip) loop on the rat's skull.

4.2.3 *Blood Sampling Protocol*

On sampling days, 0.5 ml blood samples were withdrawn every 5 to 10 minutes and immediately centrifuged for 1 min (15,600 x g). Aliquots of the plasma supernatant were quick-frozen on dry ice and stored at -80°C for later radioimmunoassay. The remaining plasma and red blood cells were resuspended in bacteriostatic 0.9% saline containing 10 IU

Figure 2. View of a conscious, freely-moving, catheterized rat prepared for serial blood sampling. The chronic indwelling catheter accesses the rat's right atrium via the internal jugular vein, and the distal end is exteriorized on top of the head to maximize the rat's freedom of movement. Protruding from the white dental cement cap are the exterior head fixtures for the catheter. The sampling tubing visible in the picture is attached to a short metal tube which, just below the cap and superficial to the skull, bends posteriorly and connects with the distal end of the flexible catheter tubing. The catheter then runs subcutaneously behind the ears and ventrally to the chest area where it enters the right jugular vein and travels to the heart. The freely-rotating black spring suspended from the cage-top encircles and protects the sampling line from the rat, and is loosely attached to the cap via a modified alligator clip and a metal loop held fast in the dental cement.

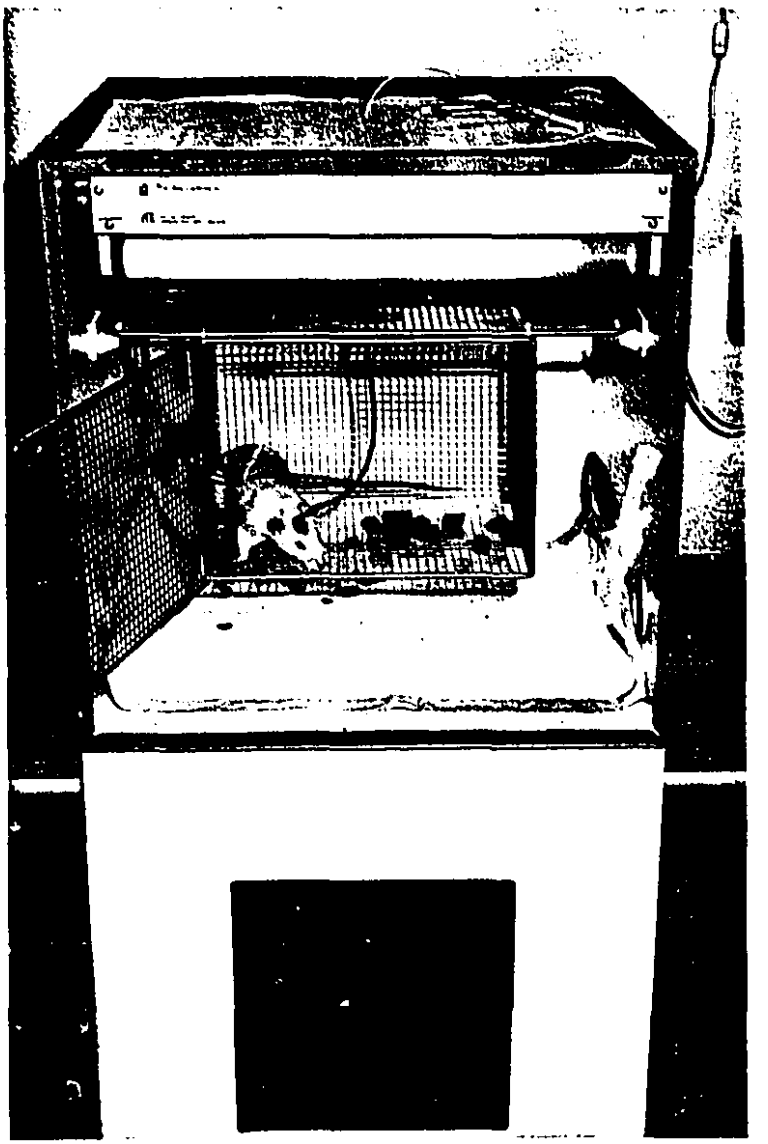

Figure 3. View of a catheterized rat in its cage within an isolation box, prepared for serial blood sampling. Note that the sampling line emerges from the black wire coil at the top of the cage and extends through a small hole in the top of the isolation box, where it is attached to a syringe used for the repeated withdrawal and injecion of liquids (usually blood). At the top rear of the box, (not visible behind the light fixture) is a fan which ensures adequate air circulation when the box door is closed. The one-way observation mirror in the door of the box appears in this photo as a large black square.

pituitary LH output monitored. For such a pituitary responsiveness challenge, the dose of LHRH employed must be supraphysiological in order for the LH secretory response to be associated unambiguously to the exogenous LHRH stimulus rather than a coincident endogenous LHRH pulse. The LHRH dose used in these studies was 500 ng/kg body weight, which has previously been shown to be an intermediate dose with repect to LH release in adult castrated rats four weeks post-orchidectomy {Grosser, 1987}. The LHRH (LHRH Acetate Salt, Sigma Chemicals Ltd., St. Louis, MO) was prepared in saline (1000 ng/ml), snap-frozen in small aliquots, stored at -80°C, and kept frozen until 45 min before injection.

Immediately following the last of a series of serum samples collected to determine "pre-LHRH" mean LH levels, 500 ng/kg LHRH was injected i.v. and its immediate entry into the rat's circulation ensured by quickly returning the resuspended red blood cells from the previous sample via the intracardiac catheter. Plasma sampling continued every 5-7.5 minutes for at least 30 minutes during which time LH concentrations ascended to a maximum and began to fall. In previous pituitary responsiveness studies with more extended post-bolus serial sampling {Grosser, 1987} it has been shown that the area under the LH concentration curve is proportional to the LH increment (LH increment = the post-LHRH LH maximum minus the pre-LHRH LH mean). Thus, to compare the effect of different androgen treatments on pituitary responsiveness, the magnitude of the LHRH-induced LH increment was calculated for each rat and averaged per treatment group.

4.3.1.2 *Naltrexone challenge*

To test the hypothesis that an increase in LH pulse amplitude might be secondary to a decrease in opioid inhibition of LHRH pulse amplitude, animals were challenged with the opioid antagonist, naltrexone. Nineteen adult male rats were divided into two groups. On

heparin per ml and returned to the animal through the sampling line following the withdrawal of the subsequent blood sample.

4.3 EXPERIMENTAL PROTOCOLS

4.3.1 *Plasma LH Pattern in Androgen-treated Rats*

The feedback effects of dihydrotestosterone on plasma LH patterns were studied in castrated adult male rats given 2 mm (n=7), 8 mm (n=5), 12 mm (n=5), or 20 mm (n=6) DHT implants, and compared with those receiving empty 20 mm implants (n=9) or 3 mm T implants (n=7). The latter group was included as a control for positive feedback effects on LH pulse amplitude. Three weeks following castration and steroid replacement, the basal pulsatile pattern of LH was determined from plasma samples collected via the chronic indwelling catheter every 5 minutes for 3 hours, starting at 10:00 a.m.. The plasma LH concentration was subsequently determined by radioimmunoassay (RIA).

4.3.1.1 *Pituitary Responsiveness*

While steroid-induced changes in LH pulse frequency may be unambiguously attributed to feedback effects upon the frequency of hypothalamic LHRH release, it is not possible (on the basis of the LH pattern alone) to distinguish between a hypothalamic and pituitary site of action for changes in LH pulse amplitude. In an attempt to assess whether or not androgen effects on LH pulse amplitude might be due to changes in the pituitary's ability to secrete LH in response to an incoming LHRH signal, rats equipped with an indwelling cardiac catheter were challenged with an intravenous LHRH bolus and subsequent changes in

day 0 the rats were orchidectomized and those in the control group were given a 20 mm empty implant (n=10), while the test animals received a 2mm DHT implant (n=9). On day 20-22 these rats were serially sampled every 5 minutes for 2 hours starting at 10:00 am. Naltrexone (1 mg/kg) was then administered through the jugular catheter and the sampling continued every 5 minutes for another hour. The acute increase in LH concentration observed following antagonist administration was quantified as the naltrexone-induced LH increment, i.e. the post-naltrexone LH maximum minus the pre-naltrexone LH mean, and compared between treatment groups.

4.3.2 *Plasma Testosterone Pattern in Intact Male Rats*

To characterize the fluctuations in plasma testosterone concentrations and the associated LH levels, normal adult male rats were serially sampled over an extended period (8 hours) at 10 minute intervals. One group of rats was sampled during the day (n=5), while two other groups were sampled starting 4 hours before the transition from day to night (15:00-23:00; n=9), and night to day (03:00-11:00; n=6), to test for the presence of light-associated changes in testosterone and LH levels. If an animal was sampled more than once (for an 8 hour period each time), then these sampling sessions were at least 3 days apart.

During a subset of these sampling sessions (n=6) an additional 10 μl aliquot of plasma was collected every hour and frozen for later use in a Lowry protein assay {Lowry et al, 1951; Waterborg & Mathews, 1984}. In conjunction with behavioural observations, these data yield a crude index of the effect of such an extended frequent-sampling protocol on the rat. Total plasma protein did not decline significantly over the 8 h sampling period (see figure 4) (Dunnett's test, $p<0.05$). The rats behaved normally during the sampling sessions, i.e. their activity varied between eating, sleeping, grooming, and exploring.

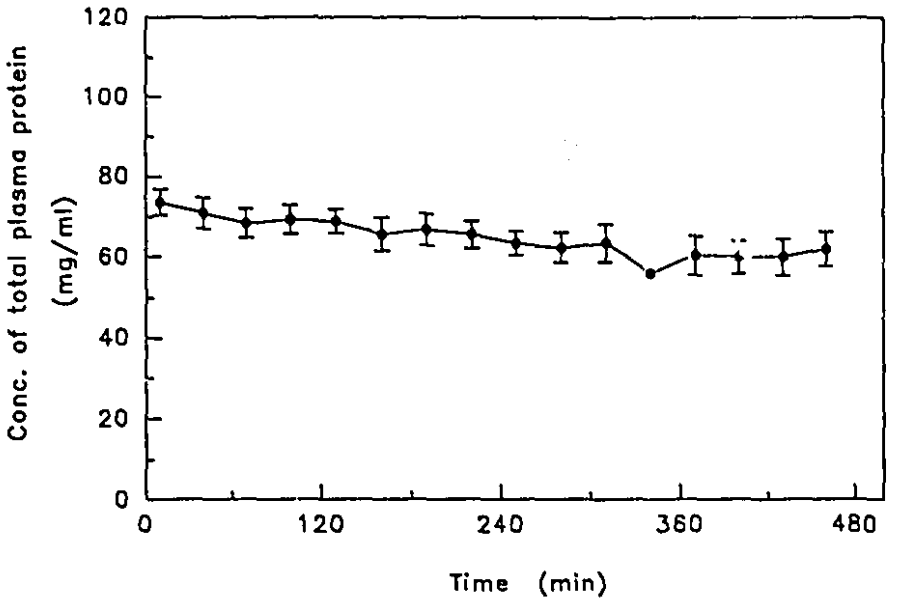

Figure 4. Total plasma protein concentration in adult male rats (n=6). A small volume of plasma (250 μl) was removed every 10 minutes for 8 h to provide samples for LH and T assays. Protein concentration was determined from an additional plasma aliquot removed every 30 minutes. Total plasma protein did not decline significantly over the 8 h serial sampling period ($p \leq 0.05$).

4.4 ASSAYS

4.4.1 *Radioimmunoassay for Luteinizing Hormone*

LH concentrations were determined in duplicate in all serial plasma samples obtained. Plasma hormone concentrations were measured by radioimmunoassay using (several) rat LH assay kits kindly provided by NIDDK and the National Hormone and Pituitary Program of the University of Maryland School of Medicine. NIDDK reference preparations for the standard curves were rat LH-RP-2 or LH-RP-3 which the kit literature indicates are "equipotent" as LH standards. The rat LH supplied for iodination purposes were from batches I-6, I-7, and I-8, and the antiserum used was rabbit anti-rat LH S-9 or S-10.

The radioimmunoassay procedure outlined by NIAMDD was modified to obtain a consistent improvement in the sensitivity (i.e. 95% confidence levels) of the assays. The standard curve was prepared in a 2% BSA/PBS solution (BSA, Sigma, St-Louis, MO), and anti-LH antiserum was added to standards and experimental samples on assay day 1 and incubated overnight at 4°C. On day 2 tracer (see "Iodination of LH") was added, and the incubation continued at 4°C until day 5. After the addition of the second antibody (goat anti-rabbit IgG; Daymar, Toronto, Ontario) and a further 4°C incubation until day 6, a 2 ml wash solution (0.2% BSA/PBS) was added, the assay tubes centrifuged at 2000 x g (Beckman J-6B centrifuge; 3000 rpm) for 25 minutes, and the supernatant decanted and discarded. Pelleted radioactivity was counted for 1 minute/tube (LKB-Wallac CliniGamma 1272), and the standard curve interpolated using a spline smoothing function.

These assays were initially done in the Obstetrics & Gynecology RIA lab at the Royal Victoria Hospital, Montreal. Upon relocation to the McIntyre Medical Building of McGill University, the bound/free separation step in the above protocol required modification, as it no longer generated precise measurements of the LH in the test samples, i.e. the duplicates were frequently highly variable. A number of variations on the established protocol were tested (see Appendix 2), and the procedure finally adopted differed from the above-described protocol as follows: the wash solution contained 5% PEG, and was added to all the tubes on day 5, immediately after the second antibody was added to all the tubes. The samples were then centrifuged as usual, without further incubation.

Large volumes of serum were periodically collected from male rats (intact, or hypophysectomized and orchidectomized, or simply orchidectomized 2-4 weeks previously) for use as quality control pools in glycoprotein and testosterone radioimmunoassays. 2% BSA/PBS was used, if necessary, to dilute the hormone pools down to a concentration

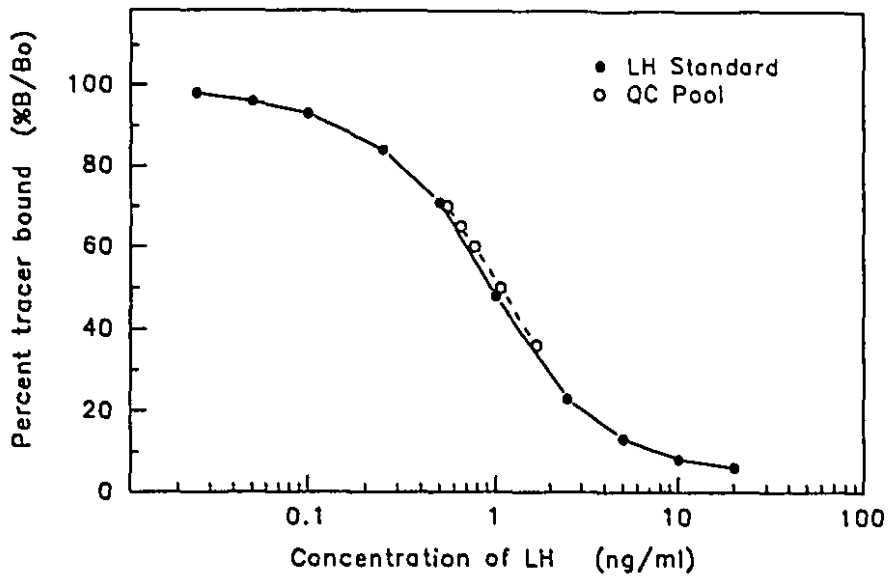

Figure 5. A sample standard curve for the LH radioimmunoassay using the cold LH standard, LH-RP-3 (solid line). Progressively larger dilutions of a quality control pool (QC pool; dashed line) co-graph in parallel to the linear portion of the standad curve, indicating that there is no assay interference from the serum or dilution buffer. (B/B_0 = cpm of tracer bound in the presence/absence of cold LH).

detectable on the linear portion of the assay standard curve. Experimental samples were similarly diluted, if necessary. Figure 5 shows that several concentrations of a typical quality control pool (pool "Orch. A" diluted 1:1, 1:2, 1:3, 1:4, and 1:5) yield a dilution curve parallel to the LH standard curve, indicating that this dilution procedure does not interfere with the measurement of LH in the sample.

The sensitivity (95% confidence limits) of the LH radioimmunoassay was 0.07 ng/sample, and the limit of detection (80% B/B_0) was 0.26 ng/ml. Coefficients of variation (CV=standard deviation/mean) were calculated from quality control pool samples included throughout in each assay. In LH assays of orchidectomized rat serum, the intra- and inter-assay CVs were 8% (n=15) and 9% (n=20) respectively at 50% B/B_0; at 80% B/B_0 the

intraassay CV averaged 9% (n=9) for those assays in which samples from intact rats were analysed. All plasma samples obtained from the animals in one sampling session were analyzed in the same assay. All samples were assayed in duplicate and only those with a CV of less than 15% were accepted. For each assay LH values were normalized against an overall average value obtained from quality control pools present in all assays.

4.4.1.1 *Iodination of LH*

The LH iodination antigen supplied by NIAMDD was iodinated by the chloramine-T method of Hunter & Greenwood {1962} as described by Chard {1987}. [^{125}I]-LH was separated from unreacted iodide on a Sephadex column (Sephadex G-25; "PD-10" Pharmacia, Baie D'Urfe, Quebec) and stored in 2% BSA/PBS at -20°C until use. [I^{125}]-sodium iodine was obtained from Amersham (IMS-300; Oakville, Ontario).

4.4.2 *Testosterone Radioimmunoassay*

Testosterone concentrations were determined in 50 µl aliquots of plasma from intact rats. Steroids and 1000 cpm of added [1,2,6,7-^{3}H]-testosterone (Amersham, Oakville, Ontario) were ether-extracted from the plasma, dried under nitrogen (40°), and dissolved in 500 µl of 0.1% gelatin/PBS. Fifty µl of sample were counted to evaluate recovery from the extraction procedure. Duplicate 200 µl samples and the standard curve were incubated overnight at 4°C with anti-testosterone antibody (Biolog Co., Montreal, Quebec) and 5000 cpm [1,2,6,7-^{3}H]-testosterone. The next day, after the addition of dextran-treated charcoal (0.2% Dextran-T-70, Pharmacia; 2% Norit-A charcoal, Fisher Scientific), all assay tubes were centrifuged for 30 minutes at 2000 x g (Beckman Model J-6B) to precipitate the charcoal-bound steroid, decanted, and the pellet was counted (LKB-Wallac RackBeta 1219).

The assay detection limit was 8.3 pg/tube (85% B/B_0). The intra- and inter-assay CV at the 50% B/B_0 level over 34 assays were 11% and 30% respectively.

4.5 DATA ANALYSIS

4.5.1 *Pulse analysis*

Assay noise inherent to the RIA technique can introduce fluctuations into the pattern of hormone levels measured over time. While the shape of a noise-derived hormone peak may suggest its ex vivo origins, a more objective criterion is usually applied to the data to identify those peaks large enough to be considered more than just assay noise. In this thesis the word "pulse" is used specifically in reference to a peak in hormone concentration thought to result from endogenous episodic stimulation of secretion.

The threshold method of pulse detection, which defines a pulse as a 20% increase in hormone concentration (20% $\approx$ 3 times the intraassay coefficient of variation) {Santen & Bardin, 1973}, was refined to include the requirement that the decrement following the peak should also fulfill this threshold criterion {Van Cauter et al, 1981}, and the threshold adjusted depending on the estimated rate of erroneous peak detection considered acceptable {Veldhuis et al, 1985}. Thus in the analysis of hormone profiles from orchidectomized rats, a pulse was identified when both the associated increment and decrement were greater than 2 times the intrassay CV, and in intact animals, where hormone levels are lower and the risk of false positive errors higher {Veldhuis et al, 1985}, a multiple of 4 times the intraassay CV was applied.

Within each steroid treatment group, the following attributes of the identified pulses

were recorded for each rat: the nadir concentration (of the nadir preceding the pulse), the amplitude, the pulse frequency, and the mean serum hormone concentration over the entire basal sampling period. Mean pulse frequency was calculated by dividing the time between the first and last pulses in a sampling session by the number of pulses for that session; records without pulses were excluded from the frequency calculation and records with only one pulse were assigned an interpulse interval equal to the duration of the sampling period.

4.5.2 *Statistics*

Mean group values are expressed as the mean of the individual mean values ± the standard error of the mean. Multiple means were analyzed by analysis of variance (ANOVA) and multigroup comparisons using Newman-Keuls multiple range test, or Dunnett's test. Pairs of means were compared using Student's T-test. The level of significance was taken as $p \leq 0.05$. Two software programs were used for the statistical calculations:

1. "CSS (Complete Statistical System): Statistica"

   StatSoft Inc., 1991, Tulsa, OK

2. "Pharm/PCS (Pharmacological Calculation System)" Version 4.2

   R.J. Tallarida & R.B. Murray

   MicroComputer Specialists, 1990, Philadelphia, PA

RESULTS

5.1 The Feedback Effects of DHT on LH in the Orchidectomized Male Rat

The androgen dihydrotestosterone (DHT) can suppress the high plasma LH concentrations that arise following the castration of an adult male rat {Verjans et al, 1974; Verjans & Eik-Nes, 1976; Smith & Davidson, 1974}. Testosterone (T) too, has such negative feedback effects {Gay & Dever, 1971; Negro-Vilar et al, 1973a; Kalra et al, 1973; Bloch et al, 1974; Kalra & Kalra, 1982} and, paradoxically, can also induce a further increase in LH levels in orchidectomized rats when the steroid dose is low {Swerdloff & Walsh, 1973; Kalra et al, 1973; Bloch et al, 1974; Verjans et al, 1975}. As DHT is a metabolite of T, and is generated in tissues considered important for the positive and negative feedback effects of T upon LH, it is possible that DHT is mediating some or all of the effects attributed to T. The present experiment characterizes the means by which DHT effects the regulation of LH pulsatility, thus permitting a comparison with similar data already available for T {Summerville & Schwartz, 1981; Steiner et al, 1982; Ellis & Desjardins, 1984a; Grosser, 1987; Culler, 1990}. To this end, several groups of adult male rats were orchidectomized and treated with a range of DHT doses for three weeks, then sampled serially to generate a view of the pattern of plasma LH fluctuations over time. Pattern analysis yields an accurate measure of mean LH concentrations and LH pulse characteristics, and a challenge with exogenous LHRH provides an indication of pituitary's ability to respond to endogenous LHRH pulses. The weights of androgen-dependent tissues provide an in vivo bioassay for the relative androgenicity of the various steroid treatments.

5.1.1 *Tissue weights*

The effects of orchidectomy and graded androgen replacement on ventral prostate and

paired seminal vesicle weights are shown in figure 6. Four weeks following castration without steroid replacement the weights of both accessory tissues had dropped to less than 30% that of intact rats. With the exception of the lowest dose of DHT, which did not significantly increase seminal vesicle weight, the castration-induced atrophy was reduced in both tissues by all treatments, though the greater sensitivity of the ventral prostate indicates that the potency of a given androgen treatment varies depending upon the tissue under consideration. The prevention of atrophy by DHT was dose-dependent, though even with the largest DHT dose the ventral prostate and seminal vesicle weights remained slightly but significantly below those of testes-intact animals. Higher doses of DHT can bring accessory tissue weight back to intact levels and beyond {Wesolowski & Robaire, unpublished}.

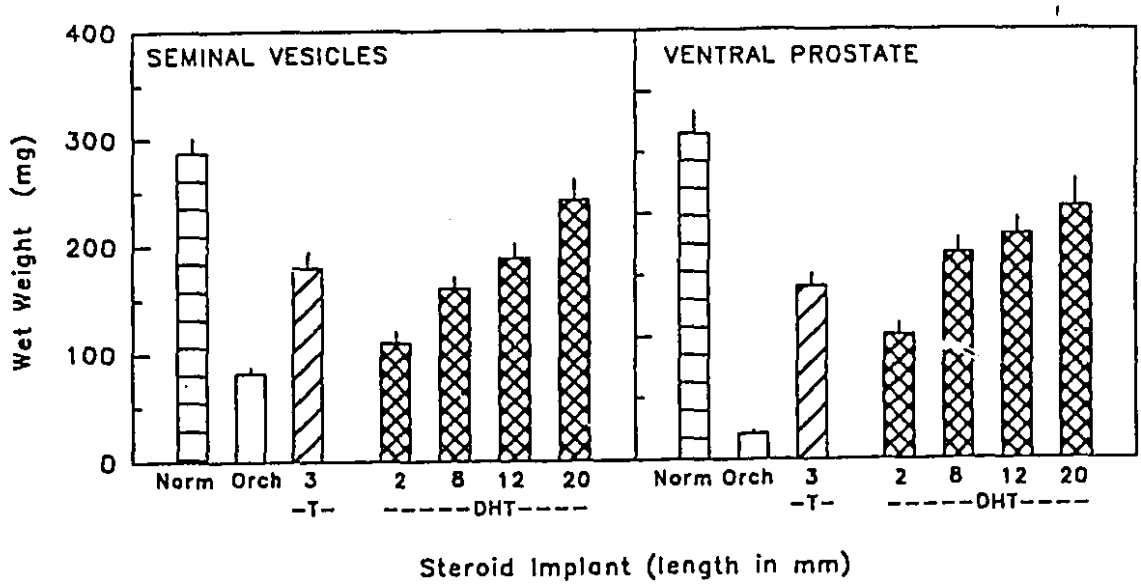

Figure 6. Wet weights of paired seminal vesicles and the ventral prostate in intact adult male rats (Norm), and 4 weeks following orchidectomy and subdermal placement of a 20 mm empty implant (Orch), a 3 mm testosterone (T), or 2 mm, 8 mm, 12 mm, or 20 mm dihydrotestosterone (DHT) implant. Represented are mean values $\pm$ SE ($n = 6, 9, 7, 11, 11$, and 9 respectively). DHT increased tissue weight dose-dependently (ANOVA, $p<0.05$). Tissue weights in all steroid-treated animals were significantly greater than in orchidectomized control animals, with the exception of seminal vesicle weight in rats bearing a 2 mm DHT implant ($p<0.05$). Tissue weights in all treated animals remained significantly lower than in normal rats ($p<0.05$).

5.1.2 *DHT dose-dependently suppresses mean LH concentration*

Figure 7 shows the effects of graded androgen replacement on mean plasma LH levels in the male rat three weeks after castration and steroid treatment. In the orchidectomized rats given an empty implant (the control group), the mean plasma LH concentration had risen to 3.84 ± 0.32 ng/ml, reaching almost 20-fold the LH levels found in testes-intact rats (0.23 ± 0.02 ng/ml; see results section 5.3.1). The T released from the 3 mm implant resulted in a mean LH concentration of 120% (4.60 ± 0.51 ng/ml), a change that was not statistically significant relative to untreated orchidectomized rats. The lowest dose of DHT did not alter the high post-orchidectomy mean LH levels, (2 mm DHT: 3.77 ± 0.47 ng/ml; 98% of control), however, with increasing implant length DHT exerted a dose-dependent negative

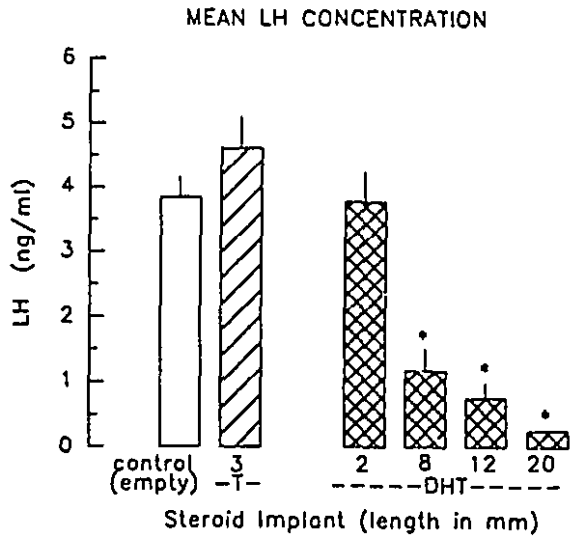

Figure 7. Mean plasma LH concentrations three weeks after adult male rats were orchidectomized and treated with an empty implant, a 3 mm testosterone (T), 2 mm, 8 mm, 12 mm, or 20 mm dihydrotestosterone (DHT) implant. For each rat LH concentrations measured in all the plasma samples collected during the 3 h sampling period were averaged. Values shown are the mean ± SE of averages from the individual rats in each treatment group (n = 9, 7, 7, 5, 5, and 6 respectively). *, significantly different from the untreated orchidectomized (control) group ($p < 0.05$).

feedback effect, lowering mean plasma LH concentration to 30%, 20%, and <7% of control levels in rats treated with 8, 12 and 20 mm DHT implants respectively. In all rats treated with a 20 mm DHT implant and in one rat carrying a 12 mm DHT implant, androgen suppression of LH was considered complete as their LH levels were at or below the limit of detection of the radioimmunoassay (0.26 ng/ml).

5.1.3 *DHT slows LH pulse frequency and can reduce pulse amplitude*

All plasma LH patterns that were above the assay detection limit were pulsatile, and were analyzed for the effects of androgen replacement on pulse parameters observed over the three hour serial sampling period. Shown in figure 8 are sample records contrasting the pattern of LH pulses over time from one rat in each DHT treatment group. Clearly, the suppression of mean LH concentration observed with larger implants of DHT (figure 7) is the net result of negative feedback effects on pulse frequency, pulse amplitude, and nadir and peak values, which decrease in a dose-dependent manner with increasing DHT dose. The mean group values for pulse frequency, amplitude and nadir are shown for each treatment group in figures 10, 11 and 12.

Of the DHT doses tested, the 8 mm implant was the first to suppress mean LH levels, and reduced pulse frequency to 63%, pulse amplitude to 69%, and nadir values to 25% of control, with the net effect of a 70% drop in mean LH. Qualitatively similar but stronger effects underlie the further attenuation of mean LH concentration in rats treated with 12 mm DHT implants. Because LH is suppressed below the limit of detection of the radioimmunoassay in those rats carrying a 20 mm DHT implant, their pattern of LH secretion is unknown, though it seems likely that an even stronger suppression of LH pulse frequency and amplitude is exerted by this larger DHT dose.

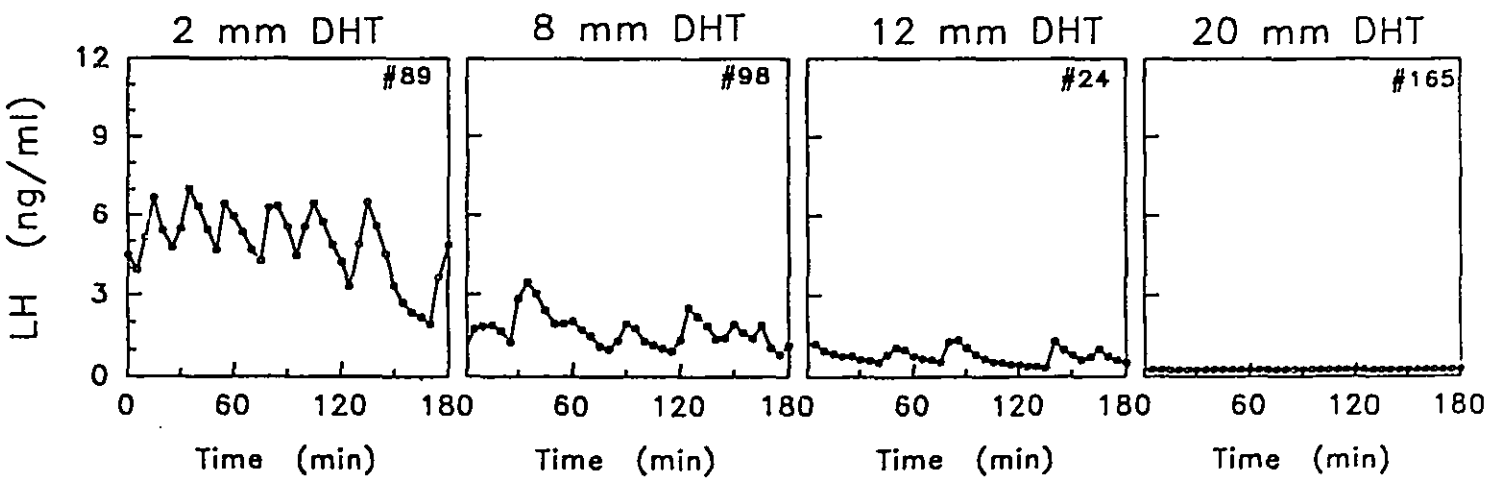

Figure 8. Samples of the plasma LH patterns in adult male rats 3 weeks following orchidectomy and subdermal placement of a 2 mm, 8 mm, 12 mm, or 20 mm dihydrotestosterone (DHT) implant. Serial plasma samples were obtained at 5 minute intervals for 3 h beginning at 10:00 a.m.. LH concentrations were determined using LH-RP-3 for the RIA standard. For purposes of comparison the LH pattern representing the 2 mm DHT-treated group is also shown in the figure 9.

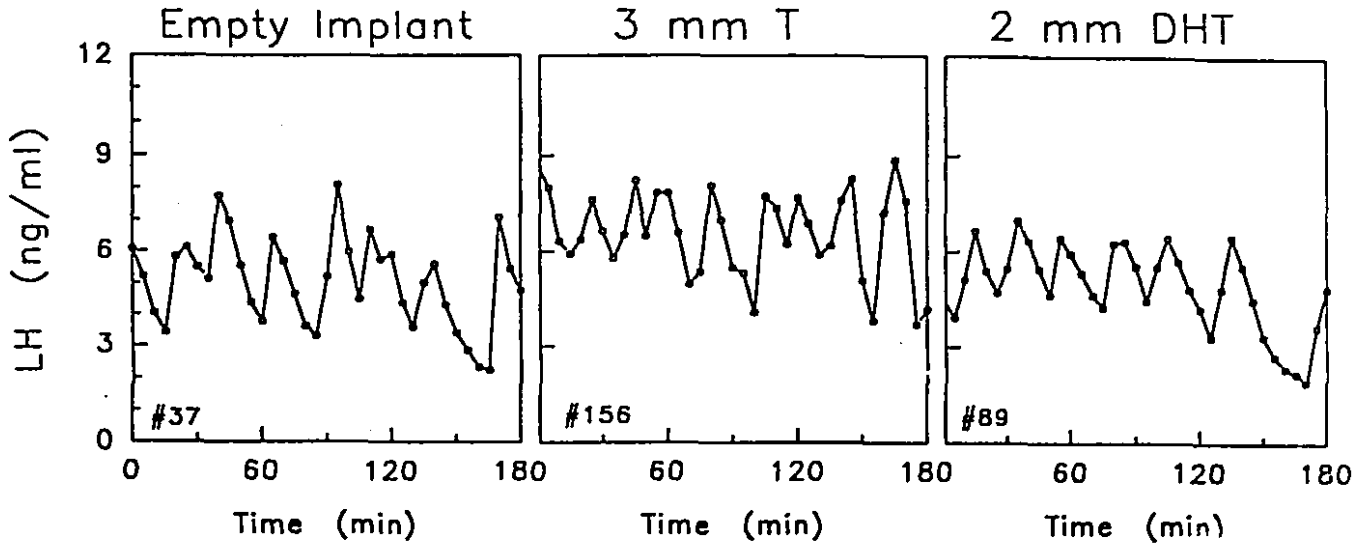

Figure 9. Samples of the plasma LH patterns in adult male rats 3 weeks following orchidectomy and subdermal placement of an empty implant, a 3 mm testosterone (T), or 2 mm dihydrotestosterone (DHT) implant. Serial plasma samples were obtained at 5 minute intervals for 3 hours beginning at 10:00 a.m.. LH concentrations were determined using LH-RP-3 for the RIA standard. For purposes of comparison, the LH pattern representing the 2 mm DHT-treated group is also shown in the preceding figure.

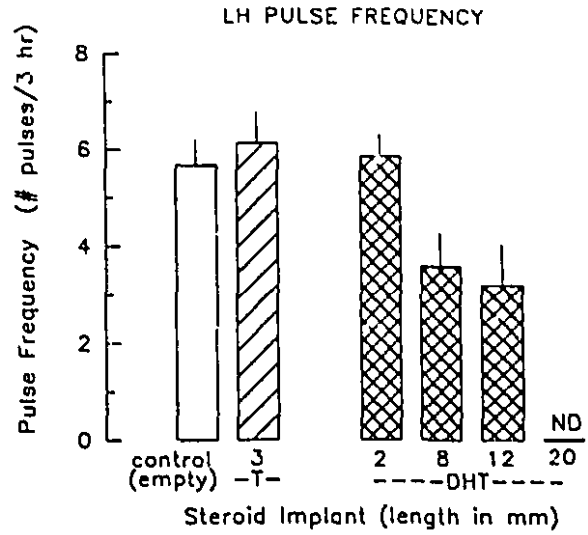

Figure 10. LH pulse frequency (# pulses/3 h), 3 weeks after adult male rats were orchidectomized and treated with an empty implant, a 3 mm testosterone (T), 2 mm, 8 mm, 12 mm, or 20 mm dihydrotestosterone (DHT) implant. Average pulse frequency was calculated for each rat as the number of complete pulses observed during the 3 h sampling period. Values are the mean ± SE of averages for the individual rats in each treatment group (n = 5-9). ND, pulses were not detectable; *, significantly different from the orchidectomized control group ($p \leq 0.05$).

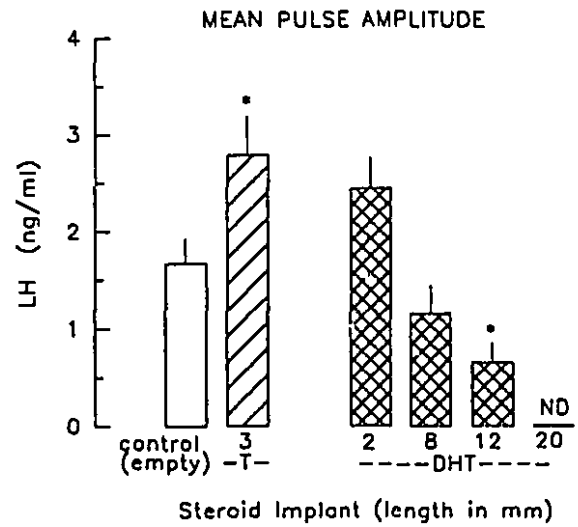

Figure 11. LH pulse amplitude 3 weeks after adult male rats were orchidectomized and treated with an empty implant, a 3 mm testosterone (T), 2 mm, 8 mm, 12 mm, or 20 mm dihydrotestosterone (DHT) implant. Average pulse amplitude was calculated for each rat as the average difference between the peak and the preceding nadir of all pulses observed during the 3 h sampling period. Values are the mean ± SE of averages for the individual rats in each treatment group (n = 5-9). ND, pulses were not detectable; *, significantly different from the orchidectomized control group.

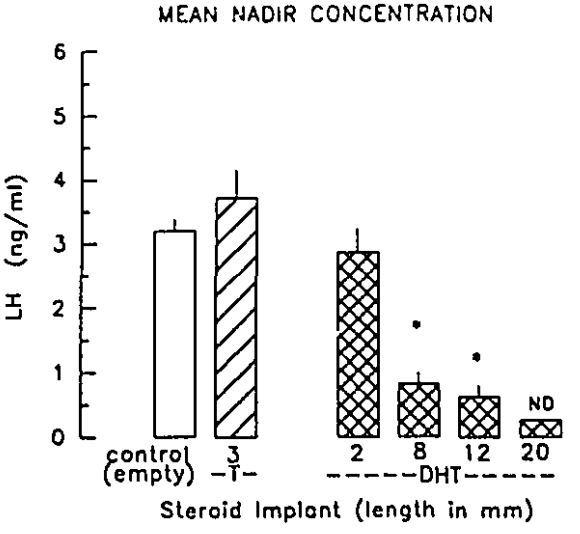

Figure 12. Mean LH pulse nadir, 3 weeks after adult male rats were orchidectomized and treated with an empty implant, a 3 mm testosterone (T), 2 mm, 8 mm, 12 mm, or 20 mm dihydrotestosterone (DHT) implant. Average pulse nadir was calculated over the 3 h sampling period for each rat, and the values represented above are the mean ± SE of averages for the individual rats in each treatment group (n = 5-9). ND, pulses were not detectable; *, significantly different from the orchidectomized control group.

5.1.4 *Low dose androgens have positive feedback effects on LH pulse amplitude*

In figure 9 the low dose DHT-treated group is represented again for comparision with samples of the T-treated and orchidectomized control patterns. Though neither of these low dose androgen treatments significantly alters mean LH levels (figure 7), changes are apparent in the underlying pulsatile patterns (figures 10 and 11); the low dose T treatment increased LH pulse amplitude (2.79 ± 0.39 ng/ml) above that observed in the control rats (1.67 ± 0.26 ng/ml). The average LH pulse amplitude was not significantly different between the two low dose androgen-treatments (2 mm DHT: 2.44 ± 0.32 ng/ml) though only in T-implanted rats was the amplitude increase significant relative to orchidectomized controls ($p \leq 0.05$) thereby leaving it uncertain whether low dose DHT mimicked the positive feedback effect of low dose T. LH pulse frequency and nadir were similar in these three groups.

5.1.5 *The pituitary secretory response to LHRH challenge*

Serum LH levels are increased following the intravenous, bolus administration of a supraphysiological dose of LHRH, as shown in figure 13. Serial blood sampling before and after LHRH injection show that the pituitary's secretory response is acute and transient, with maximal plasma LH concentrations achieved after about 15 minutes and a decline underway by 30 minutes post-injection. Animals treated with a 20 mm DHT implant had an undetectable pre-injection LH mean yet some exhibited a detectable, albeit small, LHRH-induced increase in LH indicating that even in this severe state of suppression of the hypothalamo-pituitary axis the pituitary can respond to LHRH.

In the T-treated males, the LHRH-induced LH increment was not significantly different from controls (12.1 $\pm$ 1.5 vs 12.1 $\pm$ 1.4 ng/ml LH), as shown in figure 14. In marked contrast, at the lowest dose used (2 mm implant), DHT dramatically reduced the response to 55% of control (6.63 $\pm$ 0.53 ng/ml), and 8 and 12 mm DHT implants further diminished it to 29% and 15% of control respectively. The potency of DHT in suppressing pituitary LH secretion is much greater than that of T, (even a 12 mm T implant is less effective than the 2 mm DHT implant used in these studies {Grosser, 1987})

The results presented in this section suggest that DHT inhibits the post-orchidectomy rise in plasma LH levels by the same means as T, i.e. by reducing the frequency of LH pulses (presumably a hypothalamic effect), and suppressing LH pulse amplitude, as observed in animals treated with 8, 12, and 20 mm DHT implants. Control over LH pulse amplitude may be exerted at the level of the hypothalamus, by modulating events that determine the magnitude of the LHRH pulse in the portal blood, or at the pituitary by regulating the cellular events that translate the incoming LHRH signal into LH output. That DHT acts potently at

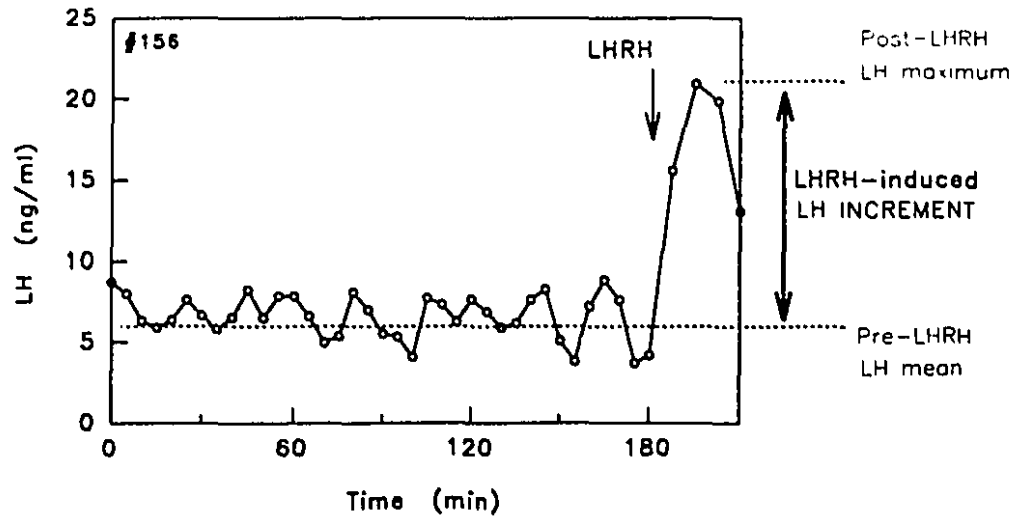

Figure 13. A sample LH pattern showing the pituitary secretory response to exogenous bolus of LHRH in a testosterone-treated rat 3 weeks after orchidectomy and implant placement. The sampling rate was every 5 minutes before LHRH injection, and every 7.5 minutes thereafter. Note that the post-LHRH LH increment is defined as the difference between the highest post-LHRH LH concentration and the mean pre-LHRH concentration.

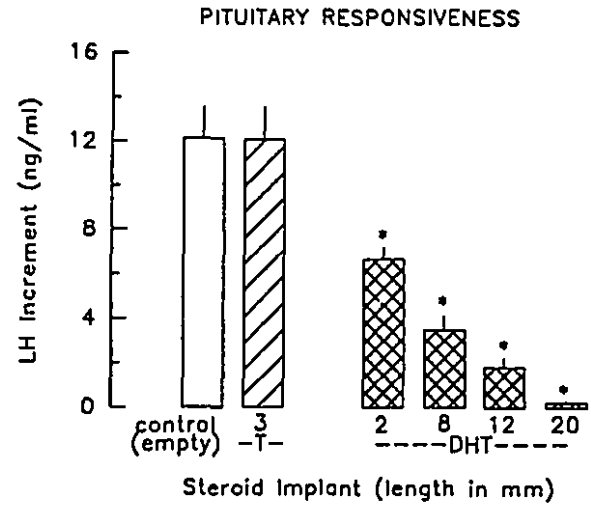

Figure 14. Pituitary responsiveness to an exogenous bolus of LHRH, in adult male rats orchidectomized and treated 3 weeks previously with an empty implant, a 3 mm testosterone (T), 2 mm, 8 mm, 12 mm, or 20 mm dihydrotestosterone (DHT) implant. The LHRH-induced LH increment is defined in figure 12. Values are the mean ± SE of data from individual rats in each treatment group (n = 6, 7, 6, 5, 4, and 6 respectively).

the pituitary level to attenuate LH pulse amplitude is suggested by the dramatic decrease in the LHRH-induced LH increment in animals receiving even the lowest DHT dose.

The low dose T-treated rats had an LH pulse amplitude greater than that observed in the untreated orchidectomized control group. While higher doses of T are known to suppress both pulse frequency and pituitary responsiveness {Swerdloff et al, 1972; Debeljuk et al, 1973; Debeljuk et al, 1974; Cheung & Davidson, 1977; Steiner et al, 1982; Grosser, 1987}, the sub-physiological levels of circulating T released from the small (3 mm) implant used in this experiment did not affect either of these parameters. If indeed the pituitary response to the exogenous LHRH bolus reflects pituitary responsiveness to endogenous LHRH pulses, then it must be concluded that the T-induced increase in LH pulse height is not effected at the level of the pituitary, (which is equally responsive in the T-treated and orchidectomized control groups), leading one to infer that T must act at the level of the hypothalamus to regulate (increase) LHRH pulse amplitude.

An analogous deduction can be made regarding the effects of low dose DHT treatment upon plasma LH. The 2 mm DHT implant did not reduce mean LH concentration, LH pulse frequency, or pulse amplitude, yet it severely suppressed the pituitary's ability to secrete LH following a challenge with exogenous LHRH. To maintain LH pulse amplitude in spite of a decrease in pituitary responsiveness, the amplitude of the incoming LHRH signal must have been larger; thus it seems that low doses of DHT are able to regulate LH pulse amplitude at the level of the hypothalamus in a positive fashion, as well as at the pituitary in a negative fashion.

5.2 THE ROLE OF OPIOIDS IN THE ANDROGEN REGULATION OF PLASMA LH

The literature holds much evidence suggesting that endogenous opioids can suppress LH pulse frequency and amplitude, and that these effects are affected at the level of hypothalamic LHRH release rather than upon pituitary's ability to respond to the LHRH signal. To determine whether or not a reduction in opioid inhibition of LHRH release accompanies the suprapituitary stimulation of LH pulse amplitude by low dose DHT inferred in the previous section, naltrexone was administered to three week orchidectomized rats treated with an empty implant or with a 2 mm DHT implant. If the hypothesis is correct, that LHRH pulse amplitude in the DHT-treated rats is higher because their LHRH neurosecretory system is subject to less suppression from endogenous opioids, then less disinhibition would result from the administration of an opioid antagonist to these rats than to the orchidectomized control animals, i.e. the increment in LH that follows a naltrexone injection would be smaller in the DHT-treated animals (than in control rats). A basal LH pattern was established to demonstrate again that an increased LHRH pulse amplitude could be inferred from the pulse characteristics of the DHT-treated orchidectomized rats. Naltrexone was then administered and the magnitude of the resulting LH increment noted.

5.2.1 *The effect of naltrexone on LH pulse parameters in DHT-treated rats*

Figure 15 shows a sample 3.5 h LH pattern for adult male rats orchidectomized and treated for 3 weeks with a 2 mm DHT implant. The basal LH pattern was established over the first 2 h, then a bolus of naltrexone and LHRH were injected intravenously, one hour apart, and the post-injection LH increment induced by each stimulant was quantitated (see Figure 15). The mean LH concentration of samples collected before naltrexone injection was

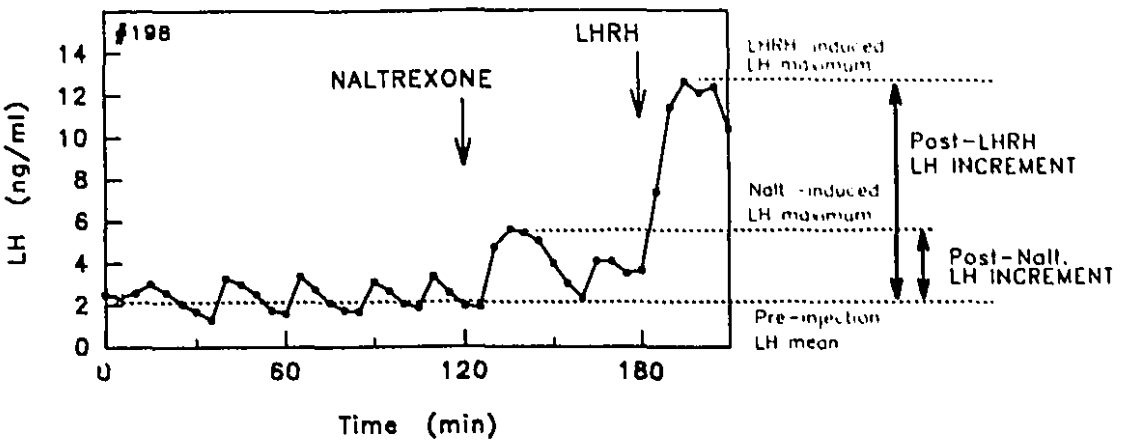

Figure 15. A sample LH pattern showing the acute LH secretory response to naltrexone and LHRH administration in an adult rat 3 weeks following orchidectomy and treatment with a 2 mm dihydrotestosterone (DHT) implant.

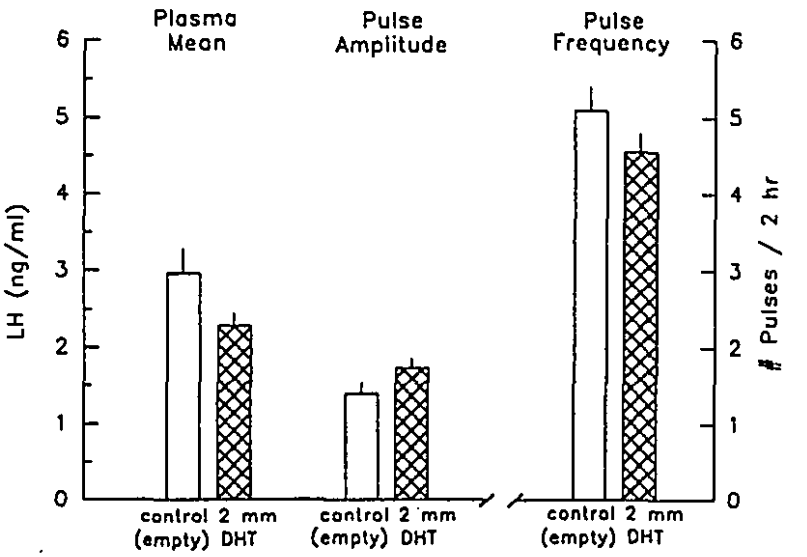

Figure 16. LH pulse analysis for the 2 h basal sampling period that preceded naltrexone injection in adult male rats 3 weeks following orchidectomy (Orch) and treatment with an empty (control; n=10) or a 2 mm dihydrotestosterone (DHT) implant (n=8). The units of LH pulse frequency are number of pulses per 2 h sampling period. Values are the mean ± SE.

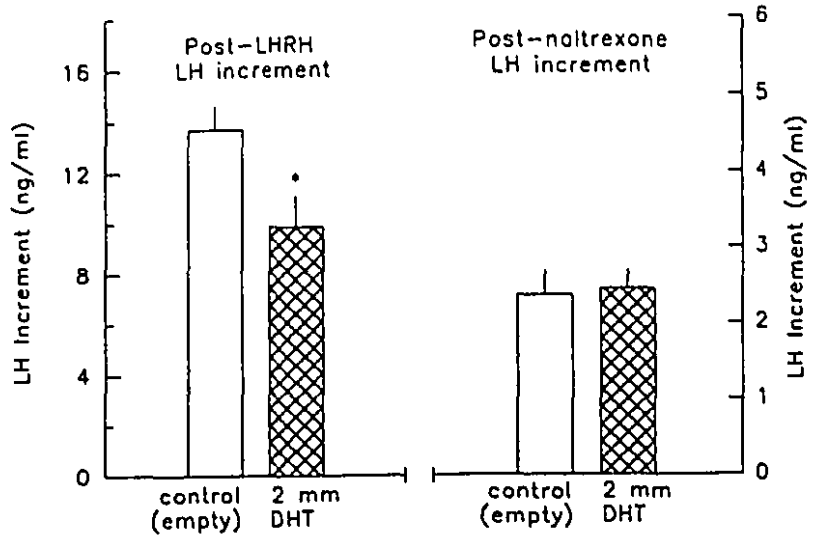

Figure 17. The LH secretory response induced by LHRH and naltrexone administration in control orchidectomized rats (n=10), and in those bearing a 2 mm dihydrotestosterone (DHT) implant (n=8). The post-LHRH LH increment and the post-naltrexone LH increment are defined in figure 15. Values are the mean ± SE of increment data from individual rats in the two treatment groups.

not altered significantly by DHT treatment, and nor was the frequency or amplitude of LH pulses occurring over the 2 hour basal sampling period (Fig. 16). However, the LH increment induced by an exogenous bolus of LHRH was significantly lower in the DHT-treated animals (Fig. 17), confirming previous results (section 3.1), which infer that DHT treatment has caused an increase in LHRH pulse amplitude.

Rats with or without a low dose DHT implant responded to the bolus injection of naltrexone with an acute and transient rise in plasma LH concentration, indicating that in both groups of animals the endogenous opioid system tonically inhibits LH secretion. The naltrexone-induced LH increment was of the same magnitude in both treatment groups (see Fig. 17).

5.3 T AND LH PULSATILITY IN THE ADULT MALE RAT

To characterize the relationship between T and its pulsatile secretagogue LH, on a moment-to-moment basis, and determine whether plasma levels of this androgen decline into the concentration range associated with positive feedback, normal (testes-intact) adult male rats were bled at 10 minute intervals for 8 h, and the plasma samples assayed for both T and LH. This approach was also used to investigate the acute effect(s) on both hormones of environmental light cues, in particular "morning" and "evening" transitions between light and darkness, as such cues have been identified as potent regulators of the hypothalamo-pituitary-testicular loop in some species {Ellis & Follett, 1983; Follett & Milette, 1982}, and may underlie the diurnal LH and T rhythms reported in rats.

5.3.1 *The nature of T and LH fluctuations in the testes-intact adult male rat*

Forty 8-hour records of hormone concentration over time (T, n=19; LH, n=21) were obtained from 8 rats each serially sampled on 1-4 occasions. Samples of concurrent T and LH patterns are shown in figures 18 and 19. Pulses were evident in all LH patterns, occurring irregularly with periods of relative quiescence interspersed among singlet (Fig 18: #8s2, #9s1, #13s1) or small groups of pulses (Fig 18: #11s1; Fig 19: #17s3). Generally, an LH peak rose quickly, going from nadir to peak within 10-20 minutes, then declined to baseline within the next 20-50 minutes.

In 17 of the 19 T patterns, at least 2 (and as many as 8) strikingly short sharp pulses of testosterone were identified; figure 18 (#11s1) and figure 19 (#19s2) are examples of the more active testosterone patterns. Testosterone pulses varied in amplitude and frequency and regularity between animals, and more suprisingly, between sampling sessions within the same

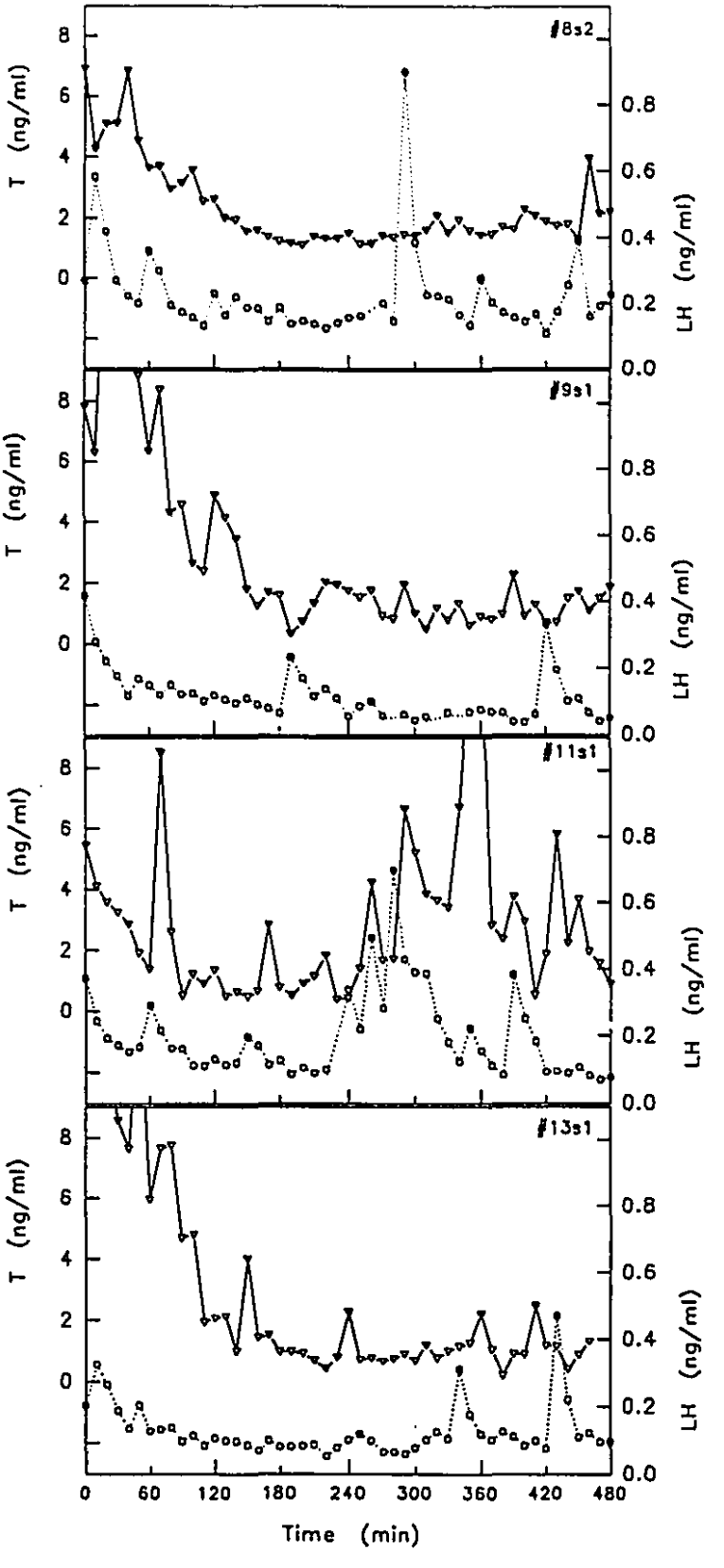

Figure 18. Samples of concurrent plasma T (testosterone; solid lines) and LH (dotted lines) in four testes-intact adult male rats bled every 10 minutes for 8 h during their daylight hours. LH concentrations were determined using the LH-RP-3 RIA standard.

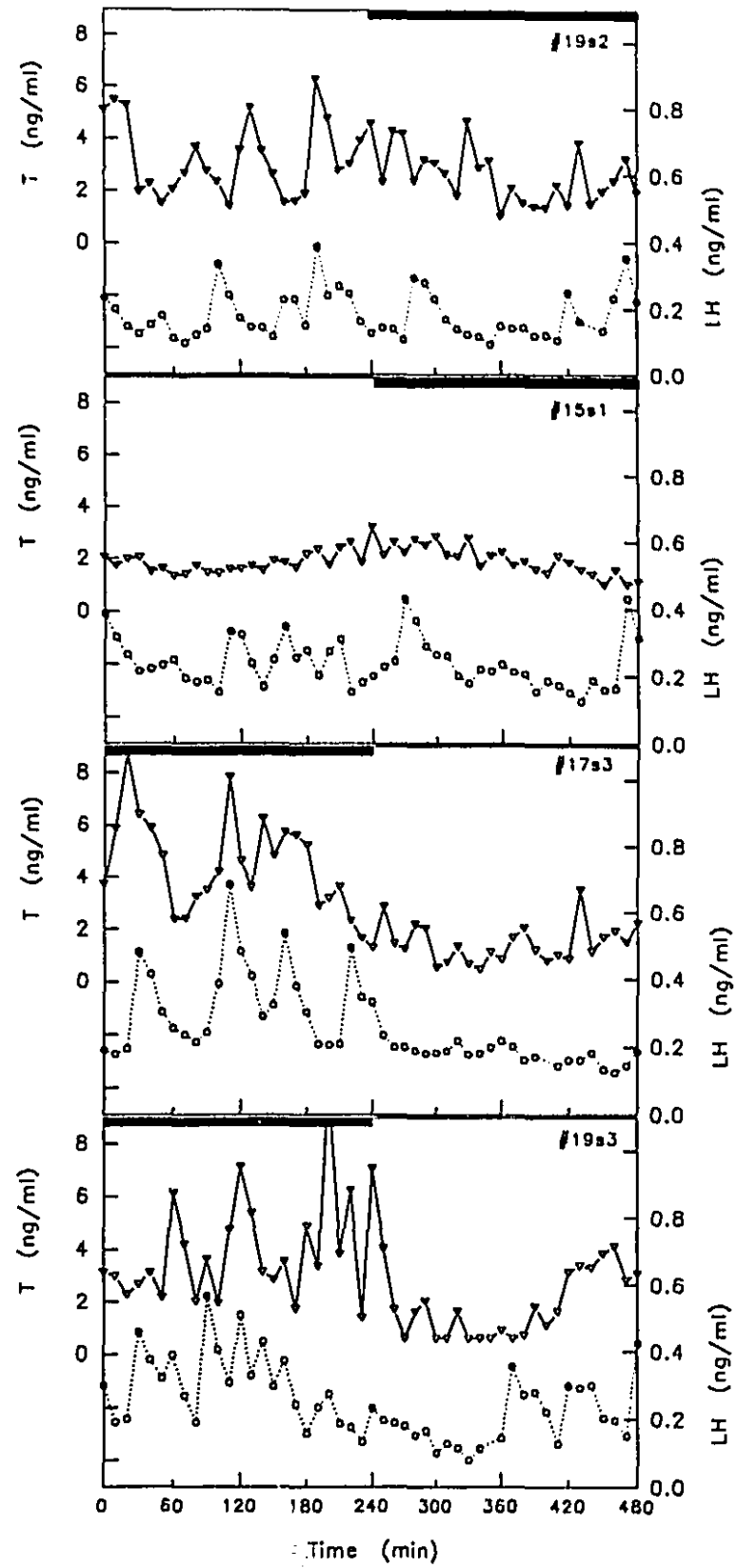

Figure 19. Samples of concurrent plasma T (testosterone; solid lines) and LH (dotted lines) concentrations in four testes-intact adult male rats bled at 10 minute intervals for 8 h. The solid bars at the top of each panel indicate the dark hours (lights off at 19:00, lights on at 07:00). LH concentrations were determined using the LH-RP-3 RIA standard.

animal. Generally, pulses of serum T rose within 10-20 min to peak values 0.6-8.6 ng/ml above the nadir, and then fell exponentially, reaching basal levels 30-40 min later.

Where several high amplitude testosterone elevations occurred in quick succession (e.g. 3-4 peaks in 3-4 h) the bases of the pulses sometimes coalesced, resulting in a T "episode," i.e. a long duration (>3 h) low amplitude (<3 ng/ml) rise in the baseline, on which the testosterone pulses were sometimes superimposed (Fig 18: #11s1; Fig. 19: #17s3). In several patterns, testosterone episodes occurred in the absence of detectable testosterone pulses (figure 19, #15s1), though the unsteadily rising baseline suggests that pulses might actually have occurred but could not be distinguished individually as they were immediately succeeded by another rise and/or were of low amplitude (see Fig. 19, #15s1, 150-420 min). Periods of relatively stable baseline levels also occurred between T pulses, though they were relatively infrequent (Fig. 18: #8s2; Fig. 19: #15s1, 0-150 min). Thus it appears that the nature of plasma T patterns in the intact adult male rat is highly variable, both within and between individual animals, reflecting the capacity of the rat testes for a wide range of secretory activity, from short sharp pulses to almost steady baseline secretion.

For each 8-hour hormone record an average plasma concentration was calculated, and for LH the overall mean was 0.228 ± 0.015 ng/ml (n=21), while mean T levels were 2.40 ± 0.15 ng/ml (n=19). Testosterone pulse height was variable both within and between individuals (2.40 ± 0.26 ng/ml), and nadir values (1.18 ± 0.13 ng/ml) were, on average, 52% of mean T concentration within a given sampling session. The frequency distribution of nadir values in figure 20 shows that for up to 22% of the pulses identified, the T concentration in the trough preceding a pulse was less than 30% of the mean T level for that animal; this is in the range of plasma T levels that can stimulate LH pulse amplitude and mean concentration in orchidectomized rats.

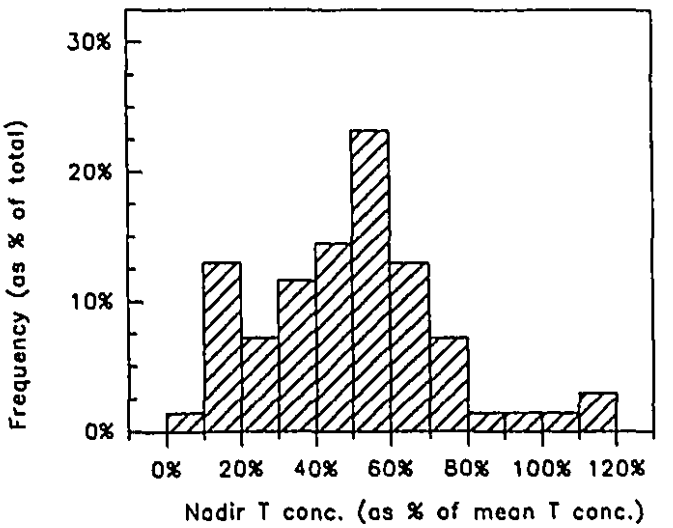

Figure 20. Frequency distribution of nadirs in testosterone concentration expressed as a percent of mean testosterone level in each rat. Note that almost one quarter of the nadirs are in or below the concentration range associated with positive feedback (i.e. 20-30% of mean T levels).

5.3.2 *The relationship between LH and T pulses*

The average number of testosterone pulses (3.9 ± 0.5/8 hr; n=19) did not differ significantly from the mean LH pulse frequency (4.4 ± 0.3/8 hr; n=21). However, it is important to note that though these pulse frequencies are similar, a visual examination of the twenty-one 8-hour patterns does not provide clear evidence of a consistently close temporal association between LH and testosterone pulses. The oscillations are not consistently related to the LH pulses, i.e. not all T pulses appears to be causally related to a distinct rise in LH concentration, at least not with a defined and consistent lag time. Nonetheless, many of the LH peaks are followed by a rise in plasma T, and episodic (broad-based) elevations in T are usually preceded by a train of LH pulses.

The relationship between the amplitude of LH pulses and the corresponding peak in circulating testosterone levels is ambiguous. This is illustrated by the hormone patterns in

figure 19 (#19s2 and #15s1) in which two rats show similar LH patterns, yet the associated testosterone patterns are quite different, representing the extremes of active and quiescent testicular secretory activities. Even within a given sampling session in a particular rat, the magnitude of the T pulses does not relate consistently to the amplitude of the preceding LH peak. For example, in figure 18 #8s2 there is a strikingly high amplitude LH pulse that is ineffective in eliciting an acute increase in T secretion, yet 2 hours later a more moderately sized rise in LH is followed by a distinct pulse of T. Thus it seems that there is variability in the magnitude and time lag of the Leydig cell response to LH, suggesting that factors other than LH contribute to the acute modulation of testosterone levels. Such other factors could include blood flow, nervous input, and the endocrine or paracrine effects of inhibin, FSH, LHRH, growth factors, and prostaglandins {Hall, 1988; Saez et al, 1987; Robaire & Bayly, 1989; Khan et al, 1993}.

5.3.3 *Effects of changes in lighting*

The records shown in figure 19 provide two examples from different lighting protocols. A striking drop in testosterone and LH concentrations is visible in most of the 8 hour records encompassing the morning dark-to-light transition. To quantify this decline, the mean hormone concentrations for the first 4 hours of sampling (pre-transition) were compared to those for the last 4 hours (post-transition) for this sampling period, and also for the 8 hour session covering the evening light to dark transition (Fig. 21). The morning T and LH levels fall significantly to 55% and 78% of their "pre-dawn" levels, respectively; in contrast, the 4 hour means for either hormone are not significantly different in the 4 hours of darkness following lights-off, relative to the previous 4 hours of light.

To characterize more precisely the temporal changes in T and LH concentration

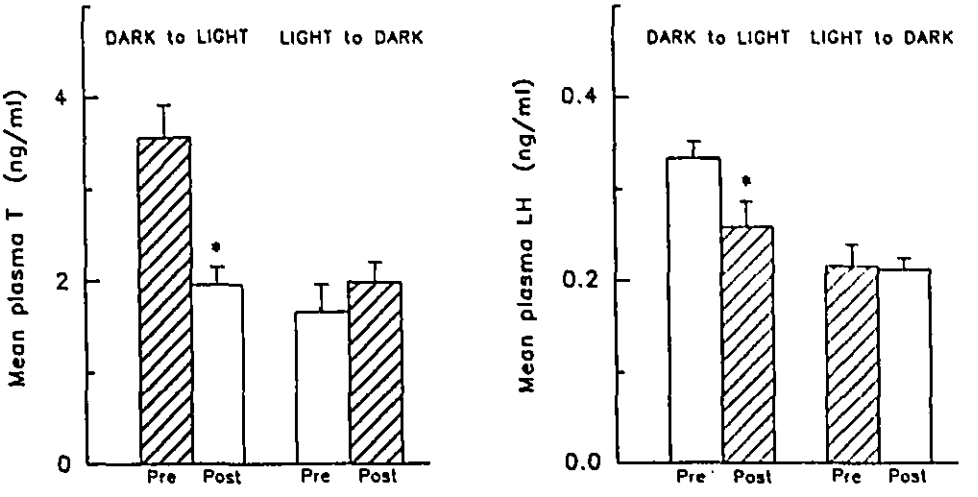

Figure 21. Average plasma T (left panel) and LH (right panel) concentrations in intact adult male rats during the 4 hours preceding ("Pre") and following ("Post") a transition in lighting from dark to light and from light to dark. Rats were serially sampled every 10 minutes for 8 hours starting 4 h before their cage lights came on at 7 a.m., or 4 hours before their cage lights went off at 7 p.m.. Pre- and post- transition hormone levels are shown as mean values $\pm$ SE for each rat group. *, significantly different from the pre-transition period mean value ($p < 0.05$).

associated with transition periods, the data were reanalyzed: the 8 hours of sampling data was divided into 40 minute intervals, with 6 falling on either side of the lighting transition, and hormone concentrations were averaged for each period. This interval of 40 minutes was chosen in order to smooth over fluctuations due to individual pulses apparent at 10 min sampling intervals, without masking gradual overall trends in hormone levels by using too large an interval. It is evident from figure 22 that the morning decrease in testosterone has begun to occur by the first 40 minute period following lights-on, and is significant for both testosterone and LH by the second period. At least for testosterone, the downward trend appears to begin even before "lights on," suggesting that the trigger for this decline might be a (light-entrained?) circadian rhythm, rather than the exposure to light per se. The 40 minute analysis of data from the light to dark records confirms the lack of an acute effect of this lighting transition on LH and T levels.

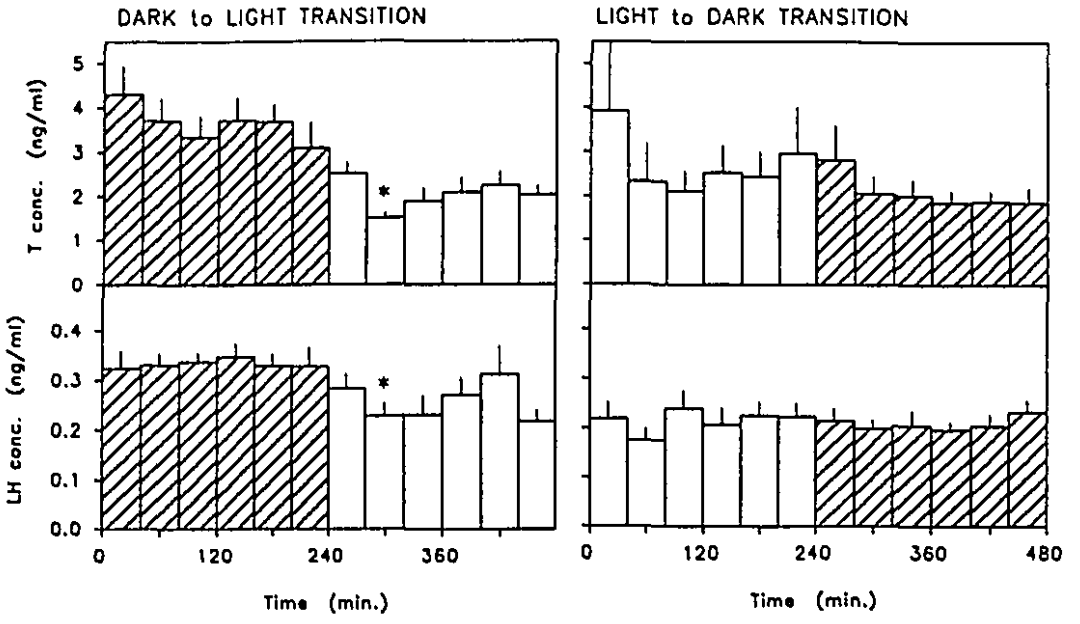

Figure 22. The effects of dark/light and light/dark transitions on serum concentrations of testosterone (T) (upper panels) and LH (lower panels) in intact adult male rats. For each rat, hormone concentration data gathered within a 40-minute period was averaged, and each bar represents the mean ± SE of these 40 minute averages for rats experiencing a dark to light transition at 7 a.m. (left panels; n=9), or a light to dark transition at 7 p.m. (right panels; n=6). Hatched bars indicate the dark periods. The asterisks indicate the first posttransition hormone level that is significantly different from pretransition hormone levels, ($p < 0.05$).

DISCUSSION

The work in this thesis focuses on the regulation of the testicular production of testosterone, and on the role of its major androgenic metabolite, dihydrotestosterone, in the neuroendocrine feedback loop that coordinates reproductive processes in the male. The results presented in the preceding section examine the means by which DHT exerts it's influence on pulsatile LH secretion, and investigate the involvement of hypothalamic and pituitary sites and neuroregulatory mechanisms underlying this control in the orchidectomized rat model. The present discussion will relate these observations to the role of testosterone in the positive and negative modulation of LH release in the orchidectomized rat and in the intact animal. The importance of the phenomenon of positive feedback for the normal operation of the hypothalamo-pituitary-testicular loop is addressed in a study that also evaluates the role of LH and other factors as regulators of testicular T secretion.

6.1 NEGATIVE FEEDBACK BY ANDROGENS

Mean LH levels in the 3-week orchidectomized rats carrying only steroid-free implants (controls) were 20-40 times higher than in intact rats, due to the increased frequency and amplitude of secreted pulses, and this is consistent with results reported by other investigators {Steiner et al, 1982; Sodersten et al, 1983; Ellis & Desjardins, 1984a}. Nadir values of LH were also greatly elevated in these control rats, as expected in association with this high frequency of episodic LH secretion, due to the brevity of the interpulse period relative to the disappearance rate of the hormone; there may also be an increase in basal LH secretion, but this can be neither concluded nor excluded on the basis of these data (see Appendix 3).

The role of DHT in the feedback loop between the testes and the brain-pituitary axis has not yet been established for the intact mammal. Studies investigating the effects of DHT on mean LH concentration in the orchidectomized rat {Swerdloff et al, 1972; Naftolin & Feder, 1973; Verjans & Eik-Nes, 1976}, rams {Schanbacher, 1984}, and man {Santen, 1975; Ando et al, 1978; Winters et al, 1979a} and in female rat {Beyer et al, 1972} have observed a DHT-associated suppression of LH concentration. Results presented here are consistent with those reports; in the adult orchidectomized rat a suppression of mean LH levels was observed following a 3 week treatment with moderate to high doses of DHT (8 to 20 mm implants; 19 - 48 μg DHT/day). In addition, this effect was found to be dose-related.

Analysis of the LH patterns in DHT-treated rats clearly shows that DHT induces a dose-dependent reduction in both the frequency and amplitude of the LH pulses, and that these effects on pulse parameters underlie the suppression of mean LH concentrations. While this is the first study examining the effect of DHT upon the LH pulse parameters in the male rat, the suppressive effects of this T metabolite on LH frequency and amplitude have been observed in the orchidectomized ram and the intact man {Clarke et al, 1991; Santen, 1975; Winters et al, 1979b}.

Pituitary responsiveness to exogenous LHRH was also suppressed by DHT, as reported by other investigators studying the male rat {Debeljuk et al, 1974; Verjans & Eik-Nes, 1976; Nansel et al, 1979; Nansel & Trent, 1979} and this response was also dose-dependent. It is interesting to compare these results on pituitary responsiveness with DHT's effects on LH pulse parameters. The potency of DHT to alter the pituitary response was markedly greater than its effect on either LH pulse frequency or amplitude; for example the 8 mm DHT implant reduced frequency and amplitude to 62% - 78% of control, whereas

pituitary responsiveness dropped to 28%, indicating that the latter is much more sensitive to DHT-induced negative feedback.

Altogether, the information on LH pulse parameters and pituitary responsiveness can provide some insight into the site(s) of feedback regulation of DHT. If one accepts that the frequency with which LH pulses are released from the pituitary is dictated by the frequency of LHRH secretion into the portal vessels {Dierschke et al, 1970; Carmel et al, 1976; Belchetz et al, 1978; Clarke & Cummins, 1982; Levine et al, 1982}, then this metabolite of T appears to mediate its suppression of LH concentration in part by an effect at the level of the hypothalamic LHRH pulse generator. The change in pituitary responsiveness, i.e. the potent inhibition of pituitary LH release following an exogenous LHRH bolus, implies that DHT reduces the responsiveness of the gonadotropes to endogenous LHRH pulses. Thus, it seems that DHT to affects both hypothalamic and pituitary determinants of the pulsatile LH pattern.

Theoretically, in the absence of an effect on pituitary responsiveness, changes in LH pulse amplitude must be the result of a hypothalamic effect, as it is pituitary responsiveness and LHRH pulse amplitude which together determine the amplitude of the LH pulse. If, however, a reduction in LH pulse amplitude is associated with a decline in pituitary responsiveness, it may be assumed that a pituitary mechanism is involved, but an additional hypothalamic site of action cannot be excluded. In all three DHT doses eliciting a reduction in LH pulse amplitude, a decline in pituitary responsiveness was also observed, indicating that the effect on LH amplitude was mediated at least partly at the level of the pituitary but also perhaps upon LHRH pulse amplitude. Thus it appears that at least in orchidectomized rats, DHT affects the suppression of serum LH concentration at two levels: by reducing LHRH

The pituitary responsiveness protocol applied in these experiments uses a single dose of exogenous LHRH that is supraphysiological and submaximal in the control orchidectomized rats, and the LHRH-induced LH increment is considered a reflection of pituitary responsiveness to physiological stimulation. The limitations of such a single dose study suggest a certain caution in this interpretation, however, as it cannot detect treatment-induced changes in the slope of the LHRH dose response curve. Nonetheless, for the purpose of this thesis, the pituitary response to the exogenous LHRH bolus is considered a measure of pituitary responsiveness to the endogenous hormone; this assumption is supported by preliminary data (not shown) indicating that the slope of the LHRH dose-response curve is indeed parallel in orchidectomized rats with and without a DHT implant.

The mechanisms and sites of DHT-induced LH suppression are thus similar to those associated with T-induced negative feedback. The administration of T to castrated males slows the high post-castration rate of LH pulse frequency in rats {Steiner et al, 1982; Clifton & Steiner, 1986; Grosser, 1987}, rams {D'Occhio et al, 1982}, bulls {Gettys et al, 1984}, monkeys {Plant, 1982; Plant & Dubey, 1984} and man {Winters et al, 1979a; Santen, 1981}. The two to three fold increase in pituitary responsiveness observed following castration {Debeljuk et al, 1974; Verjans & Eik-Nes, 1976; Whitehead et al, 1982} can be reduced by testosterone replacement {Debeljuk et al, 1972; Schally et al, 1973; Verjans & Eik-Nes, 1976; Grosser, 1987}. T also suppresses the high post-orchidectomy LH pulse amplitudes {Kalra & Kalra, 1982; Steiner et al, 1982; Grosser, 1987} although this has been determined only for doses which also reduced pituitary responsiveness, so it is not possible to deduce whether or not T can inhibit LHRH pulse amplitude {Grosser, 1987}. Thus both T and DHT act on the same determinants of pulsatile LH release in effecting the suppression of mean LH levels in the orchidectomized rat: pituitary responsiveness, LHRH pulse frequency,

and possibly LHRH pulse amplitude. These results are consistent with the hypothesis that the negative feedback actions of T at the hypothalamus and/or pituitary might be mediated by its 5α-reduced metabolite, DHT.

6.2 POSITIVE FEEDBACK REGULATION BY ANDROGENS

In orchidectomized rats, low doses of testosterone or its proprionate have been shown to stimulate mean LH to concentrations significantly above the already high LH levels found in non-steroid treated orchidectomized animals ("supra-stimulation") {e.g. Swerdloff & Walsh, 1973; Bloch et al, 1974; Verjans et al, 1975}. In two other studies which examined the effects of such low dose treatment on LH pulse parameters, T augmented LH pulse amplitude (but not frequency) {Steiner et al. 1982; Grosser, 1987}. As no other LH pulse parameters measured were positively affected by low dose T treatment it appears that this increase in amplitude is the basis of the supra-stimulated mean LH levels. In the present study too, at a dose too low to negatively affect LH pulse frequency and pituitary responsiveness, T had a positive feedback effect on LH pulse amplitude, stimulating it to levels significantly greater than those seen in orchidectomized animals, though at this dose of T the supra-stimulation of amplitude was not sufficient to cause a significant increase in mean LH concentration.

Of the DHT treatments used in this study, only the smallest implant (2 mm DHT implant) effected changes in the LH pattern that suggest DHT can positively regulate LH release; this low DHT dose increased LH pulse amplitude to a height that was statistically not different from that observed in the rats subject to the positive feedback effects of 3 mm T implant. However, nor was this increased amplitude significantly higher than in untreated

orchidectomized rats, and thus these data provide no *direct* evidence of DHT-mediated positive feedback effects on LH release.

However, an interesting inference can be made that provides *indirect* evidence that both T and DHT are capable of eliciting positive feedback via an effect on LHRH pulse amplitude. This inference rests upon two assumptions: 1) that treatment-induced changes in the magnitude of an LH increment evoked by an exogenous supraphysiological LHRH bolus, reflect a reduced pituitary responsiveness to endogenous LHRH signals, and 2) that if pulse frequency is constant, then an altered LH pulse amplitude must be the result of changes in LHRH pulse amplitude and/or pituitary responsiveness. Given that the low dose DHT implant potently reduces pituitary responsiveness, yet does not suppress LH pulse amplitude, then it must be inferred that *LHRH* pulse amplitude is increased (otherwise LH pulse amplitude would have dropped). In contrast, pituitary responsiveness was not altered by low dose T treatment, however it did increase LH pulse amplitude, and thus by inference must have augmented LHRH pulse amplitude (or else LH amplitude would have remained at control levels). Thus, one may infer that both DHT and its precursor, T, are (at least at low doses) able to stimulate LHRH pulse amplitude, though only in the case of T did this increase the size of the LH pulses secreted from the pituitary.

Endogenous opioid peptides also play a role in the regulation of the LH pulse pattern, and these effects appear to be mediated upon LHRH release rather than upon pituitary responsiveness (see introductory section "Opioid regulation of LH release"). The acute LH secretory response elicited by naltrexone treatment of control orchidectomized rats and those treated with a low dose DHT implant indicate that a tonic inhibitory opioidergic influence is present in both groups of animals. It is less clear whether there is a change in endogenous opioid input to the LHRH system with DHT treatment: if the steroid treatment lowered

pituitary responsiveness (as discussed on p. 106) then the similar magnitude of the naltrexone-induced LH increment in the test and control animals implies that the opioid influence on these two groups is different; if the pituitary responsiveness data is not indicative of a change in responsiveness to endogenous LHRH stimulation, then there may be no effect of DHT upon opioid regulation of LHRH release. Clearly, further studies are necessary to distinguish between these possibilities.

These results suggest that the spectrum of effects which both T and DHT can elicit in the adult orchidectomized rat is the same, and include at least the reduction of LH (LHRH) pulse frequency, and pituitary responsiveness (negative feedback), and perhaps also the stimulation of LHRH pulse amplitude (positive feedback). For both T and DHT, the suppression of LHRH pulse amplitude may also contribute to the suppression of LH. These data are consistent with the simple hypothesis that the regulation of pulsatile LH secretion may be achieved in part or in whole, by DHT after its local production from the circulating pro-hormone, T.

6.2.1 *There is a dose-dependent balance between the positive and negative effects of androgens on pulsatile LH secretion*

The above analysis, based on measurements of LH pulse amplitude and frequency and pituitary responsiveness, suggests that there are three main androgen-regulated determinants of the pulsatile LH pattern in the orchidectomized rat: LHRH pulse amplitude, LHRH pulse frequency, and pituitary responsiveness. The nature and magnitude of androgen effects on

each of these determinants sums to yield the plasma LH pattern detected during serial sampling (see figure 23). An increase in mean LH concentration, above the already high post-orchidectomy levels, indicates a predominence of the (putative) stimulatory androgen effect on LHRH pulse amplitude, and occurs only with low doses of androgen (e.g. in orchidectomized animals bearing a 3 mm T implant) (see figure 23, panel B vs A). In animals showing a suppression of mean LH concentration, i.e. those with high serum levels of androgen (e.g. 8 and 12 mm DHT groups), the negative effects on LHRH frequency and pituitary responsiveness outweigh any possible positive androgen influence on LHRH pulse amplitude (figure 23, panel D). Intermediate androgen concentrations might produce no net change in mean LH concentration, while the underlying pattern of pulses reflects counterbalancing positive and negative influences on LHRH pulse parameters and pituitary responsiveness {e.g. Grosser, 1987} (figure 23, panel C).

The key to the dose-dependency of the positive and negative feedback effects of androgens on mean LH levels, may lie in the relative androgen-sensitivity of the three mechanisms determining LH pulse charactistics. LH frequency and pituitary responsiveness are unaffected by the 3 mm T implant, while LH amplitude is stimulated, indicating that of the various neuronal inputs to the LHRH neurosecretory system that affect LHRH pulse amplitude and frequency, those mediating the T-induced *increase* in pulse amplitude are more sensitive to plasma T. The sensitive positive feedback effects are apparently not strong, however, as they are overcome once the circulating T levels exceed the threshold for suppression of pituitary responsiveness and pulse frequency.

Given that DHT is a more potent androgen than T {Kovacs et al, 1984; Grino et al, 1990}, it may be that the local DHT concentration is the most important determinant of the relative sensitivity of the various pituitary and hypothalamic regulatory mechanisms to T-

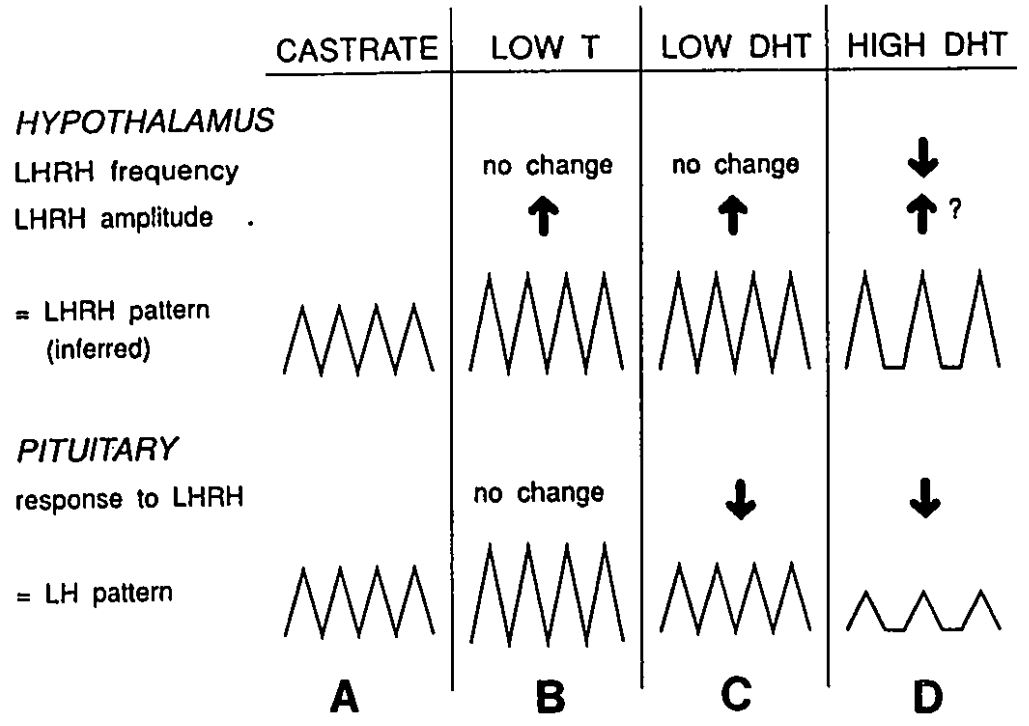

Figure 23. Schematic illustration of the influence of androgens on the three main androgen-regulated determinants of the pulsatile LH pattern. The LHRH pulse pattern reaching the pituitary via the hypophseal portal blood, and the associated serum LH pattern are represented in panel A for a castrated rat that has not received replacement steroid treatment. Based on the LH patterns observed in orchidectomized rats treated with testosterone or a range or DHT doses, the regulatory effects of increasing androgen doses upon LHRH pulse frequency and amplitude and pituitary responsiveness are shown in panels B, C, and D, and the sum of these actions are represented as LH and LHRH (inferred) pulse patterns (see text for details).

induced changes; those regions "more sensitive" to T may simply have a higher local 5α-reductase activity and thus a higher local concentration of the more potent androgen. This would predict that the CNS region(s) mediating the increase in LHRH pulse amplitude has a high 5α-reductase activity relative to those areas involved in the inhibition of LHRH pulse frequency and pituitary responsiveness. Unfortunately, this prediction is difficult to test directly; though microdissection studies have identified regional differences in hypothalamic 5α-reductase activity {Selmanoff et al, 1977; Melcangi et al, 1985}, the site(s) of amplitude

and frequency regulation have not yet been identified with a precision that would permit androgen-sensitivity to be correlated with 5α-reductase activity.

Alternatively, the administration of exogenous DHT should remove any advantages conferred by a relatively high local 5α-reductase activity, increasing the relative response of those determinants (e.g. pulse frequency and pituitary responsiveness) that are less sensitive to T treatment. To my knowledge, the experiments presented in section 3.1 are the first in vivo dose-response studies assessing the effects of DHT on the pattern of LH in the rat, and provide an opportunity to assess the relative potencies of DHT and T on a number of parameters of LH secretion. These results have been summarized in Table 2, with the data on the various LH parameters expressed as a percent of the mean values for the orchidectomized control animals. Previously in this laboratory, Grosser {1987} used a similar protocol to study the dose-dependent effects of T on LH parameters, and this work provides a basis for determining the relative potencies of T and DHT.

implant length & steroid within	control (empty)	3 mm T	2 mm DHT	8 mm DHT	12 mm DHT
steroid released (μg/day)	0,0	9,0	4,8	19	29
LH frequency	100%	108%	103%	63%	56%
LH amplitude	100%	167%	146%	69%	40%
pituitary responsiveness	100%	100%	55%	29%	15%
mean serum LH	100%	120%	98%	30%	20%

Table 3. Summary of data from section 3.1, on serum LH pulse parameters and pituitary responsiveness in adult male rats orchidectomized and given androgen implants 3 weeks previously. These data are expressed as a percent of the orchidectomized animals given an empty implant (control).

LH (LHRH) pulse frequency slowed equally under the influence of similar doses of DHT (8 mm implant, 19 μg DHT/day) and T (5 mm implant, 15 μg T/day) {Grosser, 1987}, indicating that the conversion of T to DHT is probably occurring quite readily in those sites mediating androgen action on the LHRH pulse generator. The potent inhibition of pituitary responsiveness by even the smallest DHT implant (2 mm DHT implant ≡ 4.8 μg DHT/day) suggests that 5α-reductase activity is very low in the pituitary of orchidectomized T-treated rats, as this degree of suppression was matched only by an implant releasing 7 times more testosterone (12 mm T implant ≡ 36 μg T/day; {Grosser, 1987}). There is apparently no aromatase activity in the pituitary, so this low efficacity of T cannot be due to its local metabolism to estradiol {Martini, 1982}.

These comparisons support the postulate that the array of positive and negative feedback effects mediated by T are the sum of a stimulatory androgen input upon LHRH pulse amplitude, which is sensitized to low levels of T by virtue of relatively high local 5α-reductase activity, and negative influences upon LHRH pulse frequency and pituitary responsiveness, which become apparent only at higher doses of T due to relatively low rates of local metabolism to DHT. Alternatively, differences in sensitivity to the positive and negative T effects upon the LH pulse pattern may be determined by cell-specific characteristics other than 5α-reductase activity.

6.2.2 *Other steroidal mediators of T effects*

Dihydrotestosterone is not necessarily the only T metabolite involved in mediating the actions of T. The CNS and anterior pituitary are able to form small amounts of 5α-

androstan-3ß-,17ß-diol (3ß-diol) and 5α-androstan-3α,17ß-diol (3α-diol) from DHT {Massa et al, 1972}. Whereas 3ß-diol is not converted back to DHT to a significant extent, (due to the formation and elimination of more polar metabolites), an equilibrium is established between the substrate and product of the 3α-hydroxysteroid dehydrogenase enzyme such that significantly greater levels of 3α-diol are found in the serum of orchidectomized rats given DHT {Brawer et al, 1983}. In intact rats, these diols account for 25-40% of the 5α-reduced metabolites found in the anterior pituitary and hypothalamus {Martini, 1982}. It is not currently possible to distinguish between the roles of DHT and 3α/ß-diols as mediators of T- or DHT-induced effects in vivo, as specific and effective inhibitors of the hydroxysteroid dehydrogenase enzymes catalyzing their interconversion are not yet available. However, following the administration of radioactive 3α-diol, DHT is the principal labelled steroid recovered in the nuclei of the rat ventral prostate, suggesting that at least in this tissue it is DHT rather than 3α-diol that mediates androgen action {Bruchovsky, 1971}.

The aromatase enzyme, which irreversibly converts T to estradiol, is also present in the hypothalamus, but is apparently absent in the anterior pituitary of the male rat {Naftolin et al, 1972; Roselli et al, 1984; Martini, 1982}. Estrogens are also capable of regulating LH secretion in males {Verjans et al, 1974; Swerdloff et al, 1972; Sherins & Loriaux, 1973}, and thus the feedback actions of T on LH release are probably the sum of both estrogenic and androgenic effects. This hypothesis could be tested in castrated T-treated males rats, by selective blockade of aromatase, or of the estrogen receptor, which would eliminate estrogenic contributions to T-associated effects, or conversely, by blockade of the androgen receptor which would select for T effects mediated by estradiol. Preliminary experiments were conducted with a relatively pure antiandrogen, flutamide, but were not informative as the

insolubility of the compound limited the dosage to levels that were too low to significantly affect LH levels or accessory tissue weights in intact rats.

6.2.3 *A physiological role for positive feedback?*

While stimulatory effects of low levels of androgens on LH pulse amplitude and mean plasma concentration have been demonstrated in the orchidectomized rat, it is unknown if this positive feedback is important for homeostasis within the hypothalamo-pituitary-testicular loop, or even if such low levels occur in the normal (testes-intact) male rat. It may be hypothesized that positive feedback is as important as negative feedback for endocrine homeostasis in male reproduction: for example while rising T concentrations shut down LH production (negative feedback), troughs in T levels may lead to increased LH production not only passively (by virtue of a lack of negative feedback, as seen in orchidectomized animals) but by actually stimulating an increase in LH release from the pituitary (positive feedback). As a first approach to address this issue the plasma testosterone concentrations in normal (testes-intact) adult male rats were characterized to determine whether, in the course of a day's fluctuations, they fall as low as 20-30% of their mean levels, this being the plasma testosterone level created by the low dose implants able to elicit a positive feedback effect on LH pulse amplitude and mean concentration. Indeed endogenous T serum concentrations fall to this level one-quarter to one-fifth of the time in the intact male rat, and thus the data are consistent with a daily role for androgen-induced stimulation of LH release. Alternatively, or in addition, the positive feedback phenomenon may be physiologically relevant following

more extended periods of low LH secretion, e.g. in individuals recovering from secondary hypogonadism, or undergoing puberty.

6.3 FLUCTUATIONS IN TESTOSTERONE CONCENTRATION IN THE INTACT RAT

The serial sampling data acquired shows clearly that the adult male rat is unlike other mammalian species in which there is a strikingly prompt and consistent release of T following an LH pulse, as in the mouse {Coquelin & Desjardins, 1982}, rabbit {Rowe et al, 1975}, ferret {Sisk & Desjardins, 1986}, ram {Sanford et al, 1974; Lincoln, 1976; D'Occhio et al, 1982}, bull {Katongole et al, 1971; Smith et al, 1973}, and monkey {Steiner et al, 1980; Plant 1981}. In the rat, as in man {Naftolin et al, 1973; Baker et al, 1975; Spratt et al, 1988}, the precise temporal relationship of LH to T secretion has been harder to define. This study demonstrates that testosterone levels in the male rat exhibit a pulsatility that is irregular in frequency and amplitude.

Keating and Tcholakian {1979} suggested that a circadian T rhythm existed and was entrained to the light/dark cycle. In the only frequent sampling study to consider the influence of light on testosterone and LH levels, Ellis and Desjardins {1982} noted no difference in the spectrum of LH and testosterone profiles from the daytime and nighttime sampling sessions. To determine whether or not acute changes in T and LH levels were associated with the morning and evening shifts between lightness and darkness, rats were sampled specifically during the periods immediately preceding and following these transitions. The hormone status in the rats did not change significantly following the transition from light to dark, implying that the presence or absence of light itself has no acute impact on LH and T

secretion in adult male Sprague-Dawley rats. During the sampling session when the animals were first exposed to morning light, a trough in testosterone and LH levels occurred over approximately 100 minutes, first becoming significant within 40-80 min after the lights-on. This morning decline appeared to slightly precede the light period, again suggesting that it is not the dark to light transition that directly triggered the decrease; alternatively, the coincidence between lights-on and the trough in T and LH may be a part of an endogenous circadian rhythm that is entrained to the light/dark schedule.

To test this hypothesis, trends in LH and T concentration would have to be determined again in rats which are subjected to a prolonged nighttime during the usual morning light period; if the decline in hormone levels occured at the usual time, then it could be attributed to a circadian rhythm rather than to exposure to light per se. Furthermore, if upon shifting the time of lights-on, the decline in hormone levels is similarly shifted, then it is likely that the rhythm is entrained to the light-dark cycle. As rats are nocturnal animals, known to sleep during light hours, and especially during the first hours of day-light {Weihe, 1987}, it would be consistent with this period of decreased locomotor (and mating) activity for there to be a decline in T levels, as these behaviours are not appropriate during the rat's rest period.

Given the current findings of pulsatile and episodic fluctuations inherent in the endogenous serum pattern of testosterone, and the inability of quantitatively physiological but constant levels of exogenous T to yield normal LH levels when administered to normal or orchidectomized rats {Verjans et al, 1975; Damassa et al, 1976; Steiner et al, 1982}, the qualitative nature of the androgen signal should be accommodated in future studies where physiological endocrine conditions are important; i.e. the popular constant-dose administration

protocols could be replaced with those that generate episodic and pulsatile fluctuations in serum T concentrations.

APPENDICES

APPENDIX 1

MODIFICATIONS OF THE CATHETERIZATION PROCEDURE

Numerous refinements of the atrial catheterization procedure were introduced when many of the animals developed a "one-way" block of the cannula; though fluid injection was possible, blood could not be withdrawn through the catheter. Interestingly, there were batches of animals in which this did not occur at all, and others in which the rate of one-way blockage was as high as 80% (i.e. 12 of 14 animals). This block was sometimes associated with the formation of a beige tissue mass at the atrial catheter tip, and thus was attributed to a thrombogenic response to the presence of the catheter. In an attempt to reduce the rate of one-way block, one or more of the following modifications was included in the catheterization procedure:

1) during all catheterizations the catheters were handled more carefully to minimize their contact with immunogenic materials, such as lint-laden paper towelling.

2) the catheters were autoclaved;

3) the catheter length was adjusted to improve its position in the atrium;

4) a blunt-ended tip was used instead of a bevelled tip;

5) the bevelled tip was refined to reduce sharp/jagged edges;

6) extra holes were placed in the catheter tubing just above the tip, to permit fluid to bypass the occluding tissue mass at the tip;

7) the catheters were coated with 2% TDMAC-heparin (Polysciences Inc., Warrington, PA; tridodecylmethyl ammonium chloride is a complex used to impregnate surfaces with the natural anti-thrombogen, heparin);

8) post-surgery the catheters were flushed more frequently (e.g. daily)...or less

frequently (e.g. every 7-10 days).

As none of these modifications, introduced individually or in combinations, consistently influenced the rate of one-way catheter blockage, experiments were continued using the original procedure except that the minimum recovery interval between surgery and sampling was reduced to 5 days.

In contrast to the two-way block of the atrial catheters (an infrequent but consistent drawback throughout all these studies), the problem of the one-way block was absent or rare in the first few batches of animals catheterized, became frequent and persistent over a period of 3 years (regardless of season), then completely disappeared in the last few batches of rats. During each of these periods the original catheterization procedure was the only or the main approach used, thus I believe that the increased rate of occurance of the one-way block is associated with a heightened sensitivity of the rats to the catheter tip, rather than to a problem with the catheterization procedure.

APPENDIX 2

MODIFICATIONS OF THE LH RADIOIMMUNOASSAY

The LH radioimmunoassays were initially done in the Obstetrics & Gynecology RIA lab at the Royal Victoria Hospital, Montreal. Upon relocation to the McIntyre Medical Building of McGill University, the bound/free separation step in the original protocol required modification, as it no longer generated precise measurements of the LH in the test samples, i.e. the duplicates were frequently highly variable. Interestingly, there was no such problem with the standard curve samples; the "coefficient of variation" remained comfortably below 15%, even when they were diluted in serum rather than buffer. A visual inspection of the tubes indicated that pellets were being partially or wholly lost during the decantation step. Thus, when new buffers and re-optimized antisera dilutions yielded no improvement, a variety of modifications were introduced in an attempt to improve the separation of free and antibody bound hormone:

1) substituted second antiserum then in use at former RIA lab;

2) increased centrifugation time, and speed;

3) the concentration of BSA (acting here as a carrier protein) in the wash solution was increased;

4) buffers were remade with filtered, glass-distilled (1x) water rather than deionized water;

5) precipitated bound LH using a 20% solution of PEG (polyethylene glycol 8000) instead of a second antibody; (this improved the duplicates, but specific binding dropped from the usual 30-45% to 3-4%, a well-known drawback of PEG precipitation) {Chard, 1987};

6) precipitated bound LH using a combination of the second antibody and PEG methods {Peterson & Swerdloff, 1979}.

Without compromising specific binding, the latter modification greatly improved the agreement between sample duplicates, (and shortened the 6-day assay by one day), so the following procedure was adopted. On assay day 5 the second antibody is added and the tubes hand-shaken as usual. Immediately thereafter (without overnight incubation) 1 ml of wash solution containing 5% PEG is added and tubes hand-shaken again. The tubes are then centrifuged and counted as previously, with no additional time required for incubation.

APPENDIX 3

SOME CAVEATS IN PULSE ANALYSIS

Pulse analysis is not without its caveats, and before interpreting regulator-induced changes in pulse parameters some technical limitations should be recognized.

1) Because serum LH concentrations are measured intermittently rather than continuously, it is unknown whether the concentration taken to be the highest value for a given pulse, is indeed the true LH maximum, or a sub-maximal LH concentration occurring within one sampling interval before or after the actual peak. The observed peak concentration and calculated amplitude values are thus probably underestimates of the actual values. The same argument may be made regarding nadir values, again suggesting that amplitude values are probably underestimated.

2) Nadir and amplitude values, based on serum or perfusate pooled over period that is relatively long (e.g. 10 minutes) compared to pulse duration, will be muffled by the preceding and subsequent levels of hormone. Thus techniques such as push-pull perfusion and microdialysis, in which the low yield of sample (per unit time) necessitates a somewhat prolonged collection period, may yield a relatively "compressed" pattern of hormone secretion compared to the actual signal received at the target tissue.

3) When the frequency of LH pulses is slow enough to permit post-pulse LH levels to return to baseline (as implied by a plateau of two or more nadir-level points between pulses), nadir values represent basal LH secretion, and amplitude values reflect the height of the LH pulse secreted from the pituitary. In serum LH patterns showing a high pulse frequency such that the interval between the beginning of sequential pulses

is shorter than the duration of the pulses, adjacent LH peaks coalesce at their bases yielding a nadir value that is elevated above the level of basal LH secretion; in this case the pulse amplitude calculated using this nadir value will be an underestimate of the pituitary LH secretory event. Between intact and orchidectomized animals the disappearance half-life of LH appears to be relatively constant {Gay & Midgley, 1969}, so it is pulse frequency that is most likely to introduce a treatment-dependent error in amplitude measurements.

Thus the interpretation of treatment-induced changes in LH pulse parameters should be made with these cautions in mind, that nadir values do not necessarily indicate the basal level LH secretion, and that the possibility of LH pulse amplitude being underestimated arises with higher pulse frequencies, and increases with decreasing sampling frequency.

The pulse analysis protocol used in this thesis is based on the same theory as the program "Ultra" {Van Cauter, 1981}, which yields results comparable to those obtained using other pulse detection paradigms, such as Cluster and Detect {Urban et al, 1988}. Alternate methods are becoming available with Veldhuis's development of the deconvolution approach to pulse analysis; this program generates more information from a given pulsatile serum hormone pattern, although it requires accurate data on hormone half-life in order to generate reliable results {Evans et al, 1992}.

ORIGINAL CONTRIBUTIONS

ORIGINAL CONTRIBUTIONS

1. The continuous administration of the testosterone metabolite, dihydrotestosterone, suppresses mean serum LH concentrations in the adult male orchidectomized rat; this negative feedback effect is the net result of inhibitory actions of the steroid on the frequency of pulsatile LH release, and the amplitude of individual pulses.

2. Dihydrotestosterone dramatically decreases the LH secretory response to exogenous LHRH, even at relatively low steroid doses, suggesting that this androgen effects the inhibition of LH pulse amplitude at least in part by altering the outcome of LHRH interaction with the LHRH receptor on pituitary gonadotropes.

3. As is the case for its precursor, testosterone, the sites of action of dihydrotestosterone-induced negative feedback upon LH secretion include both the pituitary and the hypothalamus (where LH (LHRH) pulse frequency is modulated), suggesting that these two androgens have similar mechanisms of action.

4. A positive feedback effect of low doses of testosterone upon LH pulse amplitude is effected without increasing pituitary responsiveness, suggesting that it is an augmented LHRH pulse amplitude that is reflected in the larger LH amplitude; dihydrotestosterone also appears to have a stimulatory suprapituitary effect on LH pulse amplitude, as LH pulse amplitude is maintained in the presence of low steroid doses that greatly decrease pituitary responsiveness.

5. The testes of the intact adult male rat are capable of secreting both brief high amplitude bursts, and prolonged low-amplitude episodes of serum testosterone. Pulsatile testosterone release may or may not occur concurrent with episodic secretory activity, and variability in the net serum pattern of testosterone is a prominent and reproducible characteristic both within and between individuals.

6. While the active and quiescent periods of LH and testosterone secretory activity coincide overall, the well-defined one to one relationship of LH to testosterone pulses that is common in other male mammals is lacking, suggesting that in the male rat, testicular steroidogenesis is subject to other acute modulatory influences in addition to LH.

7. Consistent with the possibility of a physiological role for androgen-induced postive feedback in the normal male rat, the low levels of serum testosterone that elicit a positive feedback response in the orchidectomized steroid-treated rat, are attained between almost one-quarter of the pulses in the intact animal.

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