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HORMONAL MODULATION OF LEYDIG CELL MEMBRANE LUTEINIZING HORMONE RECEPTORS

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Ph.D. 1986
HORMONAL MODULATION OF LEYDIG CELL MEMBRANE LUTEINIZING HORMONE RECEPTORS

DISSERTATION

PRESENTED IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE DOCTOR OF PHILOSOPHY IN THE GRADUATE SCHOOL OF THE OHIO STATE UNIVERSITY

BY

MOHAMED OSMAN HUSSEIN

B.V.Sc., M.S., M.S.

THE OHIO STATE UNIVERSITY

1986

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Department of Physiology
HORMONAL MODULATION OF LEYDIG CELL MEMBRANE LUTEINIZING HORMONE RECEPTORS

By
Mohamed Osman Hussein, Ph.D.
The Ohio State University
Professor John J. Curry, Adviser

SUMMARY/PURPOSE: To elucidate the role of anterior pituitary hormones on control of the Leydig cell membrane luteinizing hormone (LH) receptor density. To explore the possibility that the anterior pituitary hormonal control on Leydig cell membrane luteinizing hormone receptor density is steroid-hormone-mediated.

CONCLUSIONS: LH induction of LH receptors is very rapid with a peak at 30 minutes after LH injection. Prolactin priming effects on LH induction of LH receptors showed a latent period of 6 to 12 hours after which a positive effect persisted for about 24 hours and declined thereafter. Follicle stimulating hormone (FSH) and growth hormone (GH) have no priming effect on LH induction of LH receptors. Testosterone and estrogen do not mediate the prolactin-dependent LH induction of LH receptors and show a negative effect on LH receptor levels.

Adviser's Signature
DEDICATION

TO THE SUDANESE TAXPAYERS,
FOR PAYING TOO MUCH FROM
THE TOO LITTLE THEY HAVE
TOWARDS MY EDUCATION
ACKNOWLEDGMENTS

My sincere thanks to Dr. John J. Curry, III in appreciation for his guidance and advice that made all the rough times easier to endure. My warm, grateful thanks to Dr. William Zipf in appreciation for his guidance and support that made all this possible, it was a pleasure working with and learning from his expertise. My sincere thanks to Mrs. Judith D'Angelo for her skillful secretarial ability and her cheerful smile in spite of all those frustrating deadlines.

Also, my thanks to my committee members; Dr. Jack A. Rall, Dr. M.T. Nishikawara, and Dr. Syed Saiduddin for their questions and suggestions that helped to improve the quality of my work.

Finally, my warm regards to all the friends who cared during the hard times and shared the joy of the happy ones.
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INTRODUCTION

THE TESTES: HISTORY AND PHYSIOLOGY

The word "testis" is a Latin word meaning "witness" and the reason the gonads were given that name is unclear but may reflect an ancient belief that these organs are witnesses to the sexual intercourse act in which they take no part or because among the Romans a legal witness was required to be an adult male with testes while boys, castrates and women could not be witnesses. Among the Greeks the testes were called "didymi", meaning twins because they were normally paired in the scrotum.

The study of the structure and function of the testes historically has been an important aspect of studying human and animal biology through the investigation of the effects of orchiectomy on reproductive functions. The first observations on the functions of the testes were probably incidental to accidental castration, but even before the beginning of history it has been known that removal of the testes affected both the anatomy and behavior of men and other
male animals. Castration of men was used as a punishment for sexual offenses or adultery in Assyria (1500 B.C.), The Babylonian Code of Hammurabi (2000 B.C.), Egyptian Law (1200 B.C.) and ancient China. Eunuchs (i.e. prepubertal castrates) were mentioned in both the Old and New Testaments of the Bible and were used as guards for women in harems as well as providing male soprano singers for the Catholic Church instead of the forbidden female singers. Castration of animals was practiced as early as when animals were first domesticated during the Neolithic age (700 B.C.) to make male domestic livestock easier to handle and less prone to yield tough or tainted meat if kept beyond puberty.

Anatomic description of the testes was provided by Aristotle (400 B.C.), semen production in the testes, reported by deGraaf (1668), sperm observed by Leeuwenhoek (1678) and given the name "spermatozoa" by vonBaer (1827). [Burgor and deKretser (1981)] The hormone-producing function of the testes was demonstrated in what is usually considered the first experiment in endocrinology conducted by Berthold (1849) showing that the regression of the secondary sexual characteristics due to castration in a rooster could be prevented by the reimplantation of part of the testes. The testes physiological functions consist of spermatogenesis and steroidogenesis with both functions very closely related
because an adequate level of androgen production is necessary for spermatogenesis formation and their successful delivery requires normal sexual behaviour as well as the development of the secondary sexual characteristics which are also under the control of androgens.

The Interstitial [Leydig] Cells:

These cells were first described in humans by vonKolliker (1841), in other mammals by Leydig (1850) and their function of producing the male sex hormones was suggested by Bouin and Ancel (1903) and Setchell (1978). In most animals they are found in the 3-sided spaces between the cylindrical seminiferous tubules along with nerves, blood and lymph vessels. They have an ultrastructure which is consonant with their function of steroid synthesis such as large amounts of smooth endoplasmic reticulum, plentiful mitochondria, a prominent Golgi complex, centrioles and lipid droplets. [Burger and deKretser (1981)]

The Leydig cells produce most of the testosterone in the circulation but only 20% of plasma dihydrotestosterone probably due to the action of 5-alpha-reductase enzyme from the Sertoli cells on testosterone secreted by the Leydig cells [Setchell (1978)]. Peripheral metabolism of testosterone is responsible for the 80% remainder of plasma
dihydrotestosterone. 65-75% of plasma estradiol is due to conversion of testosterone, while the remainder is secreted by the Leydig cells [Setchell (1978)]. Leydig cell steroid production has been shown to be under a variety of hormonal regulations, including luteinizing hormone [LH], follicle stimulating hormone [FSH], prolactin [PRL], growth hormone [GH] and estrogen [Lipsett (1980)]. The primary action of FSH is on the seminiferous epithelium with specific binding sites on the spermatogonial cell membrane and the basal aspect of the Sertoli cell [Davies (1981)]. FSH actions on the Sertoli cells are mediated through activation of adenylate cyclase, resulting in elevated rate of synthesis of RNA and stimulation of specific protein synthesis [Davies (1981)]. Increased mitotic activity, plasminogen activator, estradiol and androstenedione production are noted effects of FSH on Sertoli cells function [Davies (1981)]. The role of FSH in steroidogenesis during puberty in the rodent appears to be by initiating an increase in LH receptors on the Leydig cells and inducing testicular sensitivity to LH [Chen et al. (1977)]. Studies on human beings suggest that the induced response of plasma testosterone to human chorionic gonadotropin was related to the plasma concentration of FSH. [Leinonen et al. (1982)]
Leydig cells also have receptors for prolactin and prolactin injections increase the number of testicular receptors for LH [Aragona et al. (1977)]. Prolactin induces increased testosterone synthesis by augmenting the pool of esterified cholesterol in the Leydig cells [Welsh, et al. (1986)]. On the other hand prolactin reduces the loss of LH receptors that ordinarily occurs after hypophysectomy and also LH receptor loss due to exposure to doses of luteinizing hormone that are large enough to desensitize the Leydig cells [Zipf et al. (1983)]. Hypoprolactinaemia induces a decrease in plasma gonadotropins and testosterone. It also increases testicular atrophy which is accompanied by a loss of LH binding sites in testicular tissue [Aragona et al. (1977)]. Similar results are also induced by hyperprolactinemia indicating the antigonadal effects of high levels of prolactin [Boyer et al. (1974), Bartke et al. (1977)]. In studies of hamsters with gonadal atrophy, 300 ug/day prolactin injections restored testosterone serum levels and testicular weight to significantly higher values than controls [Bartke et al. (1975)].

Administration of growth hormone to boys with delayed puberty, due only to growth hormone deficiency, induces pubescence indicating a synergistic role, probably through its effect on testicular responsiveness to LH and number of LH receptors in the Leydig cells [Laron and
Sarel (1970)]. The role of estrogens in Leydig cell function is difficult to determine since estrogens apparently counteract the effects of luteinizing hormone. Estradiol is produced within the Leydig cells, which also have estradiol receptors, allowing a unique situation for a regulatory role for estradiol in Leydig cell function. Although large doses of estradiol inhibit testosterone synthesis [Huhtaniemi et al. (1980)], neither estradiol synthesis nor a normal intracellular concentration of estradiol is essential for enhanced Leydig cell function. All hormones seem to affect the Leydig cells by increasing or decreasing their sensitivity and responsiveness to LH which binds and acts primarily to stimulate steroidogenesis [Nozu et al (1981)]. The major effect of LH upon steroidogenesis is to increase the availability of steroid substrate by accelerating the metabolism of cholesterol and cholesterol entry into the mitochondria. LH also increases the conversion of 20- and 22-alpha-hydroxycholesterol to pregnenolone, a testosterone precursor, through the action of cholesterol-esterase and cholesterol-side-chain cleavage enzymes [Huhtaniemi et al. (1981)]. These actions are mediated through LH binding to specific, high affinity surface membrane protein receptors leading to increased adenylate cyclase activity followed by a rise in intracellular cyclic AMP. The intracellular cyclic AMP
binds to the regulatory protein subunit of protein kinase which dissociates releasing its catalytic subunit that activates the phosphorylation of protein substrates in the acute phase of hormone action [Dufau et al. (1971)]. Chronic hormone actions appear to involve changes in nuclear activity and activation or modification of existing gene products of protein synthesis [Hseuch et al. (1976)].

**LUTEINIZING HORMONE RECEPTORS**

*Nature*

Significant progress has occurred recently regarding the purification of plasma membrane receptors but there is still lack of sufficient amounts of purified receptor material for better analysis. Covalent affinity labeling methods have been employed extensively to characterize the hormone binding components of plasma membrane receptors [Massague et al. (1980), Yip et al. (1980), Ji & Ji (1980), Rebois et al. (1981)]. Application of these methods to the luteinizing hormone receptors has not produced a uniform picture of the hormone binding component [Ji & Ji (1980), Rebois et al (1981)]. Although the affinity labeling method is specific, its use has been limited to impure membrane systems. A 15,000-fold purification of luteinizing hormone receptors was performed and its molecular weight determined to be 200,000 by solubilization and affinity chromatography from
Identification of the LH receptor and characterization of its properties by radiolabeling of bovine corpus luteum plasma membranes was reported [Padian & Bahl (1977)]. A polypeptide hCG receptor with a molecular weight of 110,000 has been purified from rat ovary by using an immobilized hormone antibody procedure [Metsikko and Rajaniemi (1980)]. Using a covalent affinity crosslinking method, Rebois et al. (1981) reported a molecular weight of 100,000 for a choriogonadotropin-binding polypeptide. Solubilization of rat ovarian luteinizing hormone receptors by TritonX-100 and subsequent purification by chorionogonadotropin antibodies immobilized to agarose was reported by Metsikko and Rajaniemi (1982). Their study revealed an overall luteinizing hormone receptor unit having a molecular weight of 200,000 but of an unknown composition, along with the identification and purification of the LH binding unit of the receptor with a molecular weight of 90,000. Ji and Ji (1981) used a photoaffinity cross-linking procedure to couple $^{125}$I-hCG with the label on either the alpha or beta subunit to procine granulosa cell receptors. They reported a molecular weight of 81,000 to 58,000 for each subunit and suggested a cross-linking to a protein of similar size. If the stoichiometry of the holoreceptor was 1:1:1 for the consistent bands, then its molecular weight would be
From these studies it is apparent that the LH/hCG receptor is a complex protein or glycoprotein with two or more components. Whether the receptor is a monomer macrounit of 200,000 molecular weight or a dimer of two 90,000 molecular weight subunits associated with a non-hormone cross-linking unit remains to be firmly established. A major question which has yet to be adequately addressed is the valency of the receptor. Some studies suggest that the receptor is monovalent while other studies reported that multiple components of the receptor are capable of binding the hormone [Ascoli (1985)]. Investigations are needed to determine if these components represent (a) proteolytic fragments of a single hormone binding unit, (b) true subunits which each bind a different site on the hCG molecule, or (c) subunits that bind the same site on hCG thus making the receptor multivalent.

In vitro studies of the fate of the hormone receptor complex showed binding of radiolabeled human LH to the cytoplasm of interstitial cells [deKretser et al. (1969)] and internalization of the hormone receptor complex, probably by a receptor-mediated endocytosis [Ascoli & Puett (1978)]. Hormone internalization may indicate the hormone can act directly at intracellular sites or it may represent a mechanism whereby the hormone receptor complex
is degraded. Huhtaniemi et al. (1978) and Rajaniemi et al. (1979) have demonstrated that hCG undergoes catabolic modification leading to the cleavage of peptide fragments identical to the hormone subunits and subsequent hydrolysis to amino acids. Studies were done on cultured Leydig tumor cells using hCG labeled in the alpha or beta subunits [Ascoli (1982)] to characterize the pathway involved in the internalization and degradation of the hormone. The results show that the hormone is internalized in the intact form and reaches its site of degradation (presumably the lysosomes) in that intact form. The pathway then appears to involve processing of one or both subunits of the intact hormone followed by subunit dissociation and further degradation of the individual subunits. The alpha subunit is quickly degraded by the cells with extracellular amino acids as the only detectable degradation products. The beta subunit is degraded more slowly with several detectable intracellular degradation products before amino acids appear extracellularly. Hormone degradation is directly proportional to the temperature and time of exposure to testicular homogenate in vitro [Grizard et al. (1982)]. It is also likely that the process of internalization of the hormone receptor complex is involved in the dose dependent loss of LH receptors induced by the injection of high doses of hCG [Dufau et al. (1978)], ovine LH [Hseuch
et al. (1976)] and luteinizing hormone releasing hormone [LH-RH] [Catt et al. (1979)].

The down regulation of LH receptors due to sequestration or internalization persists for several days, suggesting that the occupied LH receptors are processed and degraded rather than vacated and reutilized [Tsuruhara et al. (1977)].

HORMONAL MODULATION OF LH RECEPTORS:
Prolactin

The use of sensitive specific radioimmunoassays has shown that prolactin is present in the circulation of males at levels comparable to those observed in nonpregnant nonlactating females and prolactin is the hormone studied most widely for its modulating effects on other reproductive organs and hormones.

Aragona et al [1977] studied the specific binding sites for prolactin and gonadotropins on the ventral and dorsolateral lobes of the prostate gland as well as on the Leydig cells and testicular tubules in rats at different ages. They reported the greatest number of binding sites for prolactin in the ventral prostate while LH receptors were more numerous than FSH receptors in the Leydig cells but fewer in the testicular tubules. In the same study, following 20 days of treatment with subcutaneous injection of 3 mg/kg bromocriptine twice a day, serum prolactin levels were suppressed from 15 ng/ml to 4 ng/ml and LH
receptors in the Leydig cells were significantly decreased without any effects on prolactin or FSH receptor levels.

The lutotropic effects of prolactin on progesterone production and LH receptors were investigated by Holt et al. [1976] in the corpora lutea induced by injections of LH and FSH to immature female rats. Their data indicate that in the absence of prolactin, LH can induce the formation of a corpus luteum with a high number of prolactin receptors but few LH receptors and a reduced capacity to produce progesterone. They also reported that prolactin presence in the early phase of the luteinization process increased the capacity of the developing corpus luteum for LH binding and progesterone production.

Bex and Bartke [1977] induced gonadal atrophy in male hamsters by exposure to a short photoperiod of 5 hours light period per 24 hours for two months then studied the effects of a daily injection of either saline, 250 ug prolactin, 20 ug LH plus 150 ug FSH or a combination of prolactin, LH plus FSH for two and a half weeks. Their results showed that treatment with LH plus FSH has no effect on the low levels of LH receptors while treatment with prolactin alone or in combination with LH and FSH increased LH receptors to levels higher than in normal controls. Scatchard analysis of the data indicates that decreased LH binding in the short photoperiod animals is due to a reduced LH receptors number and prolactin
treatment increase LH binding by elevating LH receptor levels.

Hyperprolactinemia was induced by the transplantation of anterior pituitary glands beneath the kidney capsule of intact adult male rats by Sharpe and McNeilly [1979] to study its effects on Leydig cell functions and LH down-regulation of LH receptors. Their results showed reduced LH and FSH serum levels along with a 55% to 175% increase in testicular LH binding sites. Injections of ovine LH in control and hyperprolactinimic rats in the same study resulted in a 55% reduction in LH binding sites in both groups. This study indicated that hyperprolactinaemia does not prevent the LH-induced down-regulation of LH receptors.

Another study done by Belanger et al. [1979] indicated that hypoprolactinaemia induced by inhibition of plasma prolactin levels by 2-bromo-alpha-ergocryptine in adult male rats caused a 60% reduction in LH receptor levels and potentiated the inhibitory effects of LHRH ethylamide (LHRH agonist) on testicular LH receptors.

In a study conducted by Morris and Saxena [1980], various doses of 1, 2.5, 5, 10 and 20 µg of ovine prolactin were injected subcutaneously in mature and immature male rats for two days to determine the effects of prolactin on LH binding sites in Leydig cell homogenates. They reported a dose-related increase in LH
binding sites with various prolactin doses accompanied by a 37-67% decrease in prolactin binding sites in immature rats only. Results from mature rats showed a 1-3 fold increase in LH binding sites in response to 30 ug prolactin but a 55% decrease was noted with a 100 ug prolactin injection. These age and dose related effects of prolactin were reported to occur without any accompanying significant changes of receptor affinity, LH, FSH or prolactin serum levels.

Chan et al. [1981] reported that injection of prolactin or elevation of plasma prolactin levels by metoclopramide infusion produced a significant positive effect on testicular LH receptors but did not prevent the down-regulation of LH receptors by injection of LH in adult male rats. Both studies indicate that low doses of prolactin (< 100 ug) increase LH receptor levels while high doses of prolactin or hyperprolactinemia decrease LH receptor levels and does not prevent LH-induced downregulation of LH receptors.

Warren et al. [1982] reported that treatment of cultured rat fetal testes with gonadotropin and dibutyryl adenosine 3, 5-monophosphate enhanced steroidogenic responsiveness and did not cause LH receptor loss or desensitization.

The regulation of radiolabeled hCG binding to testicular receptors was studied in mutant mice with
congenital prolactin deficiency, in mice with prolactin deficiency induced by bromocriptine treatment and in normal untreated mice by Amador and Bartke [1982]. They observed that after injection of 0.3 or 0.9 IU/gm body weight hCG, normal mice showed a dose-related decrease in hCG binding followed by a subsequent recovery from the down-regulation. Mice with congenital deficiency of prolactin have a similar dose-response curve of receptor loss after hCG administration but recovered more rapidly from the down-regulation than normal mice. On the other hand induction of prolactin deficiency by bromocriptine injections prevented the down-regulation of hCG binding following hCG administration. The authors suggested that the differential effects of congenital and drug-induced prolactin deficiency could be related to a difference in the duration or severity of the deficiency.

Bohnet and Freisen [1976] reported that retardation of sexual development in dwarf mice with congenital prolactin deficiency can be counteracted by prolactin treatment and the effect may be mediated by the induction of hepatic and testicular prolactin and LH receptors.

Comparative studies on the binding of radiolabeled hCG to isolated Leydig cells in plated culture and in suspension were performed by Baranao and Dufau [1983] to assess the early phase of receptor up-regulation. They reported that plated cells showed a total binding capacity
up to 200% higher than that measured in suspended cells without the difference being due to differential internalization. The number of binding sites measured in suspended cells was increased by preincubation with hCG for 2-6 hours while the binding of labelled hCG to plated cells showed a secondary increase that was maximal after 3 hours of incubation with hCG. The investigators indicate that this increase in hCG binding was not prevented by preincubation with inhibitors of protein synthesis, steroidogenesis or microtubular and microfilamental function, with the difference between plated and suspension cells being maintained in the presence of phosphodiesterase inhibitor.

Hypophysectomized adult male rats with a pituitary transplant under the kidney capsule were used by McNeilly et al. [1979] to study the effects of chronic treatment with LHRH alone or combined with bromocriptine on the secretion of FSH, LH and prolactin along with the actions of these hormones on testicular LH receptors and testosterone production. They noted that the presence of a pituitary transplant alone increased prolactin levels and testicular hCG binding without increasing testicular weight. Administration of 5 to 50 ug of LHRH to rats with pituitary transplants produced an associated increase in testes weight and increased capacity of the whole testes to bind hCG above the levels of rats with pituitary
transplant alone. Administration of bromocriptine to rats with transplants and receiving 5 µg of LHRH reduced prolactin levels and testicular binding of hCG without affecting testes weight.

25 µg ovine prolactin injections twice daily for 5 days given to mature intact rats caused a significant stimulation of LH membrane receptors but no change in maximal testicular steroidogenesis or responsiveness to hCG (Purvis et al. [1978]). On the other hand, Ichii (1980) and Baranao et al. [1982] reported in rat prostate a decrease in the concentration of exchangeable cytosolic androgen binding sites and a dose-related increase in the total number of KCl-extractable nuclear receptors with a dose of 5-50 µg prolactin per day. They also reported a significant increase in total content of androgen receptors in the rat prostate with a 500 µg prolactin injection per day. The authors indicated that the high nuclear to cytoplasmic androgen receptor ratio found in prolactin treated animals may suggest an increase in the translocation of the prostatic androgen receptors. These studies indicate that prolactin injection did not affect testicular steroidogenesis but significantly influences the level of androgen receptors in the prostate.

**Follicle Stimulating Hormone [FSH]**

The mechanism of control of LH receptors by FSH was investigated by Erickson et al. [1982] using granulosa
cells from immature hypophysectomized estrogen primed rats. Freshly collected granulosa cells show a low specific binding to labeled hCG (0.18 fmole/million cells). A marked increase in hCG binding was observed when the cells were cultured in serum free media with 10 ng/ml FSH with maximal binding attained after 3 days of incubation (19.2 fmole/million cells). Cholera toxin, prostaglandin (PGE₂) and cyclic nucleotide analogues mimicked, in part, the FSH effect with maximum induction of hCG binding sites closely parallel with their maximum stimulatory effects on cyclic AMP formation. A study by Chen et al. [1977] indicated that a 40-80 ug FSH per day S/C injections in intact 15 day old male rats significantly increased the number of LH receptors, but did not alter testicular responsiveness to LH. A similar FSH treatment of immature hypophysectomized rats increased the number of LH receptors and markedly increased testicular response to LH. A combined treatment of hypophysectomized immature rats with testosterone and FSH enhanced the effect of FSH on the number of LH receptors but not the effect of FSH on testicular responsiveness to LH. On the other hand, a combined treatment with estradiol and FSH had no effect on the FSH-induced increase in LH receptors but completely inhibited the FSH-induced increase in testicular responsiveness to LH. The authors suggested that regulation of LH receptors is
distinct from regulation of testicular responsiveness to LH where testosterone may enhance FSH-induced increase in LH receptors while estradiol may be a factor in regulating testicular responsiveness to LH. A positive correlation between human testicular LH receptor number and circulating serum FSH levels was reported by Leinonen et al [1982]. These studies indicated that FSH had a positive effect on LH receptor levels and testicular responsiveness in immature intact or hypophysectomized rats.

**Luteinizing Hormone (LH)**

Chan et al. [1981] working with adult male rats reported that administration of ovine LH (100 ug) was followed by increased prolactin and LH binding within 40 to 120 minutes. This initial rise in testicular receptors was followed by a transient loss and recovery of prolactin sites and a more prolonged loss of LH receptors. Although elevation of serum prolactin levels produced a significant positive effect on LH receptors, it did not prevent the loss of LH receptors caused by administration of bovine LH. Similarly, prior treatment with small doses of bovine prolactin failed to prevent the ovine LH-induced loss of testicular LH receptors.

In contrast, Warren et al. [1982] reported that cultured fetal testes treated with gonadotropins showed an enhanced steroidogenic responsiveness without being followed by LH receptor loss or desensitization of the
cultured cells. A study on cultured Leydig cells from mature rat testes indicated that 1ng ovine LH injection increased LH receptors by 131%, while 100 ng and 1000 ng ovine LH injections reduced LH receptors to 70% and 44% of control levels respectively (Nozu et al. [1981]). It was also reported that injections of oLH in intact mature rats caused a transient acute increase in testicular LH receptors dependent on intact microfilament function and steroidogenesis. This increase may be related to some LH-induced changes in cell membrane conformation leading to plasma membrane extension and exposure of cryptic receptor sites on the membrane surface [Huhtaniemi et al. (1981)]. Jia and Hsueh [1984] reported maximum stimulation of LH receptors in cultured rat granulosa cells with 6, 10 and 2.5 ng/ml for rat LH, ovine LH and hCG respectively, while higher doses were less effective. The changes in LH receptor number were correlated with the responsiveness of granulosa cells to LH stimulation of cAMP production. LH stimulation of iodinated hCG binding sites was associated with increases in the number of LH receptors without changes in the dissociation constant (Kd) value. LH and hCG did not affect overall granulosa cell protein content but treatment with a protein synthesis inhibitor (cycloheximide) decreased LH-induced receptors by 46% suggesting the involvement of new protein synthesis. These studies indicate that LH induces
up-regulation as well as down-regulation of its own receptors in adult animal cells while in fetal cells only the up-regulation effect was noted.

Human Chorionic Gonadotropin [hCG]

The use of iodinated human chorionic gonadotropin as a ligand tracer for assessing LH receptors number is a common practice in receptor assay procedures. The effect of hCG injection on LH receptors in mature rat testicular culture was investigated by Nozu et al. [1981]. They reported both an up- and down-regulation of LH receptors by hCG evident by an increase in LH receptor number to 153% of control levels in response to 1ng hCG injection, while 10 and 100 ng hCG injections reduced LH receptors to 57% and 2% of control levels respectively.

Jahnsen et al. [1981] reported a 40% reduction of LH/hCG responsive adenylate cyclase [AC] 2 hours after hCG injection and the total loss of [AC] 24 hours later. They also reported a gradual loss of LH receptors by day 2 that reached a nadir by day 4 of hCG injection. The authors suggested that [AC] desensitization preceding LH receptor down-regulation may support the notion that uncoupling of the catalytic subunit of the [AC] from LH/hCG receptors is a requirement for subsequent receptor internalization.

A 75 IU hCG injection reduced LH membrane receptors to a nadir of 5-10% control levels within 3 days in intact adult male rats (Purvis et al 1978). While a reduction of
LH receptors to 70% of control levels has no effect on the sensitivity and response of Leydig cells to hCG and androgen production, reduction of LH receptors to 23% control levels produce a 5-10 fold decrease in Leydig cell response to hCG.

Huhtaniemi et al. [1978] reported that low doses of 3-10 IU hCG increase LH receptors 6-16 hours after injection, while higher doses decrease it. The authors indicated that the decrease in binding but not the increase is partially dependent on protein synthesis.

Huhtaniemi et al. [1981] reported that neonatal rat testes did not show hCG-induced desensitization of androgen biosynthesis, but showed up- and down-regulation of LH receptors by low and high doses of hCG that persisted for shorter periods than in adult rat tissues. They also reported that the decreased LH binding with high hCG doses is due to receptor occupancy without net loss of receptors.

Tsuruhara et al. [1977] reported that loss of LH receptors after hCG injection was dose related at a maximum of 50, 80 and 90% at 4, 2 and 1 days after treatment with 0.2, 1 and 10 ug hCG respectively. They indicated that receptor loss is due to sequestration or internalization of binding sites and its persistence for several days suggest that occupied LH receptors were processed and degraded rather than vacated and
reutilized. These studies indicate the ability of low doses of hCG (1-10IU) to increase LH receptors while higher doses (10-100IU) decrease LH receptors and Leydig cells response to LH.

**Growth Hormone [GH]**

Treatment of immature rats with 1 ug human growth hormone resulted in a 300% increase in hCG-binding sites concomitant with 68% decrease in human prolactin binding sites (Morris and Saxena [1980]).

Results obtained by Bohnet and Friesen [1976] from treatment of dwarf mice with congenital growth hormone and prolactin deficiency indicate that lack of prolactin but not of growth hormone retards sexual development in these mice. Treatment with 10 ug/day human growth hormone did not ameliorate sexual retardation in these mice. Zipf and Berntson (1981) reported that 100 ug/day GH injections started on the first day after hypophysectomy in mature male rats maintained LH receptors at 50% of control levels.

Mondschein and Schomberg [1981] reported that epididimal and fibroblast growth factors inhibited FSH-dependent induction of LH receptors in cultured ovarian granulosa cells, while platelet growth factor potentiated it, indicating that growth factors modulate hormone- dependent differentiation in endocrine target tissues.
An LH-receptor-binding-inhibitor present in ovine testis was reported by Kalla and Zarabi [1982] to be a protein of more than 10,000 daltons molecular weight that can inhibit iodinated hCG or LH binding to rat testicular LH receptors. These studies show that growth hormone increases LH receptor level in immature rats, can partially maintain LH receptor level in hypophysectomized mature rats, but does not improve retarded sexual development.

**Estrogen**

Evidence for direct inhibitory action of estrogens on human testicular steroidogenesis with loss of testicular LH receptors being one facet of this inhibitory action was reported by Huhtaniemi et al. [1980] from a study on human prostatic cancer patients.

**Progesterone:**

A study conducted by VanStraalen and Zeilmaker [1982] indicated that progesterone implants in hypophysectomized female rats had no effect on the levels of LH receptors in their corpora lutea. Their data showed that progesterone secretion by the corpora lutea is always associated with the presence of LH receptors regardless of whether serum LH-levels are detectable or not.

**Hypophysectomy:**

Hypophysectomy of mature male rats was reported by VanStraalen and Zeilmaker [1982] to produce a significant
decrease in both LH and prolactin receptor levels in Leydig cell homogenates. In the same study hypophysectomy followed by pituitary autografts prevented the rapid decline of LH and prolactin testicular receptors. Cryptorchidism produces a 70% reduction in $^{125}\text{I}-\text{hCG}$ uptake by rat testis [Sharpe (1983) and Jegou et al. (1983)].

**Photoperiod:**
Gonadal regression induced in the golden hamster by Bex and Bartke [1977] through shortening the photoperiod to 5 hours per 24 hours produced a significant decrease in LH receptor levels. Treatment of these animals with 250 ug prolactin and growth hormone or ectopic pituitary transplants increase LH binding and reverse testicular regression. They suggested that changes in the release of prolactin and possibly also growth hormone may play an important role in mediating the effects of photoperiod on testicular function.

**Diurnal Variation:**
Testicular content of LH receptors showed significant diurnal periodicity in adult male rats over a 24 hour period investigated by Huhtaniemi et al. [1982]. Maximum receptor binding measured in testicular cell homogenates was noted between 04:00 and 08:00, while lowest receptor binding was detected between 12:00 and 24:00 hours.
Assay

Gonadotropin receptors were extensively studied in normal or cancerous testicular and prostatic tissues of rats or humans, as well as in cancerous human breast tissue, ram, pig, mouse and golden hamster testicular homogenates. A large variety of techniques have been applied to the measurement of gonadotropin receptors employing different ligands such as $^{125}\text{I-hCG}$, and various methods were used to precipitate gonadotropin receptor complexes including adsorption to hydroxylapatite, precipitation with protamine sulphate, treatment with dextran-coated charcoal, sucrose density gradient, centrifugation and binding to linked antibodies. Some procedures were associated with problems such as semiquantitative precipitation, partial dissociation of receptors and poor resolution of radioactivity peaks.

Gonadotropin receptors were extensively studied in adult and immature rat testicular tissues by a large number of investigators using mostly $^{125}\text{I-hCG}$ as the binding ligand. Normal human testicular gonadotropin receptors were studied by Grizard et al. (1982) and Leinonen et al. (1982) using $^{125}\text{I-hCG}$ as the binding ligand at 35-37°C for 2-3 hours. Human prostate cancer cells gonadotropin receptors were evaluated with the $^{125}\text{I-hCG}$ binding at 35°C for 3 hours incubation period.
and separation of gonadotropin receptor complexes by centrifugation [Huhtaniemi et al. (1980)]. Gonadotropin receptors were assayed by $^{125}\text{I-hCG}$ binding at $37^\circ\text{C}$ in the ram testis [Kalla and Zarabi (1982)] interstitial pig cells [Saez et al. (1983)] golden hamster testes [Bex and Bartke (1977)] and mouse testicular homogenate [Bohnet and Friesen (1976), Dalterio et al. (1983)].
CHAPTER 1

RESEARCH PROTOCOL OBJECTIVES

(1) To elucidate the hypothesis that anterior pituitary hormones modulate Leydig cell membrane luteinizing hormone receptors number.

(2) To explore the hypothesis that the anterior pituitary hormonal control on Leydig cell membrane luteinizing hormone receptors number is steroid-hormone mediated.

SPECIFIC EXPERIMENTAL AIMS

PART ONE

To determine the appropriate experimental animal model for our studies by comparing the effectiveness of three methods of hypophysectomy: parapharyngeal aspiration, transauricular aspiration or transauricular aspiration plus radiofrequency lesioning in inducing luteinizing hormone receptor deficiency.
PART TWO

To determine the time course of low dose luteinizing hormone induction of luteinizing hormone receptors in prolactin-pretreated hypophysectomized adult male rats.

PART THREE

To investigate the persistence of the prolactin priming effect on luteinizing hormone receptor level in prolactin-pretreated hypophysectomized adult male rats.

PART FOUR

To evaluate the potential priming effects of follicle stimulating hormone on luteinizing hormone receptor level and their persistence in hypophysectomized adult male rats.

PART FIVE

To study the potential priming effects of growth hormone on luteinizing hormone receptor level and their persistence in hypophysectomized adult male rats.

PART SIX

To test the effects of testosterone and estrogen as possible mediators of the prolactin dependent LH induction of luteinizing hormone receptors in hypophysectomized adult male rats.
ANIMALS:
For all experiments, adult male Sprague-Dawley rats between 55-70 days of age were used. All animals were caged under controlled room conditions with 14 hours light and 22-24°C temperature. All animals were fed regular rat chow and water ad lib. Hypophysectomized animals were supplied with 5 gm of sugar in each 100 ml of tap water.

ASSAY PROCEDURES:
Iodination Protocol:
Iodinated human chorionic gonadotropin (hCG) was used in all experiments as a binding ligand to evaluate the number of LH receptors in testicular homogenates. Iodination of hCG was performed in accordance with radiation safety regulations set by the Children's Hospital Research Foundation. Procedure and solution preparation are shown in Appendix.

Preparation of Testicular Homogenate:
Animals were decapitated, both testes collected, decapsulated and weighed. The left testis is homogenized in 6 ml phosphate buffer in a pyrex tube using a teflon pestle at 2500 rpm. The homogenate is then centrifuged at 14000 rpm for 20 minutes and supernatant discarded. The pellet is resuspended by homogenization at 2500 rpm in
4 ml phosphate buffer (pH = 7.5). The homogenate is diluted 1:10 by adding buffer and 0.1 ml of this receptor dilution is used in each receptor assay tube.

**Receptor Assay:**

Five assay tubes are made for each testicular homogenate: three specific binding tubes and two non-specific binding tubes. Each specific binding tube contains 100 ul receptor dilution, 70 ul phosphate buffer and 30 ul iodinated hCG. Each non-specific binding tube contains 100 ul receptor dilution, 40 ul phosphate buffer, 30 ul cold hCG and 30 ul iodinated hCG at approximately 250,000 rpm. All tubes are incubated overnight at room temperature then the reaction is stopped by addition of 4 ml ice cold phosphate buffer. Tubes are centrifuged at 14,000 rpm for 20 minutes. The supernatent discarded and tube blotted dry. The tubes are washed each with 2 ml cold phosphate buffer, centrifuged at 14,000 rpm for 20 minutes, supernatent discarded, tubes blotted dry and counted.

**DATA REDUCTION AND ANALYSIS**

**Data Reduction:**

The amount of testicular homogenate receptor dilution used in the receptor assay per animal is equal to 1/400 of the whole testis homogenate. The number of LH receptors in this portion of the testicular homogenate is evaluated by the number of counts per minute which
represent the number of hCG binding sites in the pellet after overnight incubation with $^{125}\text{I}-\text{hCG}$ at room temperature. Our results indicated a range of $0.9 \times 10^7$ to $1.2 \times 10^7$ binding sites per testis for intact adult control rat.

It is difficult to compare the number of binding sites in our studies to those reported in the literature [Ascoli (1985)] due to a considerable variation in the reference base. Investigators reported the number of binding sites per milligram wet weight, milligram protein, microgram DNA or cell numbers but none was reported per testis. Jia and Hsueh (1984) reported $1 \times 10^4$ and $7 \times 10^4$ hCG binding sites per cell in cultured rat granulosa cells from intact control animals and LH treated animals respectively. Payne et al. (1980) reported a value of about $5 \times 10^4$ binding sites per cell for cultured Leydig cells from intact mature rats.

Data is presented as percentage of control in order to facilitate reliable comparison of different experimental groups to each other. Animals used were 55 to 70 days old and the LH receptor assay ligand $^{125}\text{I}-\text{hCG}$ has a variable specific activity that tends to decline slightly with age. Each experiment was conducted with animals of the same age and assayed with $^{125}\text{I}-\text{hCG}$ of the same specific activity for all groups. Relating animal groups of the same experiment as percentage of their own
control group eliminate those variables and allow a reliable comparison between different experiments.

Data Analysis:

TUKEY'S STUDENTIZED RANGE TEST

Tukey proposed Tukey's Studentized Range Test in 1952, sometimes called "The honestly significant difference test" to control the maximum experimentwise error rate (MEER) under any complete or partial null hypothesis. It is designed for pairwise mean comparisons based on the studentized range and is used when the analyst needs to control the maximum experimentwise error rate, needs confidence intervals and has an unequal sample size. It became our choice for statistical data analysis because we were constantly under the risk of losing some of the hypophysectomized animals in different experimental groups during the test period which results in unequal sample size in the groups comparison [Snedecor and Cochran (1980)].
CHAPTER 2

PART ONE

To determine the appropriate experimental animal model for our studies by comparing the effectiveness of three methods of hypophysectomy: parapharengeal aspiration [PPA], transauricular aspiration [TAA] or transauricular aspiration plus radiofrequency lesioning [TAAL] in inducing luteinizing hormone [LH] receptor deficiency.

INTRODUCTION

Classical hypophysectomy by parapharengeal or transauricular aspiration has been held to eliminate all adenohypophysial secretions. The adenohypophysis of the rat is comprised of the pars distalis and pars intermedia lying below the diaphragma sellae and the pars tuberalis lying above the diaphragma sellae. Classical hypophysectomy accomplishes removal of pituitary tissue below the diaphragma sellae, but leaves the pars tuberalis intact [Gross and Page, (1979)]. Pars tuberalis cells
contain gonadotropes [Baker and Yu, 1975, Baker et al. 1977] that can respond to the negative feedback action of gonadal steroids (Gross 1978) and probably secrete luteinizing hormone into the hypophysial portal circulation [Antunes et al. 1979], with more gonadotropes identifiable in monkey and man pars tuberalis than that of the rats [Baker 1977]. Gonadotropin secretion is not totally abolished by hypophysectomy in the rat [Lostroh 1963], but LH receptor level decreases to a nadir of 15% to 20% of control levels one week after hypophysectomy [VanStraalen and Zeilmaker, 1982; Zipf et al. 1978a]. Immunocytochemical studies reported a hypophysectomy-induced activation of pars tuberalis gonadotropin production with a small hyperplastic and hypertropic response noted as early as one week following hypophysectomy and markedly increasing with time [Gross and Page, (1979)]. These hypertrophic and hyperplastic pars tuberalis gonadotropes have been postulated to be responsible for the low level gonadotropin secretion after hypophysectomy.

Our experimental model should undergo chronic hypophysectomy to insure maximum induction of luteinizing hormone receptor deficiency. At that stage, very low gonadotropin levels in the animal may modulate the luteinizing hormone receptor level assayed for a certain experimental protocol. Our comparative study of the three
methods of hypophysectomy was undertaken to determine the most effective method for preparation of our experimental animal model. The hypophysectomy method to be used should produce a luteinizing hormone receptor deficiency even after chronic hypophysectomy for more than two weeks. We tested three methods of obtaining hypophysectomized rats to determine if they were different in basal LH receptor concentration. We evaluated the three methods for the effects of prolactin and LH injections on their basal LH receptors.

**PROCEDURE**

For one experiment intact adult male Sprague-Dowley white rats 60-70 days old were purchased [Zivic-Miller Laboratories, Inc.] and allowed to adapt for two days at 14 hours light, 24°C temperature, regular rat chow and water ad lib. Twenty rats were kept intact under these same conditions for 30 days as a control group. Two other groups of 20 each were hypophysectomized under ether anesthesia by either transauricular aspiration alone or transauricular aspiration plus radiofrequency lesioning [Radiofrequency Generator Model RFG-4. David Kopf Instruments Electrode K1388Z. Shaft is 0.8mm, tip is 0.7 mm diameter.] Hypophysectomized animals were kept under the same conditions as control except for an ad lib supply of sugar water [5 gm/100 ml water]. All animals
were decapitated, both testes were collected, decapsulated and weighed [Table 1].

In a series of six separate experiments, LH receptor level was assayed in testicular homogenates of adult male Sprague-Dawley rats of 60-70 days old that were hypophysectomized by the parapharyngeal method and compared to LH receptor levels in intact controls of the same age range to determine the effectiveness of this method on reduction of LH receptor level [Table 3]. For another experiment, adult male Sprague-Dawley rats at 60-70 days old were purchased already hypophysectomized by the parapharyngeal aspiration method [Zivic-Miller Laboratories, Inc.] (Group A). A group of intact rats of the same age was purchased [Zivic-Miller Laboratories, Inc.], 3 of these animals were kept intact as a control group and the rest were hypophysectomized under ether anesthesia by transauricular aspiration plus radiofrequency lesioning (Group B). Group A animals were divided into 3 small groups (n=5), one kept as a hypophysectomized control, one treated with 100 ug/day prolactin (s/c) for 7 days, and the other treated with 100 ug/day prolactin (s/c) for 7 days then a single 10 ug LH/injection (s/c) for 30 minutes before sacrifice by decapitation. The same procedure was used for Group B animals. [Table 2] Previous studies reported that LH receptor levels drop to a nadir 5 days after
hypophysectomy [Zipf et al. (1978)a], therefore, all hormonal treatments were started 7 days after hypophysectomy to allow enough time for induction of an LH receptor deficiency. A dose of 100 ug/day was chosen for prolactin because previous studies indicated that 50 to 150 ug/day prolactin injections are effective in unmasking the ability of low doses (10-50 ug) LH to induce LH receptors [Zipf et al. (1978)b].
# Table 1: Effects of Two Methods of Hypophysectomy on Testes Weight

<table>
<thead>
<tr>
<th>Control</th>
<th>TAA</th>
<th>TAAL</th>
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<tbody>
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<td>Average 0.493</td>
</tr>
<tr>
<td>Average</td>
<td>3.48</td>
<td>Average 0.590</td>
</tr>
</tbody>
</table>

**TAA** = Transauricular Aspiration.

**TAAL** = Transauricular Aspiration plus radiofrequency lesioning

* Testes weight in grams

Average = Mean + Standard Error of the Mean
### TABLE (2) EFFECTS OF TWO METHODS OF HYPOPHYSECTOMY ON LH RECEPTOR NUMBER

<table>
<thead>
<tr>
<th>ANIMAL GROUP</th>
<th>N</th>
<th>% CONTROL</th>
<th>S.D.</th>
<th>S.E.</th>
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<td>6</td>
<td>3</td>
</tr>
<tr>
<td>GROUP (B) HYPOX CONTROL</td>
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<td>4</td>
<td>2</td>
</tr>
<tr>
<td>GROUP (A) + PRL</td>
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<td>27</td>
<td>4</td>
<td>2</td>
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<tr>
<td>GROUP (B) + PRL</td>
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<td>25</td>
<td>7</td>
<td>4</td>
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<tr>
<td>GROUP (A) + PRL + LH</td>
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<td>53</td>
<td>4</td>
<td>2</td>
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<tr>
<td>GROUP (B) + PRL + LH</td>
<td>3</td>
<td>51</td>
<td>2</td>
<td>1</td>
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</table>

HYPOX = Hypophysectomized  
PRL = Prolactin  
LH = Luteinizing Hormone  
S.D. = Standard Deviation  
S.E. = Standard Error of the Mean  
GROUP A = parapharyngeal aspiration hypophysectomy  
GROUP B = Transauricular aspiration plus radiofrequency lesioning hypophysectomy
<table>
<thead>
<tr>
<th>ANIMAL GROUP</th>
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<th>% OF CONTROL</th>
<th>S.D.</th>
<th>S.E.</th>
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<tr>
<td>HYPOPHYSECTOMIZED</td>
<td>5</td>
<td>18</td>
<td>6</td>
<td>3</td>
</tr>
</tbody>
</table>

S.D. = Standard Deviation

S.E. = Standard Error of the Mean
DISCUSSION

When the comparative experiment between transauricular aspiration alone and transauricular aspiration plus radiofrequency lesioning was undertaken, our LH receptor assay procedure was not yet perfected. Our original intention was to use testicular weight and serum FSH levels for the comparison. The FSH radioimmunoassay was not sensitive enough to detect differences at these low concentrations, thus we compared testicular weights data. Statistical analysis of the data using Tukey's studentized range test indicated that both methods of hypophysectomy produce a highly significant \( p < 0.01 \) reduction in testicular weight to about \([14\%-17\%]\) of control group testicular weight. Compared to one another, there was no significant difference in testes weight between the two methods indicating that both procedures were equally effective.

Tukey's studentized range test was used for data analysis comparing parapharyngeal aspiration to transauricular aspiration plus radiofrequency lesioning. Both methods produced a highly significant \( p < 0.01 \) reduction of LH receptor level to 18% and 17% of control levels respectively. There was no significant difference between the two hypophysectomized control groups, hypophysectomized-prolactin treated groups or hypophysectomized prolactin-LH treated groups indicating
an equal effectiveness of both methods.

Our data on the effects of parapharyngeal hypophysectomy on LH receptor levels indicate a highly significant (p < 0.01) reduction in LH receptor level, down to 9%-27% of control levels by parapharyngeal hypophysectomy. [Table 3]

Our results from all three methods of hypophysectomy agree with previous studies [VanStraalen and Zeilmaker 1982, Zipf et al. 1978] in producing a significant reduction in testicular homogenate LH receptor levels to a nadir of 15%-20% of control level, which persisted through chronic hypophysectomy for two weeks or more.

Our comparison of the effectiveness of the the three methods indicates that all three are equally effective and there was no significant difference between animal groups hypophysectomized by any of the three methods. Depending on cost and time factors of each experiment we used either transauricular aspiration plus radiofrequency lesioning or parapharyngeal aspiration method for preparing our animal model for the experiment.
CHAPTER 3

PART TWO

To determine the time course of induction of luteinizing hormone receptors by low dose luteinizing hormone in hypophysectomized, prolactin pretreated adult male rats.

INTRODUCTION

Previous studies have shown that low dose luteinizing hormone injections (10 ug/day) induces an upregulation of luteinizing hormone receptor level within 40 to 120 minutes followed by a prolonged period of downregulation [Chan et al 1981, Huhtaniemi et al. 1981]. Higher doses of luteinizing hormone injections (10-1000 ng/day) reduced luteinizing hormone receptor levels to 44% of control level in cultured Leydig cells [Nozu et al. 1981] and in cultured granulosa cells [Jia and Hsuch 1984]. Prolactin pretreatment mitigates the ability of LH to downregulate LH receptors and unmasks the ability of low doses of LH to induce new LH receptors in the hypophysectomized male rat [Zipf et al. (1978)a].
Our experimental protocol includes a low LH dose (10 ug/day) subcutaneous injection which is expected to undergo rapid absorption due to the semi-dehydrated and lean body conditions of the hypophysectomized animals. LH effects might be directly induced by exposing preformed cryptic membrane receptors with a maximum effect occurring in a short time period or indirectly mediated through induction of new protein synthesis with the maximum effect occurring at a later time period after absorption. The determination of the period for maximum effects of LH on LH receptor level is important for our understanding of the mechanism of LH induction of LH receptors and to maximize the LH receptor assay sensitivity. Maximizing the LH receptor assay sensitivity is necessary to insure the detection of any changes in LH receptor level that might occur in response to hormonal modulation in the experimental protocol. Our experiments were thus designed for accurate determination of the time course for LH induction of LH receptors and the period of maximum effect.

PROCEDURE

Adult male Sprague-Dawley white rats 60-70 days old were hypophysectomized by transauricular aspiration plus radiofrequency lesioning under ether anesthesia. Prolactin treatment was started seven days after hypophysectomy with an optimum dose of 100 ug S/C
injection per day [Zipf et al. (1978)a] for seven days. Twenty-four hours after the last prolactin injection each animal received a single S/C injection of 10 ug LH and sacrificed 0.5, 1, 2, 3, 4, 5, or 6 hours after the LH injection. Three groups of 4-5 animals each were kept as intact control, hypophysectomized control and prolactin treated control groups. The left testis of each animal was decapsulated, homogenized and assayed for LH receptors. [Table 4] and [Fig. 1]
### TABLE (4): EFFECTS OF TIME AFTER LH INJECTION ON LH INDUCTION OF LH RECEPTORS

<table>
<thead>
<tr>
<th>ANIMAL GROUP</th>
<th>N</th>
<th>% CONTROL</th>
<th>S.D.</th>
<th>S.E.</th>
</tr>
</thead>
<tbody>
<tr>
<td>INTACT CONTROL</td>
<td>56</td>
<td>100</td>
<td>14</td>
<td>7</td>
</tr>
<tr>
<td>HYPOPHYSECTOMIZED</td>
<td>51</td>
<td>19</td>
<td>7</td>
<td>3</td>
</tr>
<tr>
<td>CONTROL</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HYPOPHYSECTOMIZED + PROLACTIN</td>
<td>21</td>
<td>30</td>
<td>5</td>
<td>2</td>
</tr>
<tr>
<td>CONTROL</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1/2 HOUR POST LH INJECTION</td>
<td>46</td>
<td>56</td>
<td>10</td>
<td>4</td>
</tr>
<tr>
<td>1 HR POST LH INJECTION</td>
<td>8</td>
<td>51</td>
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<td>3</td>
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<tr>
<td>2 HR POST LH INJECTION</td>
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<td>3 HR POST LH INJECTION</td>
<td>7</td>
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<td>2</td>
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<td>5 HR POST LH INJECTION</td>
<td>7</td>
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</tr>
<tr>
<td>6 HR POST LH INJECTION</td>
<td>3</td>
<td>20</td>
<td>4</td>
<td>2</td>
</tr>
</tbody>
</table>

LH = Luteinizing Hormone  
S.D. = Standard Deviation  
S.E. = Standard Error of the Mean
FIGURE (1): EFFECTS OF TIME AFTER LH INJECTION ON LH INDUCTION OF LH RECEPTORS

I.C = Intact Control
H.C. = Hypophysectomized control
PRL.C = Prolactin Control

The numbers at the bottom of the bars (N) represent the number of animals in each experimental group.
Figure 1

BINDING % OF CONTROL

HOURS AFTER LH INJECTION

(N) 56 51 21 46 8 4 7 7 3
I.C. H.C. PRLC. 05 1 2 3 5 6
DISCUSSION

Hypophysectomy by transauricular aspiration plus radiofrequency lesioning produced a significant ($p < 0.01$) reduction of testicular LH receptors down to $(19\pm6\%)$ of control values. Prolactin treatment alone induced a non-significant increase of LH receptors up to $(30\%)$ of control values. Prolactin treatment in combination with 10 ug LH injection further induced LH receptors up to $(56-51\%)$ of control level for 3 hours after LH injection, with a significant ($p < 0.01$) decline of LH receptors down to $(27\%)$ of control levels thereafter. The highest peak of LH induction of LH receptors occurred at 1/2 hour after LH injection [56% of control levels].

Our results indicate that LH induction of LH receptors in hypophysectomized, prolactin pretreated adult male rats is very rapid with a peak at 30 minutes after a 10 ug LH injection (56% of control). The induction of LH receptors by low dose LH injections persists for up to 3 hours (51% of control values) after which a decrease in LH inducability becomes apparent (20% of control values) for the next hours.

Our results agree with those found by Chan et al. (1981) who reported an upregulation period from 40 to 120 minutes compared with the 30 to 180 minutes indicated in our findings. Our results also agree with those reported by Zipf et al. (1978a) indicating prolactin pretreatment
to unmask the ability of low dose LH injection to induce new LH receptors.
CHAPTER 4

PART THREE

To investigate the persistence of prolactin priming effect on LH induction of LH receptors in hypophysectomized prolactin pretreated adult male rats.

INTRODUCTION

Hypoprolactinaemia produced by suppression of plasma prolactin levels with bromocriptine injections produced a significant decrease [Aragona et al 1977] or a 60% decrease [Belanger et al. 1979] in LH receptor levels in the Leydig cells. Hyperprolactinemia induced by transplantation of anterior pituitary glands under the kidney capsules of adult male rats resulted in a 55% to 175% increase in testicular LH receptor levels [McNeilly et al. 1979]. Prolactin pretreatment increased the capacity of developing corpus luteum for LH binding in immature female rats [Holt et al. 1976]. Prolactin treatment in combination with LH increased LH receptors to levels higher than normal controls in male hamsters with gonadal atrophy induced by exposure to short photoperiods.
Prolactin injections in mature male rats produced a 1-3 fold increase in LH binding sites [Morris and Saxena 1980], or a positive effect on testicular LH receptors but did not prevent their downregulation by high doses of LH [Chan et al 1981].

**PROCEDURE**

Adult male Sprague-Dawley rats at 60-70 days old hypophysectomized by parapharyngeal aspiration were purchased [Zivic-Miller Laboratory, Inc.]. Two weeks after hypophysectomy each animal received a 100 ug/day prolactin s/c for 7 days. A group of 5 animals was injected s/c with a single dose of 10 ug LH at 2, 4, 6, 12, 24 or 36 hours after the last prolactin injection. Three control groups of 5 animals each were kept as intact control, hypophysectomized control and hypophysectomized prolactin treated control group [Table 5] and [Fig. 2].
<table>
<thead>
<tr>
<th>ANIMAL GROUP</th>
<th>N</th>
<th>% OF CONTROL</th>
<th>S.D.</th>
<th>S.E.</th>
</tr>
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<tbody>
<tr>
<td>INTACT CONTROL</td>
<td>35</td>
<td>100</td>
<td>14</td>
<td>6</td>
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<tr>
<td>HYPOPHYSECTOMIZED CONTROL</td>
<td>35</td>
<td>27</td>
<td>6</td>
<td>3</td>
</tr>
<tr>
<td>2 HR POST PROLACTIN</td>
<td>4</td>
<td>21</td>
<td>14</td>
<td>7</td>
</tr>
<tr>
<td>4 HR POST PROLACTIN</td>
<td>5</td>
<td>26</td>
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</tr>
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<td>6 HR POST PROLACTIN</td>
<td>5</td>
<td>31</td>
<td>11</td>
<td>5</td>
</tr>
<tr>
<td>12 HR POST PROLACTIN</td>
<td>5</td>
<td>56</td>
<td>10</td>
<td>5</td>
</tr>
<tr>
<td>24 HR POST PROLACTIN</td>
<td>5</td>
<td>51</td>
<td>7</td>
<td>3</td>
</tr>
<tr>
<td>36 HR POST PROLACTIN</td>
<td>4</td>
<td>51</td>
<td>18</td>
<td>9</td>
</tr>
</tbody>
</table>

S.D. = Standard Deviation  
S.E. = Standard Error of the Mean  
LH = Luteinizing Hormone  
N = Represents the Number of Animals in each Experimental Group.
FIGURE (2): EFFECT OF TIME AFTER THE LAST PROLACTIN INJECTION ON LH INDUCTION OF LH RECEPTORS

I.C. = Intact Control
H.C. = Hypophysectomized Control
PRL.C = Prolactin Control

The numbers at the bottom of the bars (N) represent the number of animals in each experimental group.
Figure 2

BINDING % OF CONTROL

HOURS AFTER LAST PROLACTIN INJECTION

(N) 35 35 25 4 5 5 5 5 4
DISCUSSION

The parapharyngeal aspiration hypophysectomy was effective in producing a significant \( p < 0.01 \) reduction in LH receptor levels down to 27±6% of control values. Prolactin treatment alone did not produce a significant change in LH receptor level from the hypophysectomized group (30±2% of control values). The low dose 10 ug LH injection given 2, 4 or 6 hours after the last prolactin injection did not induce any significant increase in LH receptor levels from hypophysectomized values (21-31% of control values). LH injection given at 12 hours after the last prolactin injection produced a significant \( p < 0.01 \) increase in LH receptors level up to 56±5% of control values. A similarly significant \( p < 0.01 \) increase in LH receptor levels was noted with LH injections given 24 and 36 hours after the last prolactin injection (51±3% of control values).

Our results agree with those reported by Bex and Bartke (1977), Morris and Saxena (1980) and Chan et al. (1981) in reporting a significant increase in LH receptor level with a prolactin treatment in combination with low dose LH injections. Our results further indicate that the priming effect of prolactin treatment on LH induction of LH receptors shows a no effect period of 6-12 hours during which no significant increase in LH receptor levels was noted in response to low dose LH injections. The
prolactin priming showed a significant effect at 12 hours, persisted for 24 hours then started to decline slightly afterwards. There is no evidence in the literature of a similar investigation of the persistence and time course of prolactin priming effects on LH induction of LH receptors to compare to our results.

The study suggests that prolactin priming effect on LH induction of LH receptors may occur through an intermediate step. The nature of this intermediate step could possibly be either through another intermediate hormonal pathway or through induction of a new population of LH receptors that involves new protein synthesis. These possibilities and questions are addressed in Part (6).
CHAPTER 5

PART FOUR

To evaluate the potential priming effect of FSH on LH receptors and their persistence in hypophysectomized adult male rats.

INTRODUCTION

The effects of FSH on LH receptor levels and testicular responsiveness to LH was investigated in immature intact and immature hypophysectomized male rats [Chen et al. (1977)] and in cultured granulosa cells from female rats [Erickson et al. (1982)]. Chen et al. (1977) tested for the effects of 40-80 ug/day injections of FSH alone and in combination with either testosterone or estradiol on LH receptors level and testicular responsiveness to LH stimulation. They reported that intact immature rats showed a significant increase in LH receptors without a change in testicular responsiveness while the immature hypophysectomized rats showed a significant increase in both LH receptor number and testicular responsiveness. Cellular binding of hCG as an
indicator of LH receptor level was used by Erickson et al. (1982) to study immature hypophysectomized rats from which estrogen primed granulosa cells were cultured in a serum free media and incubated with 10 ng/ml FSH. They reported a marked increase in hCG binding with a maximum after 3 days of incubation.

Our studies with prolactin had demonstrated the crucial nature of the timing and possible need for LH to induce LH receptors. Therefore, these studies were designed to re-evaluate the potential effects of FSH on mature Leydig cells that might have been missed by earlier studies not incorporating these important factors.

PROCEDURE

Adult male white Sprague-Dawley rats at 60-70 days old hypophysectomized by parapharyngeal aspiration were purchased [Zivic-Miller Laboratories Inc]. Seven days after hypophysectomy, a 100 ug/day S/C injection of FSH was administered for seven days. Animals were given a single 10 ug S/C injection of LH at 1/2, 3, 6, 12 or 24 hours after LH injection. Four additional groups were kept as intact, hypophysectomized, plus LH or plus FSH control groups. [Table 6] and [Fig. 3]
### TABLE (6): EFFECTS OF FSH PRIMING AND TIMED LH INJECTIONS ON LH RECEPTORS NUMBER

<table>
<thead>
<tr>
<th>ANIMAL GROUP</th>
<th>N</th>
<th>% of CONTROL</th>
<th>S.D.</th>
<th>S.E.</th>
</tr>
</thead>
<tbody>
<tr>
<td>INTACT CONTROL</td>
<td>35</td>
<td>100</td>
<td>12</td>
<td>5</td>
</tr>
<tr>
<td>HYPOPHYSECTOMIZED</td>
<td>35</td>
<td>19</td>
<td>6</td>
<td>3</td>
</tr>
<tr>
<td>CONTROL</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HYPOPHYSECTOMIZED</td>
<td>10</td>
<td>19</td>
<td>6</td>
<td>3</td>
</tr>
<tr>
<td>+ LH CONTROL</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>FSH CONTROL</td>
<td>4</td>
<td>20</td>
<td>2</td>
<td>1</td>
</tr>
<tr>
<td>LH INJECTION</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1/2 HOUR POST FSH</td>
<td>4</td>
<td>23</td>
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<tr>
<td>3 HOURS POST FSH</td>
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<td>12 HOURS POST FSH</td>
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<td>24 HOURS POST FSH</td>
<td>4</td>
<td>18</td>
<td>2</td>
<td>1</td>
</tr>
</tbody>
</table>

S.D. = Standard Deviation  
S.E. = Standard Error of the Mean  
FSH = Follicle Stimulating Hormone  
LH = Luteinizing Hormone  
N = The Number of Animals in Each Experimental Group
FIGURE (3): EFFECTS OF FSH PRIMING AND TIMED LH INJECTIONS ON LH RECEPTORS NUMBER

I.C. = Intact Control
H.C. = Hypophysectomized control
FSH. E. = Follicle stimulating hormone control

The numbers at the bottom of the bars (N) represent the number of animals in each experimental group.
Figure 3

BINDING % OF CONTROL

(N) 35 35 10 4 4 4 4 4 4

HOURS AFTER LAST FSH INJECTION
DISCUSSION

Statistical analysis using Tukey's studentized range test indicate that parapharyngeal hypophysectomy reduced LH receptors level significantly (p < 0.01) to about 19±6% of control values in these experiments. LH or FSH treatment alone was not able to induce any significant change in LH receptors level (19±6% and 20±2% respectively). FSH treatment plus a single dose of LH had no significant effect on LH receptor level at any time point investigated between 1/2 and 24 hours.

Results reported by Chen et al. (1977) indicated that a 40-80 ug/day FSH injection in immature intact male rats significantly increased the number of LH receptors without altering testicular responsiveness to LH. A similar treatment in immature hypophysectomized rats markedly increased both the number of LH receptors and testicular responsiveness to LH. Erickson et al. (1982) reported a marked increase in hCG binding with a maximum after 3 days of incubation of cultured granulosa cells with 10 ng/ml FSH in a serum free media.

Although these studies indicate a positive effect of FSH on LH receptor level in immature rats, our results showed no significant effect of FSH on LH receptor level in a mature hypophysectomized rat.

It is concluded that FSH affects the LH receptor level and testicular responsiveness to LH during the
immature stages to assist maturation, but the role and effects of FSH declines to a non-significant level after the animal attains maturity. Thus the specific response of immature cells to FSH is not seen again in the mature Leydig cells.
CHAPTER 6

PART FIVE

To study the potential priming effects of GH on LH receptor level and their persistence in hypophysectomized adult male rats.

INTRODUCTION

The effects of human growth hormone injections on hCG-binding sites and human prolactin binding sites in immature rats were studied by Morris and Saxena [1980]. They reported a 300% increase in hCG binding sites in response to 1 ug GH injections. Dwarf mice with congenital growth hormone and prolactin deficiency were studied by Bohnet and Friesen (1976) to investigate the effect of growth hormone and prolactin treatment on their sexual development. Their results indicated that treatment with 10 ug/day GH did not improve sexual retardation in these mice. Our results from the prolactin experiments (Part 3) indicated the important role of time of hormonal treatment and LH crucial role in induction of LH receptors. These experiments were designed to account
for these factors and since no study was conducted on the effects of growth hormone on LH induction of LH receptors in a mature hypophysectomized male rat model, our experiments were undertaken to investigate that role.

**PROCEDURE**

Adult male Sprague-Dawley rats at 60-70 days old hypophysectomized by parapharyngeal aspiration were purchased [Zivic-Miller Laboratories, Inc.]. Seven days after hypophysectomy, animals were injected S/C with 100 ug/day of GH for seven days. Animals were given a single S/C injection of 10 ug LH 1/2, 3, 6, 12 or 24 hours after the last GH injection and sacrificed for assay 1/2 hour after LH injection. An intact, hypophysectomized, plus LH or plus GH control groups were also maintained. [Table 7] and [Fig. 4]
TABLE (7): EFFECTS OF GH PRIMING AND TIMED LH INJECTIONS ON LH RECEPTORS NUMBER

<table>
<thead>
<tr>
<th>ANIMAL GROUP</th>
<th>N</th>
<th>% CONTROL</th>
<th>S.D.</th>
<th>S.E.</th>
</tr>
</thead>
<tbody>
<tr>
<td>INTACT CONTROL</td>
<td>35</td>
<td>100</td>
<td>12</td>
<td>6</td>
</tr>
<tr>
<td>HYPOPHYSECTOMIZED CONTROL</td>
<td>35</td>
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<td>6</td>
<td>3</td>
</tr>
<tr>
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<td>3</td>
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<td>1/2</td>
</tr>
<tr>
<td>LH INJECTION</td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1/2 HOUR POST GH</td>
<td>4</td>
<td>29</td>
<td>4</td>
<td>2</td>
</tr>
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<td>3 HOURS POST GH</td>
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<td>24</td>
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</tr>
<tr>
<td>6 HOURS POST GH</td>
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<td>26</td>
<td>4</td>
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</tbody>
</table>

S.D. = Standard Deviation
S.E. = Standard Error of the Mean
GH = Growth Hormone
LH = Luteinizing Hormone
FIGURE (4): EFFECTS OF GH PRIMING AND TIMED LH INJECTIONS ON LH RECEPTORS NUMBER

I.C. = Intact Control
H.C. = Hypophysectomized Control
G.H. C' = Growth Hormone Control

The numbers at the bottom of the bars (N) represent the number of animals in each experimental group.
Figure 4

Binding % of Control

(N) 35 35 10 4 4 4 4 4 4

I.C. H.C. H.C. II G.H.C. 0.5 3 6 12 24

Hours After Last GH Injection
DISCUSSION

The parapharyngeal aspiration hypophysectomy produced a significant decrease in LH receptor levels down to $19 \pm 6\%$ of control levels. LH treatment alone did not induce any significant change in LH receptor levels ($19 \pm 6\%$ of control levels). GH treatment alone showed a slight increase in LH receptor level from $19 \pm 6\%$ for hypophysectomized to $28 \pm 1\%$ of control levels which was not significant. GH treatment combined with a low dose LH injection failed to induce any significant change in LH receptor level at all time points investigated between 1/2 to 24 hours ($29-26\%$ of control levels).

Results obtained by Bohnet and Friesen (1976) from treatment of dwarf mice with congenital growth hormone and prolactin deficiency indicate that treatment with 10 ug/day human growth hormone had no effect in improving sexual retardation in these mice. On the other hand, Morris and Saxena (1980) reported that treatment of immature rats with 1 ug human growth hormone resulted in a 300% increase in hCG-binding sites. Our results are in contrast to Morris and Saxena (1980) but agree with those of Bohnet and Friesen (1976) who reported no significant effect of growth hormone on LH receptor level. Zipf et al. (1978) reported that ovine GH injections (150 ug/day) started immediately after hypophysectomy for 7 days were effective in maintaining LH receptor concentration near
normal levels in male rats. Our results indicate that starting GH injections 7 days after hypophysectomy did not show any GH capability to affect LH induction of LH receptors unlike that shown by prolactin. GH effects may be similar to FSH effects in increasing LH receptors level in the immature animal to assist sexual maturation but decline to a non-significant level after maturation is attained.
To test for effects of testosterone and estrogen as possible mediators of prolactin-dependent luteinizing hormone induction of luteinizing hormone receptors in hypophysectomized adult male rats.

INTRODUCTION

A study in human prostatic cancer patients was conducted by Huhtaniemi et al. (1980) to evaluate the action of estrogens on human testicular steroidogenesis and testicular LH receptors and reported an inhibitory action of estrogens on testicular responsiveness with loss of LH receptors. A combined treatment of hypophysectomized immature rats with estradiol or testosterone plus FSH was done by Chen et al. (1977) to study the role of testosterone and estradiol on the effect of FSH on the number of LH receptors and the testicular responsiveness to LH. Their results indicated that testosterone enhances the effect of FSH on LH receptors but not the effect of FSH on testicular response to LH.
while estradiol has no effect on FSH induced increase in testicular responsiveness to LH.

Previous studies have reported a direct luteinizing hormone effect on plasma levels of gonadal steroid in both male and female animals [O'Shaughnessy and Payne (1982)]. On the other hand, prolactin has no effect on plasma concentrations of gonadal steroids, but has an effect on changing gonadal steroid receptors number [Holt et al. 1976, Bex and Bartke 1977, Aragona et al. 1977, Belanger et al. 1979, McNeilly et al. 1979, Morris and Saxena 1980, Chan et al. 1981].

Our experiments were undertaken initially to study the direct effects of testosterone and estrogen on potentiation of LH induction of LH receptors in the hypophysectomized mature male rats with or without prolactin priming. Further investigations were carried with both hormones to test the hypothesis that prolactin priming effects on LH induction of LH receptors might work through a pathway involving steroid production. This hypothesis was to find a physiological explanation for the essential role of prolactin in LH induction of LH receptors and to account for the latent period of 6-12 hours in prolactin priming effect on LH induction of LH receptors observed in our previous experiments (Part 3).
PROCEDURE

Adult male Sprague-Dawley rats at 60-70 days old hypophysectomized by parapharyngeal aspiration were used. Seven days after hypophysectomy, the animals were injected with 100 ug/day prolactin, 1 mg/day testosterone or 1 mg/day estrogen [Premarin] singley or in combination for seven days. These testosterone and estrogen doses were chosen because previous studies indicated that 0.4 to 1.0 mg/day is effective in maintaining normal function of primary sex glands and normal sexual behavior [Chowdhury et al. (1979)]. All animals were given a single S/C injection of 10 ug LH before being killed for assay. Two sets of experiments were performed with groups of intact and hypophysectomized control kept for each set [Table 8] and [Fig. 5 and 6].
TABLE (8): EFFECTS OF PROLACTIN, TESTOSTERONE AND ESTROGEN TREATMENT ON LH INDUCTION OF LH RECEPTORS

<table>
<thead>
<tr>
<th>ANIMAL CONTROL</th>
<th>N</th>
<th>% CONTROL</th>
<th>S.D.</th>
<th>S.E</th>
</tr>
</thead>
<tbody>
<tr>
<td>INTACT CONTROL</td>
<td>35</td>
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S.D. = Standard Deviation
S.E. = Standard Error of the Mean
LH = Luteinizing Hormone
PRL = Prolactin
FIGURE (5): EFFECTS OF PROLACTIN AND TESTOSTERONE TREATMENT ON LH INDUCTION OF LH RECEPTORS

I.C. = Intact Control
H.C. = Hypophysectomized Control
T = Testosterone
PRL = Prolactin

The numbers at the bottom of the bars represent the number of animals in each experimental group.
FIGURE (6): EFFECTS OF PROLACTIN AND ESTROGEN TREATMENT ON LH INDUCTION OF LH RECEPTORS

I.C. = Intact Control
H.C. = Hypophysectomized Control
E = Estrogen
PRL = Prolactin

The numbers at the bottom of the bars (N) represent the number of animals in each experimental group.
DISCUSSION

Parapharyngeal aspiration hypophysectomy reduced LH receptors significantly \( p < 0.01 \) to about 19+7% of control levels. Treatment with testosterone or estrogen for seven days, plus a single low LH dose did not show a significant change in LH receptor level \[12+2\% \text{ for testosterone and } 13+1\% \text{ for estrogen}\]. Treatment with prolactin plus a single LH injection produced a significant increase up to 56+2% of control levels. A combined treatment of prolactin and testosterone or estrogen for seven days plus a single LH injection did not produce a marked increase in LH receptors level \[18+1\% \text{ for testosterone and } 22+3\% \text{ for estrogen}\]. A combination of prolactin with testosterone or estrogen without LH was tested and showed a non-significant effect on LH receptors level \[22+2\% \text{ for testosterone and } 27+2\% \text{ for estrogen}\].

Results reported by Chen et al. (1977) indicated that a combined treatment of hypophysectomized immature rats with testosterone and FSH enhanced the effect of FSH on LH receptors but not the effect of FSH on testicular responsiveness to LH. In the same study, a combined treatment of estradiol and FSH has no effect on FSH induced increase in testicular responsiveness to LH. Huhtaniemi et al. (1980) reported evidence of direct inhibitory action of estrogens on human testicular steroidogenesis with loss of testicular LH receptors in
human prostatic cancer patients. Our results clearly show an inhibitory role for both estrogen and testosterone on the prolactin-dependent LH induction of LH receptors. The mechanism of this inhibitory steroid action is difficult to explain but it may indicate that an intermediate product in the steroidogenic pathway, rather than testosterone or estrogen, is the mediator of prolactin-dependent LH induction of LH receptors. Another possible explanation is that this might indicate an autocrine negative feedback of steroids at Leydig cell level via decreasing LH receptor concentration thus reducing testicular responsiveness to LH and consequently reducing testicular steroidogenesis.
CHAPTER 8

CONCLUSION

It is known that endocrine control of testicular function in the adult is composed of intratesticular and extratesticular elements. Luteinizing hormone (LH) and follicle stimulating hormone (FSH) regulate both Leydig cell and seminiferous tubule function. In turn, Leydig cell sex steroids and seminiferous tubular non-sex steroid factors modulate peripheral concentrations of the gonadotropins by their interaction at the hypothalamic and pituitary level. The main regulator of Leydig cell function is LH, but FSH also influences Leydig cells probably through an FSH mediated increase in Leydig cell LH receptors and enhancing LH stimulation of testosterone secretion. Erickson et al. [1982] suggested that cyclic AMP plays a role in the mechanism of LH-receptor regulation by FSH but indicated that the mechanism is complex and some non-cyclic AMP factors are also involved. Considerable research has been done regarding the role of prolactin in the regulation of testicular
function and modulation of LH receptors. Two important points should be mentioned here, the first one is that most of the research on prolactin effects and function was done on rodents. The second point is that the assessment of LH receptors was mostly done using labeled hCG due to its availability but hCG has a longer half-life than LH and an exaggerated binding capacity may be reflected by hCG use. Hypoprolactinaemia induced by hypophysectomy (VanStraalen and Zeilmaker [1982], Erickson et al. [1982] Bohnet and Friesen [1976]) produce a significantly decreased testicular LH receptor and testosterone production. Hyperprolactinemia induced by pituitary transplants (Sharpe and McNeilly [1979]) or by metoclopramide infusion (Chan et al. [1981]) significantly increased testicular LH receptor and testosterone production. On the other hand, hyperprolactinemia in man is often associated with hypogonadism and impotence (Boyer et al.[1974], Thorner et al. [1974]). Other studies indicate that prolactin maintains or even increases LH receptors in the Leydig cells (VanStraalen and Zeilmaker [1982]) and that the modulation is both dose and age dependent (Morris and Saxena [1980]). Prolactin is suggested to stimulate testicular function in hamsters with gonadal regression through increasing the binding and receptor levels of endogenous LH (Bex and Bartke [1977]). Prolactin was shown to regulate its own receptors as well
as LH receptors (Morris and Saxena [1980]) mediated probably through the luteotropic actions of prolactin on the Leydig cells (Holt et al. [1976]). Chan et al. [1981] pointed out that the long-term action of prolactin on the maintenance of LH testicular receptors does not influence the acute increase and subsequent loss of binding sites that follow gonadotropin stimulation of Leydig cells. On the other hand, Warren et al. [1982] reported no down-regulation of LH receptors in cultured fetal rat testis in response to gonadotropins indicating a significant difference in the response of fetal and adult Leydig cells to gonadotropin stimulation.

The actions and interactions of gonadotropins on the testicular components are very complex and may include beside the biochemical components some structural organization that can affect adjacent cellular functions [Bartke (1976) and deKretser (1982)]. Experimentation and biochemical analysis have proven that testicular steroidogenesis function is highly affected by LH receptor levels which may be modulated by a variety of hormones such as prolactin, FSH, growth hormone, estrogen, testosterone, progesterone as well as by LH itself. In addition to this differential hormonal modulation, physiological variations in gonadotropin levels due to environmental factors such as diurnal and photoperiod variations can induce autoregulatory change in testicular
LH receptor levels and the steroidogenic process [Huhtaniemi et al. (1982) Bex and Bartke (1977)].

Our experiments were designed for a more detailed investigation of the hormonal modulation of total LH receptors in the testicular homogenate of a hypophysectomized adult male rat model. Assessment of the regulatory influence of prolactin, follicle stimulating hormone, growth hormone, estrogen and testosterone on the number of LH receptors were studied at specific dose ranges and timed injection intervals to further understand the dose-related, time-dependant effects of these hormones on the total LH receptors population in a testicular homogenate.

Our investigations about LH modulation of its own receptor level indicated that LH induction of LH receptors is very rapid showing a significant peak at 30 minutes after LH injection, which persisted for up to 3 hours after which a non-significant inductibility was noted.

The mechanisms responsible for the ability of low dose LH injections to induce new LH receptors are not clearly understood. This phenomenon can be blocked by pretreating the animals with aminoglutethimide (a compound that blocks steroid biosynthesis by preventing the conversion of cholesterol to pregnenolone) [Huhtaniemi et al. (1981)]. In addition, it was reported that cycloheximide (a protein synthesis inhibitor) had no
effect on LH induction of LH receptors. These results imply that LH induction of LH receptors is not due to an increase in receptor synthesis and is related to the ability of LH to stimulate steroid biosynthesis. These findings support the hypothesis of Suter et al. (1980) who proposed that LH induction of LH receptors is due to the externalization of preformed receptors that occurs during the LH-induced exocytosis of steroid-containing secretory granules. Other investigators [Huhtaniemi et al. (1981), Baranao and Dufau (1983)] have proposed that LH induction of LH receptors is due to general changes in membrane conformation leading to exposure of cryptic membrane receptors. More work needs to be done in this area before a clear understanding of the molecular events involved in LH induction of LH receptors is attained.

Our studies on prolactin priming effects on LH induction of LH receptors showed a no effect period of 6 to 12 hours after which a significantly positive effect of prolactin on LH induction of LH receptors was noted and persisted for about 24 hours then declined thereafter. A possible hypothesis for the prolactin no-effect period of 6-12 hours could be that prolactin effect is induced through an intermediate hormonal pathway involving one or more hormones from the steroidogenic pathway. Our investigations of testosterone and estrogen as candidates for this mediation reported negative results. The
remaining members of the steroidogenic pathway need to be tested for this role before the hypothesis is disregarded.

Another possible hypothesis is that prolactin effect is induced by increased protein synthesis and new prolactin receptor population. A Leydig cell culture preparation with protein synthesis inhibitors is required to test the validity of this hypothesis.

It could also be hypothesized that the prolactin no-effect period is due to prolactin-induced steroidogenesis via androgen receptors on the Leydig cell membrane [Guliza et al. (1983)] leading to a steroid inhibitory action on LH ability to induce LH receptors. Experiments using different doses of prolactin need to be designed to establish a dose-time relationship between prolactin injections and the time duration of the no-effect period with a possible correlation to serum steroid levels. [See Fig. 7]

Our experiments on the probable priming effects of FSH and GH on LH induction of LH receptors indicated no significant effect on LH receptors level at any time period between 1 to 24 hours after either FSH or GH injections started 7 days after hypophysectomy and continued for 7 days at 100 ug/day doses.

Testing the hypothesis of a probable role of testosterone or estrogen as prolactin mediators in LH
FIGURE (7): DIAGRAMATIC REPRESENTATION OF THE HYPOTHETICAL PATHWAYS INVOLVED AS POSSIBLE EXPLANATIONS FOR THE PROLACTIN NO-EFFECT PERIOD ON LH INDUCTION OF LH RECEPTORS
induction of LH receptors did not show any significant mediatation effects of testosterone or estrogen on LH receptor level to support the hypothesis. However, an inhibitory effect of both testosterone and estrogen on LH receptors was observed. The mechanism of this inhibitory steroid action is difficult to explain but it may indicate that an intermediate product in the steroidogenic pathway, rather than testosterone or estrogen, is the mediator of prolactin-dependent LH induction of LH receptors. Another possible explanation is that this might indicate an autocrine negative feedback of steroid at Leydig cell level via decreasing LH receptor concentration thus reducing testicular responsiveness to LH and consequently reducing testicular steroidogenesis.

These studies demonstrate the ability of small doses of LH to increase LH receptor level and emphasize its specific dependency and association with prolactin. The studies also suggest that the more physiologic role of LH is maintenance of LH receptor level in contrast to the pharmacologic downregulation effect.
APPENDIX A

PREPARATION OF 0.05 M PHOSPHATE BUFFERED SALINE

Solution A: Place in a 500 ml volumetric flask:
1. 3.45 gm Na$_2$HPO$_4$·H$_2$O (monobasic)
2. 4.5 gm NaCl
3. 0.5 gm NaN$_3$ or 50 mg merthiolate
4. Bring to volume with deionized water

Solution B: Place in 1000 ml volumetric flask:
1. 7.10 gm Na$_2$HPO$_4$, anhydrous (diabasic)
   Note: increase weight if a hydrated form is used, e.g. Na$_2$HPO$_4$·H$_2$O
2. 9.0 gm NaCl
3. 1.0 gm NaN$_3$ (sodium azide) or 100 mg merthiolate
4. Bring to volume with deionized H$_2$O

Place about 800 ml of solution B in a 2000 ml beaker (pH 8.7) with a stir bar and pH electrode. Add solution A (monobasic) until a pH of 7.5 is obtained with a total volume of 1000 to 1200 ml.
APPENDIX B

PREPARATION OF 0.5 M PHOSPHATE BUFFERED SALINE

Solution A: Place in a 250 ml volumetric flask:

1. 14.25 gm NaH$_2$PO$_4$ (monobasic)
2. 2.25 gm NaCl
3. 0.25 gm NaN$_3$ (sodium azide) or 25 mg merthiolate
4. Bring to volume with deionized H$_2$O

Solution B: Place in a 500 ml volumetric flask:

1. 35.5 gm Na$_2$HPO$_4$ anhydrous (dibasic)
2. 4.5 gm NaCl
3. 0.5 gm sodium azide or 50 mg merthiolate
4. Bring to volume with deionized H$_2$O

Place about 400 ml of solution B in a 1000 ml beaker (pH 8.7) with stir bar and pH electrode. Add solution A (monobasic) until a pH 7.5 is obtained with a final volume of 700 to 800 ml.
APPENDIX C

PREPARATION OF ASSAY BUFFERS

BUFFER I

To make one liter of buffer I

85.85 gms of 0.25 M Sucrose
11.92 gms of 0.01 M HEPES

BUFFER II

To make 100 ml of buffer II

100 ml buffer I
0.1 gm of Bovine Serum Albumin
0.1 gm of CaCl₂

BUFFER III

To make 100 ml of buffer III

100 ml of buffer I
0.01 gm of Bovine Serum Albumin
0.05 gm of CaCl₂
APPENDIX D

PREPARATION OF BIO-GEL COLUMN

1. Prepare Bio-gel slurry with:
   - 2 gm Bio-gel
   - 0.01 gm sodium azide (NaN₃)
   - 60 ml 0.05 M NaPO₄ buffer

   Cover and let swell overnight at room temperature.

2. Pack column and clamp outlet.

3. Add 3 ml of 2% BSA solution and unclamp outlet.

4. Elute with 0.05 M NaPO₄ buffer for 10 minutes in order to coat gel.

5. Clamp outlet and store at 4°C for use within the next 2 months. If column was stored for more than 2 months, coating with BSA should be redone by repeating Steps 3 and 4.
APPENDIX E

PREPARATION OF IODINATION SOLUTIONS

CHLORAMINE-T

25 mg chloramine-T
10 ml 0.05 M NaPO$_4$ buffer

Sodium metabisulphite (Na$_2$S$_2$O$_5$)

25 mg Na$_2$S$_2$O$_5$
10 ml 0.05 M NaPO$_4$ buffer

Transfer-Rinse Solution

1 mg KI or NI
8 gm sucrose
100 ml 0.05 M NaPO$_4$ buffer

2% Bovine Serum Albumin Solution

2 gm BSA
100 ml 0.05 M NaPO$_4$ buffer

hCG Hormone Solution

1 mg hCG powder
10 ml 0.05 M NaPO$_4$ buffer
This gives 0.1 ug/ul solution.

Store 25 ul aliquate containing 2.5 ug in 1 ml vials (frozen)
APPENDIX F

IODINATION PROCEDURE

1. Put on gloves.
2. Remove top from lead pig containing $^{125}$I and place behind lead bricks.
3. Thaw 1 ml serum vial containing 2.5 ug hormone in 25 ul 0.05 NaPO$_4$ buffer.
4. Add 25 ul of 0.5 M NaPO$_4$ buffer to hormone vial.
5. Load Hamilton syringe with 1 mCurie $^{125}$I and add to hormone vial. Mix by tapping.
6. Change gloves.
7. Add 10 ul chloramine-T to hormone vial. Mix by tapping for exactly 60 seconds.
8. Add 50 ul sodium metabisulphite. Mix by tapping.
10. Take clean transfer syringe with air bubble in it and fill with reaction mixture. Slowly add the reaction mixture to top of Bio-gel column just under buffer surface.
11. Add 50 ul of rinse solution to hormone vial. Use transfer syringe with air bubble to layer rinse solution on top of Bio-gel column.
IODINATION PROCEDURE

12. Open column outlet and collect 20 fractions of 1.0 ml each in 12x75 test tubes containing 200 ul of 2% BSA solution. Count 10 ul of each fraction to determine peaks.
1. Decapsulate the left testis and remove the spermatic blood vessel.

2. Add 6 ml of buffer I.

3. Homogenize in a pyrex tube and centrifuge at 14,000 g for 20 minutes.

4. Pour into a 10 ml culture tube and centrifuge at 14,000 g for 20 minutes.

5. Decant and discard the supernatant.

6. Dislodge the pellet in 4 ml of buffer I, pour into a homogenizing tube and resuspend by homogenization.

7. Make a 1:10 dilution of the homogenate in step (6) by mixing 0.1 ml homogenate with 0.9 ml of buffer I.

8. Use 0.1 ml of this dilution for the receptor assay.

NOTE:

a) The homogenate will separate if allowed to stand. Mix well by vortexing before pipetting.

b) The homogenate is viable for 72 hours if stored at 4°C.
APPENDIX H

RECEPTOR ASSAY PROCEDURE

1. Prepare three tubes for specific binding and two tubes for non-specific binding for each sample.

2. Specific binding tubes each contain:
   - 30 ul hot $^{125}$I-hCG (containing 250,000 cpm)
   - 70 ul buffer II
   - 100 ul testicular homogenate dilution

3. Non-specific binding tubes each contain:
   - 30 ul hot $^{125}$I-hCG
   - 30 ul cold hCG
   - 40 ul buffer II
   - 100 ul testicular homogenate dilution

4. Incubate overnight at room temperature

5. Add 4 ml of cold buffer I to stop the reaction

6. Centrifuge at 14,000 g for 20 minutes

7. Discard the supernatant and blot the tubes dry

8. Add 2 ml buffer I and centrifuge at 14,000 for 20 minutes

9. Discard the supernatant and blot the tubes dry

10. Count and subtract the non-specific binding count from the specific binding counts.
LIST OF REFERENCES


