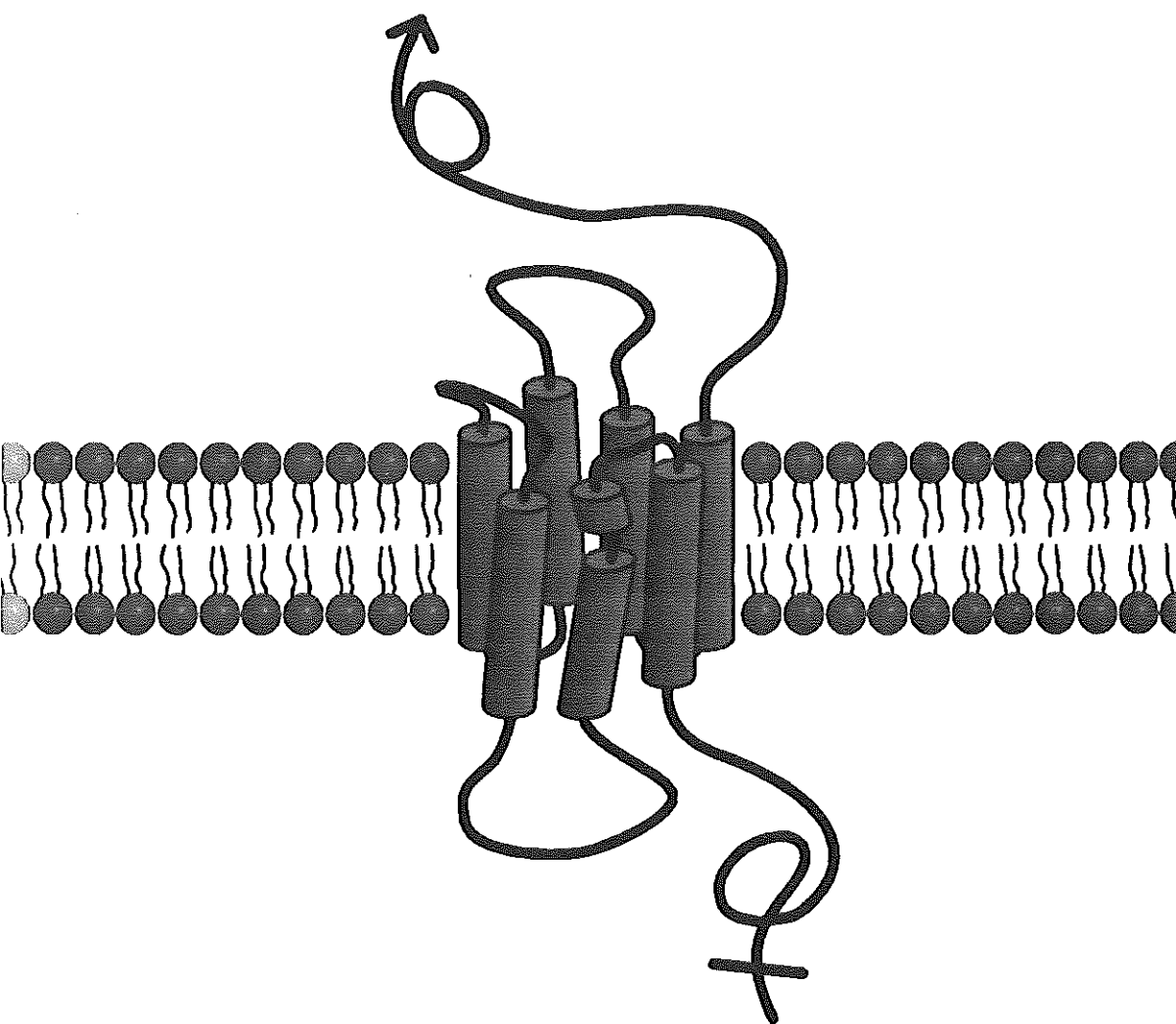


Expression and Activation of Gonadotropin Receptors



Robert Kraaij

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Expressie en Activering van Gonadotropine Receptoren

PROEFSCHRIFT

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Voor mijn ouders
Voor Marjan

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ABBREVIATIONS

ActD	actinomycin D	ICL	intracellular loop
ACTH	adrenocorticotrophic hormone	IGF	insulin-like growth factor
AIS	androgen insensitivity syndrome	IL	interleukin
AMH	anti-Müllerian hormone	i.m.	intramuscular
β ARK	β -adrenergic receptor-specific kinase	IP ₃	inositol 1,4,5-trisphosphate
Bmax	maximal binding	IU	international unit
bp	base pair	i.v.	intravenous
8-Br-cAMP	8-bromo 3',5'-cyclic adenosine monophosphate	Kd	dissociation constant
cAMP	3',5'-cyclic adenosine monophosphate	kD	kilo Dalton
CAT	chloamphenicol transferase	LCH	Leydig cell hypoplasia
cDNA	complementary deoxyribonucleic acid	LH	luteinizing hormone
CREBP	3',5'-cyclic adenosine monophosphate response element-binding protein	min	minute
C-terminus	carboxyl-terminus	mRNA	messenger ribonucleic acid
DAG	1,2-diacylglycerol	MSH	melanocyte-stimulating hormone
dbcAMP	dibutyl 3',5'-cyclic adenosine monophosphate	N-terminus	amino-terminus
DHT	dihydrotestosterone	p.c.	post-coitus
DRB	5,6-dichlorobenzimidazole riboside	PCR	polymerase chain reaction
ECL	extracellular loop	PIP ₂	phosphatidyl-inositol 4,5-bisphosphate
ED	effective dose	PKA	protein kinase A
EDS	ethane dimethyl sulphonate	PKC	protein kinase C
EGF	epidermal growth factor	PMA	phorbol 12-myristate 13-acetate
FMPP	familial male-limited precocious puberty	PMDS	persistent Müllerian duct syndrome
FSH	follicle-stimulating hormone	PMSG	pregnant mare serum gonadotropin
GAPDH	glyceraldehyde 3-phosphate dehydrogenase	PTH	parathyroid hormone
GH	growth hormone	PTHrP	parathyroid hormone related peptide
GM-CSF	granulocyte-monocyte colony-stimulating factor	R1881	methyltrienolone
GnRH	gonadotropin-releasing hormone	s.c.	subcutaneous
G protein	GTP-binding protein	SD	standard deviation
h	hour	s.e.m.	standard error of the mean
hCG	human chorionic gonadotropin	SRY	sex determining region of the Y chromosome
		SSCP	single strand conformational polymorphism
		SV40	simian virus 40
		TGF	transforming growth factor
		TM	transmembrane segment
		TSH	thyroid-stimulating hormone

Chapter 1

General Introduction

Hypothalamus-pituitary-gonadal axis

Among the many hormones that are produced by the anterior pituitary gland, luteinizing hormone (LH, lutropin), follicle-stimulating hormone (FSH, follitropin), and thyroid-stimulating hormone (TSH, thyrotropin) form the separate family of so-called glycoprotein hormones (reviewed by Gharib *et al.*, 1990). These hormones consist of two glycosylated subunits, α and β , which are associated through non-covalent interactions. The α -subunit is identical for all glycoprotein hormones, whereas the β -subunit is hormone specific. The gonadotropins, LH and FSH, are the key regulators of testis and ovary function, and are synthesized in cells called the gonadotrophs of the pituitary gland. TSH, which regulates thyroid function, is produced in the thyrotrophs. In primates and horses, a fourth glycoprotein hormone exists, chorionic gonadotropin (CG), which is synthesized in the placenta during pregnancy, and is structurally and functionally related to LH (Gharib *et al.*, 1990).

LH and FSH act on specific target cells in the gonads. LH stimulates the testicular Leydig cells and the ovarian theca and granulosa cells, whereas FSH stimulates Sertoli and granulosa cells in these respective organs. The target cells express hormone-specific receptors, which belong to the large family of G protein-coupled receptors (Loosfelt *et al.*, 1989; McFarland *et al.*, 1989; Parmentier *et al.*, 1989; Sprengel *et al.*, 1990). G protein-coupled receptors have a characteristic transmembrane domain that consists of seven membrane spanning helices. Signal transduction through these receptors is mediated by specific G proteins that transfer the signal from the hormone activated receptor to an effector protein that generates a specific second messenger signal inside the cell (Chapter 2). Receptors for the glycoprotein hormones share unique structural and functional characteristics, and form a distinct subgroup within the G protein-coupled receptor family (Chapter 2).

LH and FSH production by the pituitary gland is under the control of the central nervous system, through the secretion of the decapeptide GnRH (gonadotropin-releasing hormone) from the hypothalamus into the hypothalamus-pituitary portal blood. GnRH stimulates the gonadotrophs in the anterior pituitary to secrete LH and FSH into the general circulation. In the male (Fig. 1.1; Griffin and Wilson, 1992), LH stimulates the Leydig cells to produce testosterone, a so-called male sex hormone. In target tissues, testosterone can be converted

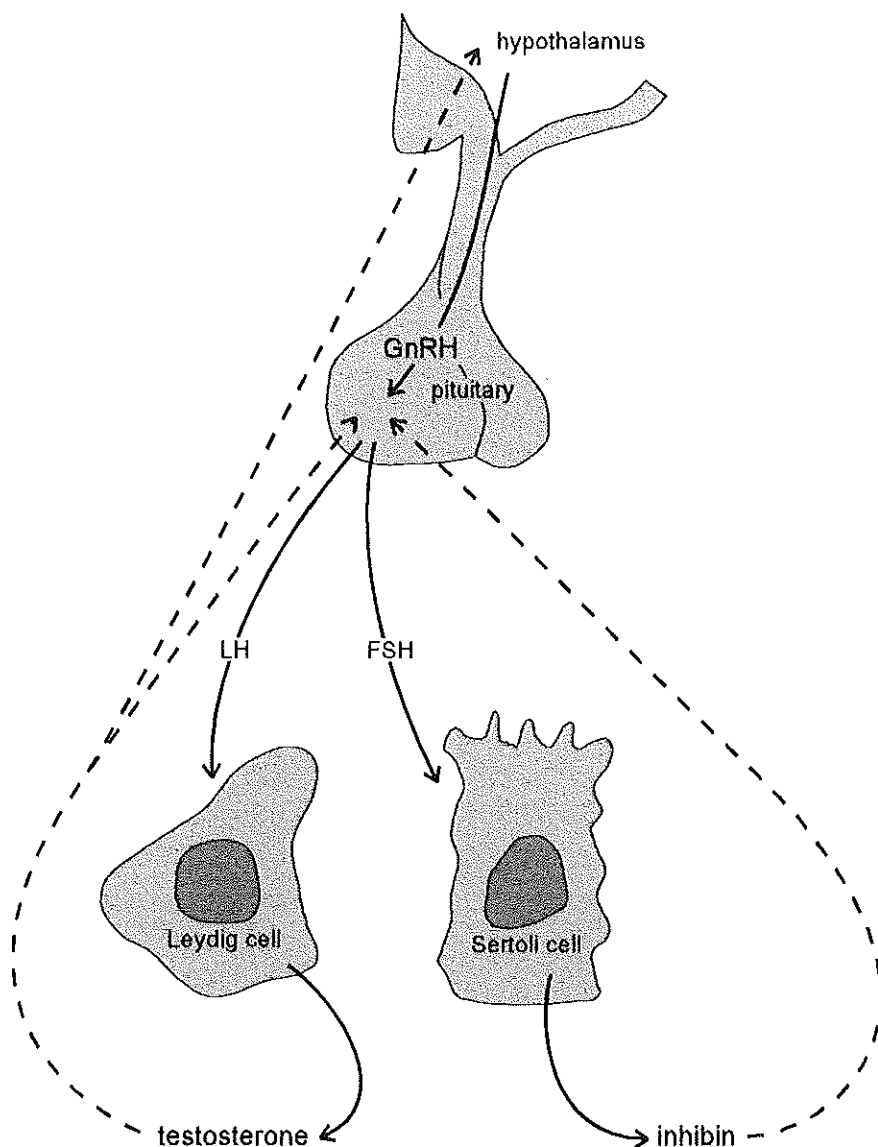


Fig. 1.1. Schematic representation of the hypothalamus-pituitary-testis axis.

Hypothalamus and pituitary gland are depicted at the top of the figure, and the testis is represented by two major cell types: Sertoli cells and Leydig cells. Stimulatory actions are shown in solid lines; negative feedback loops are shown in dotted lines. GnRH: gonadotropin-releasing hormone; LH: luteinizing hormone; FSH: follicle-stimulating hormone. Not shown are interactions between Leydig cells and Sertoli cells.

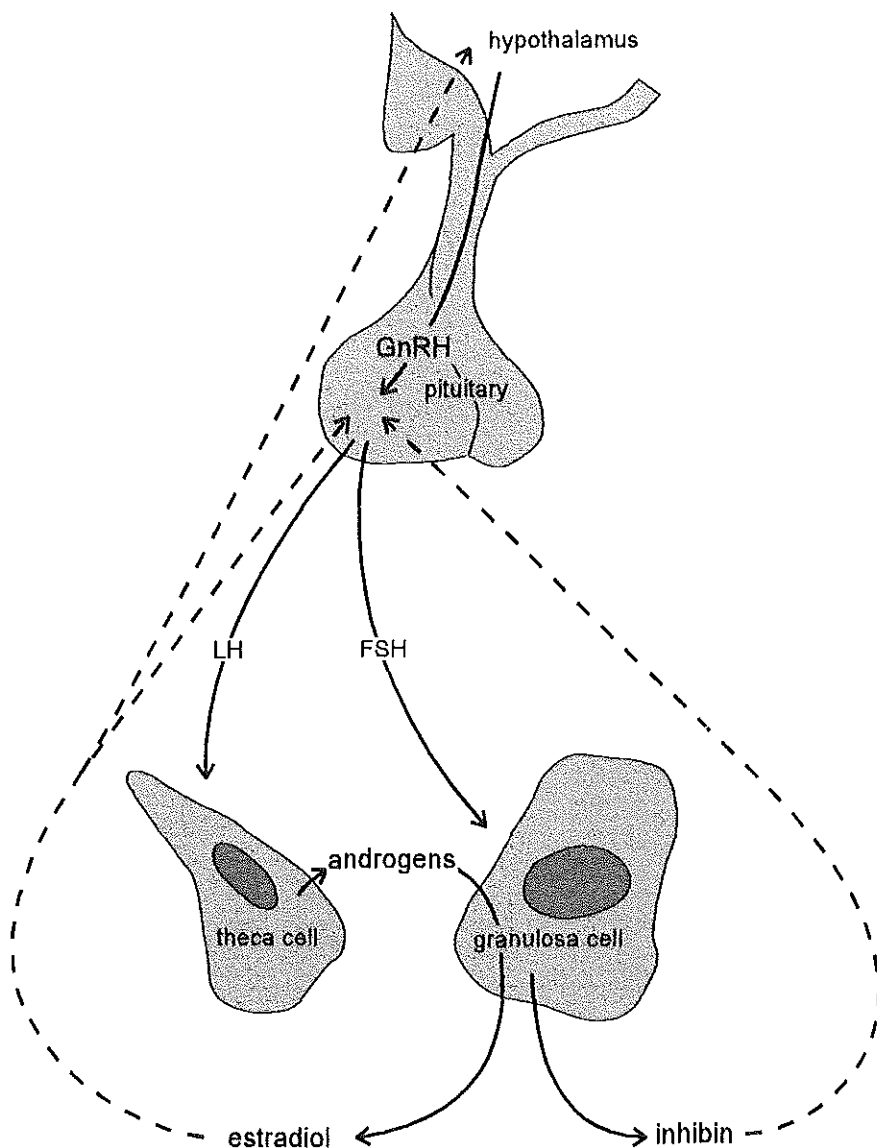


Fig. 1.2. Schematic representation of the hypothalamus-pituitary-ovary axis.

Hypothalamus and pituitary gland are depicted at the top of the figure, and the ovary is represented by theca cells and granulosa cells. Stimulatory actions are shown in solid lines; negative feedback loops are shown in dotted lines. GnRH: gonadotropin-releasing hormone; LH: luteinizing hormone; FSH: follicle-stimulating hormone.

into the more potent androgen dihydrotestosterone, through the action of 5 α -reductase. Furthermore, in Sertoli cells testosterone can be converted into the 'female sex hormone' estradiol by the FSH-induced aromatase activity (Gore-Langton *et al.*, 1980). Testosterone and dihydrotestosterone are the main sex hormones produced in the male. Besides the induction of aromatase activity, FSH regulates the synthesis of many proteins in Sertoli cells (e.g. Cheng *et al.*, 1986; Lee *et al.*, 1986). One of these proteins, inhibin, exerts a negative feedback action on FSH secretion from the pituitary, but its role in the hypothalamus-pituitary-testis axis in the adult male is not clear (Risbridger *et al.*, 1990). Testosterone remains the major negative feedback regulator in the hypothalamus-pituitary-testis axis, by inhibition of GnRH secretion from the hypothalamus and LH and FSH secretion at the level of the pituitary (Griffin and Wilson, 1992). In the female (Fig. 1.2; Carr, 1992), LH stimulates the theca cells to produce androgens, and to a lesser extent estrogens. Most of the androgens is converted into estradiol, in granulosa cells by the FSH-dependent aromatase activity. Also in extra-glandular tissues, ovarian and adrenal androgens are converted into estrogens, which serves as the major estrogens source in the female. Similar to the situation in Sertoli cells, estradiol and inhibin production by granulosa cells is under control of FSH (Risbridger *et al.*, 1990; Gore-Langton and Dorrington, 1981). Estradiol exerts negative feedback actions on GnRH secretion at the hypothalamic level, and on LH and FSH secretion at the pituitary level, whereas inhibin has a negative feedback action on FSH secretion from the pituitary (Carr, 1992; Risbridger *et al.*, 1990).

The hypothalamus-pituitary-gonadal axis is first established during fetal development, whereafter some components of the axis play a role in male sex differentiation. At later ages, maturation of the axis initiates the second step in sex differentiation: puberty. Finally, the mature axis in the adult individual regulates the reproductive functions of the gonads. The role of the hypothalamus-pituitary-gonadal axis in sex differentiation, puberty, and adult reproduction is described in the following paragraphs.

Sex determination and differentiation

At the time of conception, the sex of the fertilized egg is determined genetically. Male zygotes have an XY sex chromosome constitution, whereas female zygotes have an XX constitution. During the first developmental stages, male and female embryos develop according to a similar pattern, resulting in the formation of indifferent gonads, the mesonephric or Wolffian ducts, and the paramesonephric or Müllerian ducts. These ducts are the anlagen of most of the male and female internal genitalia, respectively. During these stages of early development, also the external genitalia are bipotential, and their differentiation is dependent on the sex specific development of the gonads (George and Wilson, 1994).

In the absence of a Y sex chromosome, the indifferent gonads develop into ovaries. Subsequently, the Wolffian ducts do not develop due to the absence of androgens, but the Müllerian ducts develop into the fallopian tubes, the uterus, and the upper part of the vagina. Finally, the external genitalia develop according to the female pattern. Male fetuses also have the potential to follow this developmental path, but expression of the *SRY* gene (sex determining region of the Y chromosome gene) induces the development of the indifferent gonads into testes (Sinclair *et al.*, 1990; Berta *et al.*, 1990). The subsequent steps in male sex differentiation are regulated by the testes through secretion of testosterone and anti-Müllerian hormone (AMH). Testosterone stimulates the Wolffian ducts to develop into epididymides, vasa deferentia, and seminal vesicles. Furthermore, testosterone is converted by 5 α -reductase activity in several target cells into dihydrotestosterone, which stimulates the development of the prostate and male external genitalia. Development of the Müllerian ducts is prevented through production by Sertoli cells of AMH, that induces active regression of the ducts (Jost *et al.*, 1973).

Incomplete sex differentiation

After correct testis development, formation of male internal and external sex organs and structures is under control of AMH and androgens. However, the production or action of these hormones may fail, which leads to partial development according to the default, female pattern. If androgen signalling completely fails, the Müllerian ducts still regress under control of AMH, but the Wolffian ducts do not develop. Furthermore, female external

genitalia develop due to the absence of androgen action. Isolated failure of androgen action has been described for the androgen insensitivity syndrome (for review see Brinkmann *et al.*, 1991; Schweikert, 1993). In the complete form of the androgen insensitivity syndrome, androgen action is abolished due to mutational inactivation of the androgen receptor that is encoded by a gene on the X chromosome. Most aspects of the androgen insensitivity syndrome are mimicked in patients with decreased androgen production. In Leydig cell hypoplasia patients, reduced androgen action is observed due to a very low testicular androgen production (Brown *et al.*, 1978). Both syndromes (androgen insensitivity and Leydig cell hypoplasia) in their most complete forms result in the clinical presentation of an external female phenotype by 46,XY individuals, who appear to have inguinal testes and a blind-ending vagina, but no internal male or female genitalia derived from the Wolffian and Müllerian ducts. In patients with the Leydig cell hypoplasia syndrome, however, partial development of epididymes and vasa deferentia has been observed, which may be due to residual testicular androgen production that exerts a local action (Chapter 6). Failure in signalling by AMH, the other testicular product involved in sex differentiation, can be caused by incorrect or absent synthesis of the hormone or the receptor, and results in the presence of Müllerian duct derivatives in individuals with an otherwise normal male phenotype (PMDS: persistent Müllerian duct syndrome; Josso *et al.*, 1991). Although the Wolffian ducts develop normally in these patients, the presence of oviducts and uterus disturbs the normal process of testicular descent. This results in cryptorchidism or aberrant descent, which affects male fertility (Mouli *et al.*, 1988; Mahfouz *et al.*, 1990).

Herein, the description of sex differentiation is brief, also because more comprehensive discussions on this topic can be found in recent theses by Ris-Stalpers (1994), and Baarends (1995).

Puberty

Puberty is the stage in the process of postnatal growth and development at which the hypothalamus-pituitary-gonadal axis matures, which leads to increased production of sex hormones and subsequent development of secondary sex characteristics, and accelerated

growth (growth spurt). Behavioural alterations and the onset of the final stages of gamete production (reproductive maturity) are important processes accomplished during puberty. Puberty can be regarded as the final step in sex differentiation.

The initial trigger for the onset of puberty is produced in the central nervous system, where the negative feedback actions of the sex hormones on the hypothalamus and pituitary become desensitized (Grumbach and Styne, 1992). This results in elevation of GnRH and gonadotropin levels, and, subsequently, in elevation of sex hormone levels. The sex hormones initiate and maintain the development of secondary sex characteristics. Furthermore, sex hormones enhance the secretion of growth hormone (GH), which results in the pubertal growth spurt (Tanner, 1989). Most actions of GH are mediated by insulin-like growth factor-I (IGF-I), which stimulates longitudinal growth through stimulation of bone tissue. Sex hormones are also able to stimulate local IGF-I secretion in the bone. At the end of the pubertal growth spurt, when growth velocity ceases, sex hormones are involved in epiphyseal fusion in the bone, thereby ending longitudinal growth (Tanner, 1989).

Physical changes for boys and girls during puberty have been documented by Marshall and Tanner (Marshall and Tanner, 1969; Marshall and Tanner, 1970). In boys, the first sign of puberty is testicular growth (at an average age of 12 years) and pubic hair development. Later on (average 13 years) the growth spurt and growth of the penis commence. Other features of physical male pubertal changes are facial and auxiliary hair development, and growth of the larynx causing lengthening of the vocal cords and subsequent lowering (breaking) of the voice. At age 16-17, the major changes of puberty are finished (Marshall and Tanner, 1970). In girls, puberty starts at an average age of 11 years. At this point, the growth spurt is most obvious, but breast development and pubic hair growth start at approximately the same time. At an average age of 13 years, the first menstrual bleeding (menarche) occurs, although the accompanying cycle of the ovary is, in most cases, anovulatory. In girls, the major changes are accomplished at an age of 14 to 15 years (Marshall and Tanner, 1969).

Delayed and absent puberty

The onset of puberty varies between individuals. In general, puberty is delayed when no pubertal signs are present at 2.5 standard deviations (SDs) after the average age of onset (age and SD expressed in years). Puberty is considered to be delayed when no signs of puberty

have occurred, for boys by 14 years of age, and for girls by 13 years (Tanner, 1989). In most cases, however, puberty will eventually commence, but in some cases puberty is truly delayed or absent. Two groups of delayed puberty can be distinguished: *hypogonadotropic hypogonadism* and *hypergonadotropic hypogonadism* (Grumbach and Styne, 1992).

Hypogonadotropic hypogonadism is caused by failure of the hypothalamus-pituitary system to produce enough GnRH or gonadotropins to initiate puberty. Disruption of the hypothalamus-pituitary system, caused by a tumor, radiation therapy in this region, or disruption of the stalk that connects the hypothalamus and the pituitary, may result in lowered or absent gonadotropin production. Also, hypogonadotropic hypogonadism can be one aspect of the phenotype of a syndrome, e.g. Kallmann's syndrome. In this syndrome, hypoplasia or aplasia of the olfactory lobes results in affected or absent smell, which is often associated with isolated GnRH deficiency. This is caused by failure of GnRH secreting neurons to migrate during development from the fetal nose to the hypothalamus (Schwanzel-Fukuda *et al.*, 1989).

Hypergonadotropic hypogonadism is caused by inability of the gonads to produce sex hormones upon gonadotropin stimulation. Since the negative feedback of sex hormones on the hypothalamic-pituitary level is intact but decreased, high levels of gonadotropins are found. Partial or complete dysgenesis of the gonads is mostly the cause of lowered gonadal responsiveness, such as is found in Klinefelter's syndrome in 47,XXY males, and more complete in Turner's syndrome in 45,X0 females (Grumbach and Styne, 1992).

Precocious puberty

When the first signs of puberty are present 2.5 SDs (years) before the average age of onset, i.e. in boys before 9 years and in girls before 8 years of age, the onset of puberty is considered to be too early, which is called precocious puberty (Tanner, 1989). Two forms of precocious puberty can be distinguished: true or complete precocious puberty, and incomplete precocious puberty. In case of the complete form, puberty is initiated through elevated GnRH or gonadotropin secretion. This may be caused by disorders, such as tumors, in the hypothalamus-pituitary system (Grumbach and Styne, 1992). In contrast, when puberty starts as a result of autonomous sex hormone production, or, in boys, by ectopic LH or hCG production (Cohen *et al.*, 1991; Englund *et al.*, 1991), then the incomplete form of precocious puberty is found. In females, this may be caused by granulosa or theca cell

tumors, that produce sufficient amounts of estrogens to initiate breast and pubic hair development (Biscotti and Hart, 1989). Autonomous androgen production in boys may be caused by Leydig cell tumors (Kaufman *et al.*, 1990). Also, adrenal tumors, producing high amounts of adrenal androgens, may cause precocious puberty in both boys and girls (e.g. Morales *et al.*, 1989).

Besides histological disorders, such as tumors that secrete excessive amounts of hormones, mutations in components of the gonadotropin signal transduction routes may lead to precocious puberty in boys and girls. In McCune-Albright syndrome patients, many disorders of hyperactive endocrine glands, including precocious puberty, can occur. The syndrome is caused by a somatic dominant mutation in the α -subunit of the stimulatory G protein G_s , which results in continuous stimulation of adenylyl cyclase (Weinstein *et al.*, 1991). Depending on the site of expression, this mutation can result in complete (pituitary oversecretion of gonadotropins) or incomplete (gonadal overproduction of steroid hormones) precocious puberty in both males and females (Grumbach and Styne, 1992; Weinstein *et al.*, 1991). Familial male-limited precocious puberty, an autosomal dominant inherited disorder, is caused by a mutation in the LH receptor. Single amino acid substitutions result in an activated receptor in the absence of ligand, which leads to autonomous LH-independent testosterone production in males and, subsequently, to precocious puberty (Kremer *et al.*, 1993; Shenker *et al.*, 1993; Chapter 5).

The adult gonad

The testis

The adult testis can be divided roughly into two compartments: the seminiferous tubules and the interstitial space in between the tubules (Fig. 1.3). The tubules contain a basal membrane that is synthesized by the peritubular myoid cells on the outside and the Sertoli cells on the inside. Through tight junctions, adjacent Sertoli cells form the blood-testis barrier that is impenetrable for large hydrophilic molecules and separates the basal side of the tubule from the adluminal side. The main function of the Sertoli cells is to support and regulate the development of germ cells (spermatogenesis). During germ cell development,

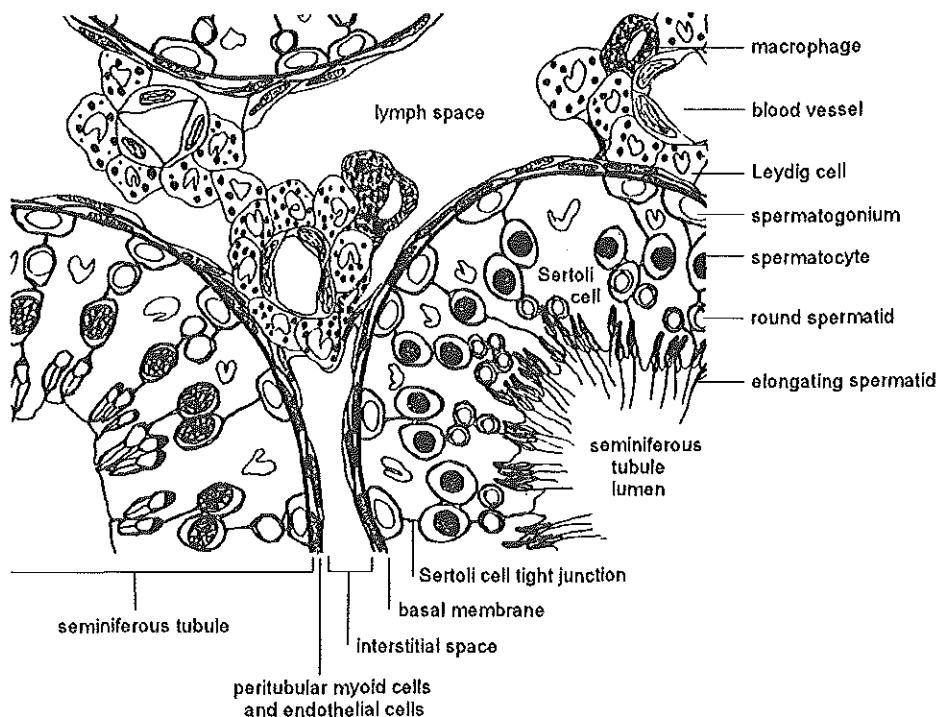


Fig. 1.3. Schematic representation of a cross-section through a part of the rat testis. Cross-section through the adult testis, showing parts of two tubules at different stages of the spermatogenic cycle (lower left and right), and the interstitial space. (From Blok, 1992).

the cells are associated with the Sertoli cells and migrate from the basal side of the tubule to the adluminal side, where they undergo meiotic and post-meiotic development, finally ending in the release of mature sperm into the lumen. Spermatogenesis is highly organized: each Sertoli cell is associated with a specific set of germ cells at different steps of their development. In the rat, a total of fourteen different germ cell combinations can be distinguished, that follow each other in time and constitute the spermatogenic cycle (Sharpe, 1994). Undifferentiated spermatogonia are located at the basal membrane, and through mitotic divisions differentiated spermatogonia are produced that enter spermatogenesis. Upon differentiation of spermatogonia to spermatocytes, the cells pass through the meiotic prophase and then undergo the meiotic divisions. This results in formation of haploid spermatids that differentiate into mature spermatozoa (spermiogenesis), which are then

released into the lumen (spermiation). Further maturation of the spermatozoa takes place in the epididymis (Sharpe, 1994).

An important cell type of the interstitial space are the Leydig cells, which produce and secrete testosterone under the control of LH. Besides the Leydig cells, blood vessels, macrophages and lymph space add up for a significant part of the interstitial space. As described in the previous paragraphs, testosterone and its metabolite dihydrotestosterone are responsible for manifestation of the male primary and secondary sex characteristics. Furthermore, testosterone, together with FSH, is also involved in regulation of spermatogenesis. When LH and FSH levels rise at the onset of puberty, spermatogenesis is thought to be initiated and maintained through combined actions of testosterone and FSH on Sertoli cells, although the importance of FSH for these processes is less clear than the importance of testosterone (Sharpe, 1994; discussed in Chapter 7). Besides the actions of testosterone and FSH, paracrine interactions between Leydig cells, Sertoli cells, and peritubular myoid cells are involved in fine-tuning of testis functions (Sharpe, 1994).

The ovary

The fetal/pre-pubertal ovary is populated by a large number of primordial follicles that consist of an oocyte, a layer of pregranulosa cells, and a basal membrane (Fig. 1.4; Hirshfield, 1991). Starting at puberty, primordial follicles grow by division and differentiation of the pregranulosa cells, and become, through different intermediate stages, tertiary follicles (Fig. 1.4). During this growth phase, mesenchymal interstitial cells differentiate and form a theca cell layer around the basal membrane. Further growth of tertiary follicles is dependent on FSH (Carr, 1992; Chappel and Howles, 1991). A cyclic pattern of secretion of the gonadotropins from the pituitary gland regulates the estrous cycle, which can be divided into a follicular phase and a luteal phase. During the follicular phase, a group of tertiary follicles is *recruited* to grow. The granulosa cell layer of these follicles further increases by granulosa cell divisions, and cavities develop that will eventually merge into one large antrum (antral follicles). During each ovarian cycle in the human, one or two antral follicles are *selected* to ovulate, and continue to grow into a pre-ovulatory or Graafian follicle (Fig. 1.4). The follicles that are not selected will degenerate, and disappear in a process called atresia that involves apoptosis (Hsueh *et al.*, 1994). The processes of recruitment and selection of follicles are thought to be regulated by actions of FSH (Carr,

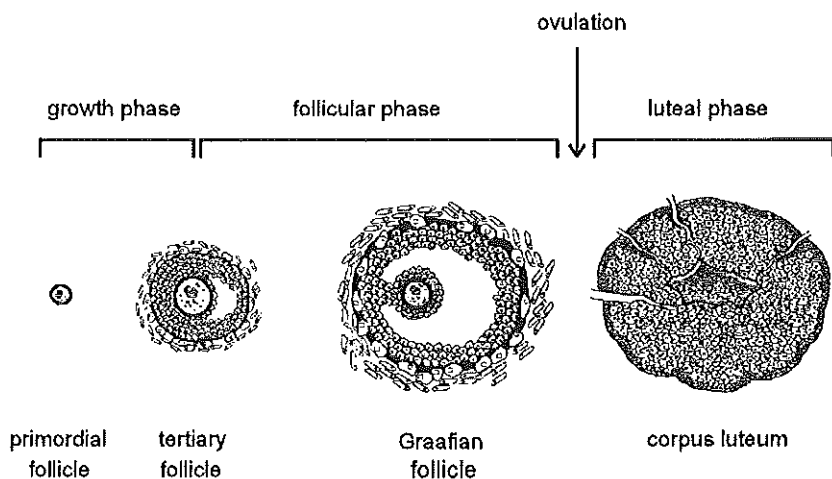


Fig. 1.4. Schematic representation of folliculogenesis.

Subsequent stages of follicular development are shown, and the different phases, as described in the text, are indicated at the top. From the outside of the Graafian follicle to the inside, the following layers can be distinguished: theca cell layer, basal membrane, granulosa cell layer, and antrum. The oocyte and its surrounding cumulus cells are depicted in the center. The corpus luteum is invaded by blood vessels, and consists of luteinized granulosa and theca cells. [Based on Carr *et al.* (1992)]

1992; Chappel and Howles, 1991). LH levels are relatively low during the follicular phase and stimulate the theca cells to produce androgens, which are converted into estradiol by the FSH-induced aromatase activity in granulosa cells. Besides its effects on aromatase activity, FSH induces the production of inhibin and the expression of LH receptors in granulosa cells (Carr, 1992; Kanzaki *et al.*, 1994). Both estradiol and inhibin exert negative feedback actions on gonadotropin levels (Fig. 1.2), but at the end of the follicular phase, when a Graafian follicle is present, the estradiol level rises and exerts a negative feedback rather than a positive feedback. This results in a surge of circulating LH, which induces ovulation of the Graafian follicle through LH receptors now present on the granulosa cells. The oocyte is released and the granulosa and theca cell layers differentiate to form a corpus luteum (Fig. 1.4). During the luteal phase of the cycle, the corpus luteum produces large amounts of progesterone and estradiol under control of LH. The high estradiol level controls the levels of LH and FSH via the feedback loop that has become negative again (Carr, 1992).

Progesterone stimulates maturation of the endometrium to become receptive to the fertilized oocyte (Carr, 1992). If the oocyte is not fertilized, the corpus luteum degenerates, the endometrium is shed during menses, and a new cycle has commenced. On the other hand, when the oocyte is fertilized, the zygote is implanted in the endometrium and fetal development commences. The developing placenta produces large amounts of hCG, a functional homologue of LH, that stimulates the corpus luteum to maintain secretion of progesterone and estradiol (Carr, 1992).

Objectives and approaches

Objectives

As described in the previous paragraphs, the hypothalamus-pituitary-gonadal axis plays an essential role in sex differentiation, puberty, and adult gonadal functions. In this axis, the gonadotropins LH and FSH are the messengers between the central nervous system and the gonads, and are considered to be the main regulators of gonadal function. Gonadotropin actions in the gonads are mediated by the gonadotropin receptors, which belong to the family of G protein-coupled receptors. Signal transduction through these receptors involves different aspects, such as ligand binding, receptor activation, G protein-coupling and activation, effector activation, and subsequent intracellular signalling. Furthermore, the activated signal transduction route is controlled by cellular processes that regulate receptor expression and receptor function. Signal generation and signal regulation are important processes in the physiological actions of gonadotropins and, thus, in the regulation of the hypothalamus-pituitary-gonadal axis. This thesis is focussed on two parts of gonadotropin signal transduction, i.e. regulation of FSH receptor expression, and mutational analysis of the LH receptor.

Approaches

In Chapter 2 an overview of G protein-coupled receptors is given, with a focus on specific characteristics of glycoprotein hormone receptors.

In Chapter 3, a conspicuous characteristic of glycoprotein hormone receptors is

described, i.e. alternative splicing of the pre-mRNAs encoding these receptors. Alternative splicing of the FSH receptor pre-mRNA can result in expression of truncated receptor proteins. Possible functions of these truncated proteins in FSH signal transduction were investigated. In Chapter 4, experiments on hormone-induced FSH receptor down-regulation are described.

Chapters 5 and 6 concern mutational analysis of the LH receptor. Mutations in the LH receptor gene were found in patients with familial male-limited precocious puberty (Chapter 5), which resulted in a constitutively activated LH receptor molecule. In Chapter 6, patients with male pseudohermaphroditism are described that have a homozygous mutation in the LH receptor gene that renders the encoded receptor completely inactive. These studies give insight into receptor function, and also into the physiological importance of LH/hCG during development, puberty, and gametogenesis.

In the General Discussion (Chapter 7) the importance of regulation of FSH receptor expression and alternative splicing in FSH signal transduction is discussed. A current compilation of different mutations in glycoprotein hormone receptors is given, and the effects of these mutations on receptor function are discussed. Furthermore, the physiological importance of LH/hCG and FSH during sex differentiation, puberty, and gametogenesis is discussed.

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Chapter 2

Gonadotropin Receptors

Part of this chapter is based on:

Regulation of gonadotropin receptor gene expression

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Introduction

The gonadotropin receptors are expressed at the cell surface and belong to the family of G protein-coupled receptors. Although these receptors share a number of structural and functional characteristics, each receptor possesses several unique features. This chapter describes general characteristics of G protein-coupled receptors, such as ligand binding, receptor activation, G protein coupling, intracellular signalling, and cellular desensitization. Besides these general characteristics, the glycoprotein hormone receptors share some specific features, such as ligand binding and expression of alternatively spliced mRNA transcripts, which will also be described in this chapter.

The family of G protein-coupled receptors

The G protein-coupled receptor family has many different members. The name of this family comes from the interaction of the receptor molecule with an intracellular G protein. The receptors share a general serpentine structure (Fig 2.1), and three domains can be recognized: a small N-terminal extracellular domain, a transmembrane domain, and a C-terminal intracellular domain, also referred to as intracellular tail (Savarese and Fraser, 1992). Although the basic structure of the various receptors is similar, the ligands or activators of these receptors range from small bioamines to large protein hormones, and even include ions and photons. The transmembrane domain consists of seven transmembrane segments, which are hydrophobic helices that span the plasma membrane in an alternating N-C or C-N orientation. These segments are connected by three extracellular and three intracellular loops (Fig. 2.1). Many G protein-coupled receptors are glycosylated at asparagine residues in the extracellular domain, and some are palmitoylated at cysteine residues in the intracellular tail. The palmitoyl moiety is thought to be associated with the lipid bilayer of the plasma membrane, thereby forming a putative fourth intracellular loop (Ovchinnikov *et al.*, 1988; Fig. 2.1).

For rhodopsin, the photoreceptor of retinal rod cells that is composed of the protein opsin and the prosthetic group 11-cis-retinal, and that is activated by photons, the three-dimen-

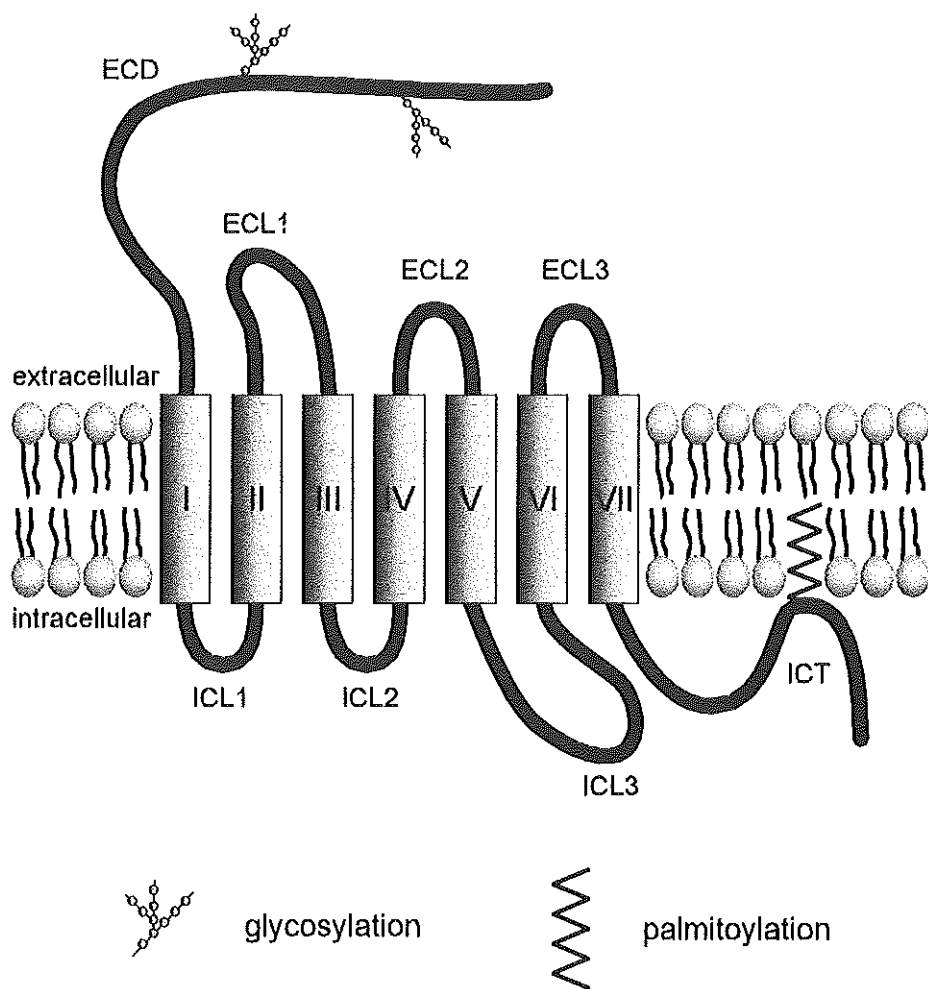


Fig. 2.1. Two-dimensional representation of the putative membrane topography of the β_2 -adrenergic receptor.

Transmembrane segments, numbered in roman numbers I to VII, are depicted as cylinders. ECD: extracellular domain; ECL: extracellular loop; ICL: intracellular loop; ICT: intracellular tail (intracellular C-terminal domain). (After Ostrowski et al., 1992).

sional structure of the transmembrane domain has been determined (Findlay and Pappin, 1986). In spite of the limited amino acid sequence homology between rhodopsin and the plasma membrane receptors, rhodopsin serves as a model for other G protein-coupled

receptors, since no three-dimensional structures are available for the other family members. This model, viewed from an N-terminal position (the extracellular side for the plasma membrane receptors; see Fig. 2.2), shows that the transmembrane segments are counter-clock-wise orientated, and form a barrel-shaped pocket in the center. This pocket accommodates retinal in rhodopsin and serves as a binding site for small ligands in the plasma membrane receptors. After photo-excitation or ligand binding, rhodopsin and the different plasma membrane receptors are activated, and in turn activate various specific intracellular signalling routes that stimulate cellular processes.

Signal transduction

Transduction of a signal from the extracellular to the intracellular side through a G protein-coupled receptor involves different steps. First, the ligand, also called first messenger, binds to the receptor through specific interaction with several side chains of amino acid residues. This interaction includes electrostatic and hydrophobic interactions and formation of hydrogen bonds. Conformational and charge changes within the binding pocket, brought about by the ligand, may rearrange interactions between the transmembrane segments, and thereby change the positions of the intracellular loops. These changes result in binding and activation of one or different members of a family of GTP-binding proteins (G proteins), that is followed by a change in the activity of effector enzymes such as adenylyl cyclase or phospholipase-C.

G proteins are hetero-trimeric proteins composed of α , β , and γ -subunits (for a review see Birnbaumer, 1992). The γ -subunit is anchored in the plasma membrane, conferring a membrane-associated location of the holo-G protein. The α -subunit contains a GTP-binding site with an associated GTPase activity. Furthermore, this subunit is able to interact with both the receptor and effector molecules. At least 16 different α -subunit genes have been identified, which are separated in three functionally distinct families: α_s , $\alpha_{q/11}$ and α_i . The main effector proteins that interact with these α -subunits are phospholipase C for the $\alpha_{q/11}$ family and adenylyl cyclase for the α_s and α_i families, where α_s stimulates and α_i inhibits the activity of the effector protein. As for the α -subunit, also for the β and γ -subunits several subtypes have been identified. The inactive G protein is an $\alpha_{GDP}\beta\gamma$ -complex, where the α -subunit is bound to GDP. Upon interaction of the α -subunit with the ligand-activated receptor, GDP is exchanged by GTP, and the α -subunit dissociates from the $\beta\gamma$ -complex.

The α_{GTP} -subunit is the active compound of G proteins that interacts with the effector protein, but also for the $\beta\gamma$ -complex interactions with effector proteins have been described. The α_{GTP} -subunit is inactivated by its endogenous GTPase activity that hydrolyses GTP to GDP, whereafter the α -subunit reassociates with the $\beta\gamma$ -complex and an inactive G protein is formed again. Although most receptors demonstrate clear specificity for the type of G protein they activate, some receptors can couple to more than one type of G protein. Receptors for the glycoprotein hormones couple to adenylyl cyclase via G_s as their main signal transduction route, but also the phospholipase C/ Ca^{2+} signalling route can be activated through G proteins of the $G_{q/11}$ type (Birnbaumer, 1992; Grasso and Reichert, 1989; Gudermann *et al.*, 1992; Van Sande *et al.*, 1990).

Two types of effector proteins can be distinguished: enzymes and ion channels, which both produce specific second messengers inside the cell. Ion channels transport ions from outside the cell to the inside, whereas the effector enzymes convert intracellular substrates into second messengers. Two important effector enzymes are adenylyl cyclase and phospholipase C. Adenylyl cyclase converts ATP into cAMP that activates protein kinase A (PKA). By means of phosphorylation of serine/threonine residues, PKA regulates the activities of specific cellular proteins. The non-genomic effects of PKA are relatively fast (minutes), whereas the effects of PKA on gene transcription (genomic effects) are relatively slow (hours). Genomic effects are mediated by the cAMP response element-binding protein (CREBP), a transcription factor that is activated by PKA (Montminy *et al.*, 1990; Lalli and Sassone-Corsi, 1994). Phospholipase C signalling involves two second messengers. Activated phospholipase C hydrolyses phosphatidyl-inositol 4,5-bisphosphate (PIP_2) into inositol 1,4,5-trisphosphate (IP_3) and 1,2-diacylglycerol (DAG), which both serve as intracellular second messengers. IP_3 binds to IP_3 receptors on intracellular Ca^{2+} stores, resulting in a release of Ca^{2+} and a subsequent rise in the intracellular free Ca^{2+} level (Berridge, 1993). DAG activates protein kinase C, which regulates the activities of cellular proteins by means of phosphorylation (Nishizuka, 1992).

Receptor activation

The process of receptor activation of G protein-coupled receptors can be divided into two steps: ligand binding and receptor activation. In addition, receptor desensitization is important in order to prevent over-stimulation of the target cell. The processes of receptor

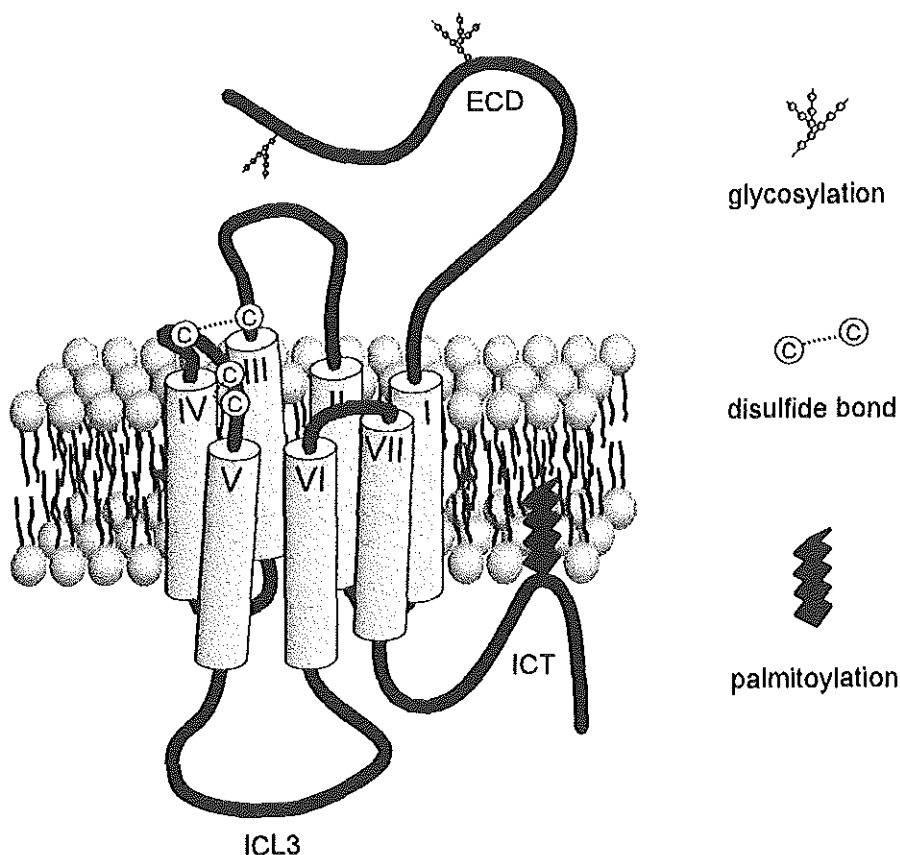


Fig. 2.2. Three-dimensional representation of the putative membrane topography of the β_2 -adrenergic receptor.

Transmembrane segments, numbered in roman numbers I to VII, are depicted as cylinders. ECD: extracellular domain; ICL: intracellular loop; ICT: intracellular tail (intracellular C-terminal domain). (After Ostrowski et al., 1992).

activation and receptor desensitization are described below, using the β -adrenergic receptor as a model. The natural ligands for adrenergic receptors are adrenalin (epinephrine) and noradrenalin (norepinephrine). Different receptor subtypes ($\alpha_{1,2}$ and $\beta_{1,2,3}$) have been identified that couple to different G proteins. Furthermore, many agonists and antagonists are available, which exert receptor subtype-specific interactions. These features have made the adrenergic receptors an excellent model system to study the structure-function relationship of G protein-coupled receptors.

After cloning of the β_2 -adrenergic receptor (Dixon *et al.*, 1986), extensive mutagenesis studies have been performed (reviewed by Ostrowski *et al.*, 1992). Deletion mutants that lack the extracellular domain were constructed and expressed in COS-7 and L cells (Dixon *et al.*, 1987). Binding and adenylyl cyclase stimulation studies on these cells showed that this part of the receptor is not involved in ligand binding and G protein activation. Furthermore, mutagenesis of the two consensus asparagine-linked glycosylation sites in the N-terminus (Fig. 2.2) showed that deglycosylation has only minor effects on ligand binding and effector stimulation (Rands *et al.*, 1990). These results showed that the N-terminal domain of the β_2 -adrenergic receptor is not directly involved in proper receptor functioning. However, the plasma membrane expression level of the deglycosylated receptors was approximately 50% lower than the level observed for the wild type receptor, indicating that glycosylation of the N-terminus is important for intracellular trafficking and/or stability of the receptor molecule (Rands *et al.*, 1990).

Cysteine residues in the extracellular loops of the β_2 -adrenergic receptor form disulfide bridges between the first and second loops and within the second loop (Fig. 2.2), which are obligatory for ligand binding (Dohlman *et al.*, 1990). Dithiothreitol reduction of disulfide bridges, or site-directed mutagenesis of the four cysteine residues in the extracellular loops, resulted in impaired ligand binding. It is believed that the disulfide bridges function to maintain the relative position of the transmembrane segments, in order to form the binding pocket (Dohlman *et al.*, 1990). Covalent incorporation of a radio-labelled ligand into the ligand binding site of the receptor showed that the ligand actually enters the binding pocket (Dohlman *et al.*, 1988). This finding was supported by experiments using the fluorescent antagonist carazolol (Tota and Strader, 1990). These authors showed that bound carazolol is inaccessible for agents that would normally quench the fluorescence, indicating that this ligand is buried in the binding pocket. In this pocket, the ligand interacts with specific amino acid side-chains of the transmembrane segments. Studies using site-directed mutagenesis in which specific amino acid residues were substituted, in combination with binding studies of different agonists and antagonists, revealed specific interactions of adrenaline with side-chains of residues located within transmembrane segments III, V, and VI (Ostrowski *et al.*, 1992). Although these direct interactions are crucial for ligand binding, it can be suggested that interplay of all transmembrane segments and connecting loops that form the binding pocket is equally important (Ostrowski *et al.*, 1992).

Studies in which effects of large deletions on receptor function were investigated, showed that the third intracellular loop and the C-terminal tail are essential for coupling of the β_2 -adrenergic receptor to G_s , whereas agonist binding was still present for receptors lacking these domains (Dixon *et al.*, 1987; Strader *et al.*, 1987). In more detailed studies, mutant receptors were constructed by exchanging different regions of the β_2 -adrenergic receptor for homologous regions of the α_2 -adrenergic receptor that couples to G_i . These experiments revealed that the third intracellular loop and its connecting transmembrane segments (V and VI) are sufficient to enable specific G protein coupling (Kobilka *et al.*, 1988). Extending these studies using chimeric α_2/β_2 receptors with smaller exchanged regions, Ligett and co-workers showed that the N- and C-terminal parts of the third intracellular loop and the N-terminal part of the intracellular tail determine specificity of G_s or G_i coupling (Ligett *et al.*, 1991). The conformation of the N-terminal part of the intracellular tail is also important for G protein coupling. The β_2 -adrenergic receptor is palmitoylated at a cysteine residue in this tail (O'Dowd *et al.*, 1989), which may result in the formation of a fourth intracellular loop (Fig. 2.2). The C-terminal tail determinant for G protein coupling resides within this putative fourth loop. Deletion of this cysteine residue that confers palmitoylation, resulted in impaired agonist-induced adenylyl cyclase stimulation (O'Dowd *et al.*, 1989). Besides the third and putative fourth intracellular loops, the other intracellular loops are also involved in proper β_2 -adrenergic receptor signal transduction (O'Dowd *et al.*, 1988). Similar to the role of the extracellular loops, the role of the first and second intracellular loops may probably be found in maintenance of the ligand binding pocket.

After ligand binding, a G protein-coupled receptor is thought to be activated by ligand-induced conformational changes. Binding of the ligand in the pocket may result in breaking of existing interactions within the transmembrane domain and subsequent formation of new interactions with the ligand. This rearrangement of interactions within the transmembrane domain results in conformational changes that are transmitted to the third intracellular loop, thereby giving access to the G protein coupling determinants. The involvement of conformational changes in the third loop in G protein activation is supported by the finding that substitution of the alanine residue at position 293 (Ala²⁹³) in the C-terminal part of the third loop of the α_{1B} -adrenergic receptor for any other amino acid residue resulted in activation of the receptor in the absence of ligand (constitutive activation) (Kjelsberg *et al.*, 1992). Through site-directed mutagenesis, mutant receptors were constructed that contained

different amino acid residues at the 293 position. Transient expression of these receptors and subsequent determination of basal IP_3 levels showed that the unstimulated wild type receptor did not elevate the basal level of IP_3 in COS-7 cells, whereas the mutant receptors exhibited agonist-independent increases of the IP_3 level, ranging from 1.2-3-fold increase (Kjelsberg *et al.*, 1992). The amino acids in this regions are important to maintain an inactive conformation, whereas substitution of the alanine residue induces a partial conformational change, resulting in constitutive activity of the receptor. This finding led to the identification of other constitutively activated G protein-coupled receptors in patients, starting with the identification of LH receptor amino acid substitutions in boys with precocious puberty (Kremer *et al.*, 1993; Shenker *et al.*, 1993), and TSH receptor amino acid substitutions in patients with hyperfunctioning thyroid adenomas (Parma *et al.*, 1993). Further analysis of such patients yielded many different receptor-activating mutations (Table 7.1), and showed a clustering of activating mutations in the C-terminal part of the third intracellular loop and the N-terminal part of the sixth transmembrane segment. This supports the observation in studies with the β -adrenergic receptor that conformational changes in the third intracellular loop activate the receptor protein. However, mutations were not only found in the sixth transmembrane segment, but also in other segments, including the second, the fifth, and the seventh (Table 7.1). This suggests that some other regions are also involved in keeping the unoccupied receptor in an inactive conformation. Besides receptor activation, single amino acid substitutions in the transmembrane domain of G protein-coupled receptors have been identified that result in receptor inactivation (Chapter 6; and discussed in Chapter 7). These substitutions may abolish the possibility to switch from the inactive to the active conformation upon stimulation with ligand, although effects on ligand binding, intracellular receptor trafficking and/or stability, and expression may also account for reduced receptor activity.

Cellular desensitization

Chronic exposure of cells to a stimulus leads to an adaptive response that attenuates the signal under continuous stimulation. This process is called cellular desensitization. At the molecular level, three different forms of cellular desensitization can be distinguished: functional uncoupling of receptor and G protein (receptor desensitization), receptor internalization, and receptor down-regulation. Herein, the term receptor down-regulation is

used with respect to decreased receptor synthesis, which generally is the slowest process in receptor desensitization.

Functional uncoupling of receptor and G protein has been studied in great detail for the β_2 -adrenergic receptor. The third intracellular loop and the intracellular tail of the β_2 - adrenergic receptor each contain a PKA phosphorylation site. Upon activation of the receptor and subsequent activation of the cAMP/PKA signalling route, the receptor is phosphorylated by PKA (Clark *et al.*, 1988). The introduction of the highly charged moieties in this region that is important for G protein coupling is thought to result in impaired coupling to G_s (Hausdorff *et al.*, 1990). Synthetic peptides, corresponding to the C-terminal part of the third intracellular loop including the PKA phosphorylation site of the β_2 -adrenergic receptor, can specifically activate G_s in *in vitro* G protein activation assays, whereas G_i is only weakly activated (Okamoto *et al.*, 1991). *In vitro* phosphorylation of the peptide by PKA resulted in reduced G_s coupling, whereas coupling to G_i was enhanced. These findings suggest that this G protein-coupling region has the potential to couple to both G_s and G_i , and that PKA phosphorylation determines the specificity of coupling (Okamoto *et al.*, 1991). At high agonist concentrations, the receptor is also phosphorylated by the β -adrenergic receptor-specific kinase (β ARK), which specifically phosphorylates ligand-occupied β -adrenergic receptors in the C-terminal tail (Benovic *et al.*, 1986; Hausdorff *et al.*, 1990). β ARK-phosphorylated receptors are specifically bound by β -arrestin, a protein that sterically hinders functional coupling of the receptor to the G protein (Lohse *et al.*, 1990).

Receptor internalization is the process by which ligand-bound receptor is sequestered away from the plasma membrane (for a review see Hausdorff *et al.*, 1990). Through endocytosis of a part of the plasma membrane, the receptors are transported into the cytoplasm, where they may be processed or degraded. Processed receptors could be recycled to the plasma membrane. A recent study reported that internalization of a mutant β -adrenergic receptor, that is impaired in its ability to be internalized, could be rescued through over-expression of β -arrestin (Ferguson *et al.*, 1996). This implies that, besides receptor-G protein uncoupling, β -arrestin may also be involved in receptor internalization.

Receptor down-regulation involves mechanisms that inhibit receptor synthesis, at the levels of transcription and posttranscriptional processing. Stimulation of DDT₁MF-2 hamster cells of vas deferens origin with β -adrenergic agonists resulted in a decrease of the β_2 -

adrenergic receptor mRNA level, reaching approximately 50% of the initial level after 20 hours (Hadcock and Malbon, 1988). Further studies on the mechanism of this process showed that the rate of β_2 -adrenergic receptor gene transcription was unaltered, and that the half-life of receptor mRNA was reduced from 12 hours in untreated cells to 5 hours in agonist-stimulated cells (Hadcock *et al.*, 1989b). The process is mediated by the cAMP/PKA signalling pathway (Hadcock *et al.*, 1989a) that induces the synthesis of β -ARB, a protein that specifically binds to β -adrenergic receptor mRNAs (Port *et al.*, 1992; Huang *et al.*, 1993).

Glycoprotein hormone receptors

Among the ligands for G protein-coupled receptors, the glycoprotein hormones LH, hCG, FSH, and TSH form a distinct subgroup, on basis of their large and glycosylated protein structure (Gharib *et al.*, 1990). After cloning of the respective receptors (Loosfelt *et al.*, 1989; McFarland *et al.*, 1989; Parmentier *et al.*, 1989; Sprengel *et al.*, 1990), it became apparent that these receptors form a distinct subgroup within the G protein-coupled receptor family, on basis of a large extracellular domain. This domain consists of leucine-rich repeats, and may have the conformation of a semi-circle of anti-parallel β -turns. This suggestion is based on the known structure of a porcine ribonuclease inhibitor that also contains leucine-rich repeats (Kobe and Deisenhofer, 1993). The extracellular domain constitutes approximately half of the receptor protein, and is encoded by 9 or 10 exons (Tsai-Morris *et al.*, 1991; Koo *et al.*, 1991; Heckert *et al.*, 1992; Gross *et al.*, 1991). Only one exon (10 or 11) encodes the transmembrane domain and the intracellular tail. This is the part of the glycoprotein hormone receptors that is homologous to the other G protein-coupled receptors, that are almost always encoded by a single exon.

The large extracellular domain of the glycoprotein hormone receptors is responsible for specific and high affinity binding of the large hormone. This has been demonstrated by expression of truncated LH receptors that consist of only the extracellular domain, which were found to be able to bind hCG with high specificity and affinity (Tsai-Morris *et al.*, 1990; Xie *et al.*, 1990). The finding that hormone binding is primarily determined by the

extracellular domain can be extended to FSH and TSH receptors. Chimeric LH/FSH and LH/TSH receptors, of which homologous parts of the extracellular domains were exchanged, were tested for their hormone specificity (Braun *et al.*, 1991; Nagayama *et al.*, 1990). These studies showed that the behaviour of the chimeric receptors with respect to hormone binding specificity was determined by the origin of the extracellular domain. Besides hormone-binding to the extracellular domain, there are indications that the hormone is able to interact with the transmembrane domain. A truncated LH receptor, lacking the extracellular domain, is able to bind hCG with low affinity, and can subsequently be partly activated (Ji and Ji, 1991). These data suggest the existence of an additional hormone binding site, which is involved in receptor activation, and indicate that some mechanistic aspects of activation of glycoprotein hormone receptors are similar to activation of the other G protein-coupled receptors, in particular interactions of the ligand with the transmembrane domain. Binding of a glycoprotein hormone to the extracellular domain of its respective receptor may be involved in proper positioning of the hormone towards the transmembrane domain.

Gonadotropin receptor expression

Regulation of gonadotropin receptor expression

FSH and LH receptors show high expression in the gonads. However, functional expression of the LH receptor in the uterus has also been reported (Ziecik *et al.*, 1986). *In vitro* stimulation of rat uterine tissue with hCG showed that this extra-ovarian LH receptor expression may be involved in LH/hCG regulated progesterone and estradiol production from the uterus (Bonnamy *et al.*, 1989; Mukherjee *et al.*, 1994). In spite of this extra-gonadal expression of the LH receptor, the expression of LH and FSH receptors in the gonads is most obvious. Little information is available about the DNA elements in the receptor genes that confer this tissue specificity, although regions in the 5' flanking sequences that are involved in tissue specific expression have been described (Heckert *et al.*, 1992; Wang *et al.*, 1992; Tsai-Morris *et al.*, 1993; Tsai-Morris *et al.*, 1994; Gromoll *et al.*, 1994). Within the gonads, the receptors are expressed in distinct cell types: in the testis, FSH receptors can only be found in Sertoli cells (Heckert and Griswold, 1991), whereas LH receptors are expressed in

Leydig cells (Segaloff and Ascoli, 1993). In the ovary the situation is slightly more complex: FSH receptors are found in granulosa cells, while LH receptors are present in both granulosa cell and theca cell layers (Camp *et al.*, 1991).

There is some information available about expression of LH and FSH receptors in fetal and postnatal gonads. During testis development in immature rats, LH receptor mRNA expression in the testis increases continuously, both as a fraction of total RNA and per total testis RNA, until a constant level is reached in adult animals (Vihko *et al.*, 1992). The FSH receptor mRNA level per Sertoli cell decreases from birth until the rats are 21 days of age, when Sertoli cells have stopped dividing. Thereafter, total testicular FSH receptor mRNA expression does not change (our unpublished observations). However, in the adult testis several components of the FSH signaling pathway are regulated: different levels of FSH receptor mRNA expression, FSH binding, FSH-dependent cAMP production, and CRE-binding protein mRNA expression in Sertoli cells were found to be associated with different stages of the cycle of the spermatogenic epithelium (Parvinen, 1993; Kliesch *et al.*, 1992; Heckert and Griswold, 1991).

In the ovary, some LH receptor mRNA species appear to be constantly expressed during fetal life, although mRNAs that encode functional receptor molecules are expressed starting from day 7 of postnatal life only (Sokka *et al.*, 1992). For LH receptor expression in the testis and FSH receptor expression in both the ovary and the testis, similar patterns were found, although the delay in expression of functional receptors was approximately 2 days (Zhang *et al.*, 1994; Rannikki *et al.*, 1995).

Hormonal regulation of expression of gonadotropin receptors has been studied both *in vivo* and *in vitro*, in cell cultures. In the testis, there are differential effects of hormone administration on the LH receptor mRNA level in fetal/neonatal and adult rats: hCG treatment of intact animals resulted in up-regulation of LH receptor mRNA expression in fetal/neonatal testis, but in down-regulation in adult testis (Pakarinen *et al.*, 1990). In the mouse Leydig tumor cell line MA-10, treatment of the cells with epidermal growth factor, phorbol ester (activation of protein kinase C), or with hCG or 8-Br-cAMP, resulted in rapid loss of LH receptor mRNA (Wang *et al.*, 1991c; Wang *et al.*, 1991b; Segaloff and Ascoli, 1993). This loss was not a result of decreased stability of LH receptor mRNA induced by any of the compounds that were used, but rather was caused by a decrease in LH receptor gene transcription rate (Nelson and Ascoli, 1992). However, homologous down-regulation of LH

receptor mRNA in primary Leydig cell cultures indicated that the main mechanism of LH receptor down-regulation involved a decrease of receptor mRNA stability (Chuzel *et al.*, 1995). This suggests that the receptor mRNA destabilizing mechanism was lost upon establishment of the MA-10 cell line from Leydig cells. FSH receptor mRNA expression in Sertoli cells *in vivo* also is down-regulated rapidly by its homologous hormone (Themmen *et al.*, 1991). As for the LH receptor in cultured Leydig cells, this involves a receptor mRNA destabilizing mechanism. However, the molecular mechanism of homologous down-regulation of LH receptor mRNA is dependent on both transcription and translation (Chuzel *et al.*, 1995), whereas for down-regulation of FSH receptor mRNA only transcription is obligatory (Chapter 3). This implies that LH receptor mRNA is specifically degraded by a protein factor, whereas for the FSH receptor this may be achieved by an RNA species.

In the ovary, studies of hormone effects on gonadotropin receptor regulation are confounded by the fact that both LH and FSH receptors are expressed in the same cell type, the granulosa cells. In cultured granulosa cells isolated from estrogen-primed rats, both LH and FSH up-regulate their respective receptor mRNAs (Piquette *et al.*, 1991; Tilly *et al.*, 1992). When follicular growth is induced in immature female rats by treatment with PMSG (pregnant mare serum gonadotropin), LH receptor mRNA expression in the growing follicles is increased, presumably through activation of FSH receptors. This action can be mimicked *in vitro*, by incubation of cultured granulosa cells with FSH (Piquette *et al.*, 1991). Immediately after the ovulatory LH peak, either in intact cycling rats or when mimicked by injection with hCG, both FSH and LH receptor mRNA levels are decreased significantly (Camp *et al.*, 1991). With the appearance of corpora lutea, LH receptor mRNA expression again increases (LaPolt *et al.*, 1990).

Thus, both in testis and ovary, mechanisms exist that result in either up- or down-regulation of gonadotropin receptor mRNA expression, depending on cyclic changes or on the developmental stage of the gonads. It must be kept in mind that short-term changes in mRNA expression may not cause changes in receptor protein levels, independent of ligand-induced receptor internalization. This is illustrated by down-regulation of receptor mRNA expression by dibutyryl-cAMP in cultured Sertoli and Leydig cells, where disappearance of mRNA was not followed by a decrease in hormone binding immediately (Themmen *et al.*, 1991; Chuzel *et al.*, 1995). Furthermore, the *in vitro* conditions under which cultured cells are treated with relatively high concentration of hormone can result in an exaggerated and

unchecked response. However, the response of cells *in vivo*, such as the response of Sertoli cells in developing testis or granulosa cells in growing follicles to FSH, probably occurs under conditions of moderate stimulation (sub ED₅₀). This *in vivo* response may be kept in check by paracrine mechanisms.

Multiple mRNA species and splice variants

Northern blot analysis of LH receptor mRNAs has resulted in the detection of several mRNAs with distinct sizes. LH receptor cDNA probes revealed at least four LH receptor mRNA species: 6.5, 4.3, 2.6 and 1.2 kb (Wang *et al.*, 1991a). Similar sizes were reported by other investigators (Camp *et al.*, 1991; Piquette *et al.*, 1991). These mRNA species can be differentially regulated. In the developing rat testis, the smaller transcripts were apparent at five days after birth, whereas all mRNA species were present from day 25 onwards (Vihko *et al.*, 1992). Treatment of pseudopregnant rats with hCG resulted in down-regulation of all detectable LH receptor mRNA species. However, in the testis of hCG-treated rats, the 1.2 kb mRNA persisted, while the other mRNAs were down-regulated (LaPolt *et al.*, 1991). Reprobing the same Northern blot with a transmembrane/intracellular domain-specific probe revealed that this 1.2 kb mRNA did not contain a portion of the transmembrane/intracellular domain (LaPolt *et al.*, 1991). Possibly, the 1.2 kb LH receptor mRNA encodes a truncated receptor protein (see below).

When the LH receptor cDNA was first cloned, investigators showed cDNA variants that are probably the result of alternative splicing (McFarland *et al.*, 1989). Hereafter, many LH receptor mRNA splice variants have been described (see Fig. 2.3). With regard to mRNA splice variants of the FSH receptor, similar results have been obtained (Fig. 2.3). Some of the variants have been found when cDNA libraries were screened for the full-length receptor cDNAs, while other research groups have used reverse transcriptase-polymerase chain reaction strategies in a more targeted effort to find other divergent receptor mRNAs (Aatsinki *et al.*, 1992).

Many of the gonadotropin receptor mRNA splice variants encode at least a partially intact hormone binding domain. Indeed, translation of one of the cloned cDNAs resulted in a truncated protein with no transmembrane/intracellular domain, but which was able to bind hCG (Tsai-Morris *et al.*, 1990). Similar observations have been reported for pig LH receptor cDNAs (VuHai-LuuThi *et al.*, 1992). The latter authors reported secretion of a portion of

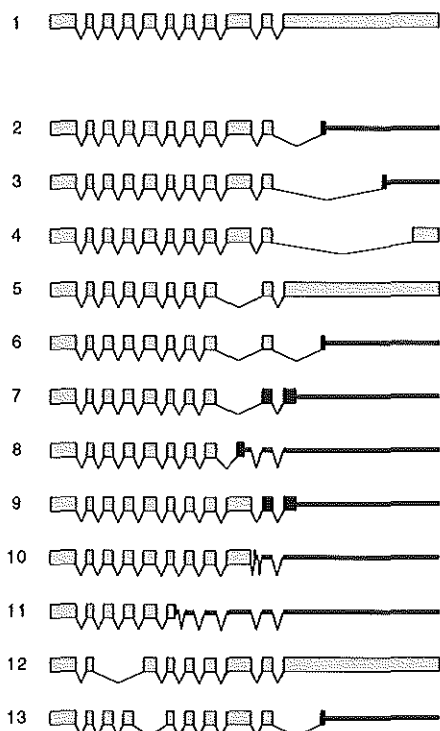
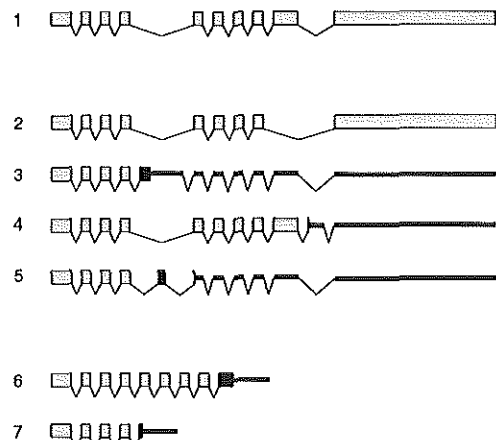
A**B**

Fig. 2.3. LH receptor (A) and FSH receptor (B) pre-mRNA splice variants.

Stippled box: original open reading frame, produced from 11 exons as indicated; black box: translated intron or exon sequences with a different reading frame; black line: untranslated intron or exon sequences. The spliced-out sequences are indicated with folded lines. (A) 1: full-length LH receptor; 2: porcine LHR-B (Loosfelt et al., 1989), rat LHR-1234 (Aatsinki et al., 1992), rat LHR-B (Bernard et al., 1990), rat LHR-B4 (Segaloff et al., 1990; Tsai-Morris et al., 1990); 3: porcine LHR-C (Loosfelt et al., 1989); 4: porcine LHR-D (Loosfelt et al., 1989); 5: rat LHR-E (Bernard et al., 1990), rat LHR-A2 (Segaloff et al., 1990); 6: rat LHR-EB (Bernard et al., 1990); 7: rat LHR-B1 (Segaloff et al., 1990); 8: rat LHR-B2 (Segaloff et al., 1990), rat LHR-2075 (Aatsinki et al., 1992); 9: rat LHR-B3 (Segaloff et al., 1990); 10: rat LHR-C1 (Segaloff et al., 1990); 11: rat LHR-C2 (Segaloff et al., 1990); 12: rat LHR-1950 (Aatsinki et al., 1992); 13: rat LHR-1759 (Aatsinki et al., 1992). (B) 1: full-length FSH receptor; 2: human FSHR-E (Gromoll et al., 1992); 3: human FSHR-5-10 (Kelton et al., 1992); 4: rat FSHR-1 (Chapter 3); 5: rat FSHR-2 (Chapter 3); 6: ovine FSHR-HK18 (Khan et al., 1993); 7: ovine FSHR-151A1 (Khan et al., 1993). 6 and 7 are the result of both alternative splicing and alternative polyadenylation. (From Themmen et al., 1994).

the truncated LH receptor molecules, but other investigators studying similarly truncated cDNAs have not confirmed this observation (Xie *et al.*, 1990). Actually, there are no indications that mRNAs encoding truncated, but hormone binding, receptors are expressed at significant levels. If they are, these mRNAs may not be translated under physiological conditions. In the ovary, Sokka *et al.* (1992) have shown the existence of alternatively spliced LH receptor transcripts missing the transmembrane/intracellular domain encoding exon. They propose that the expression of these transcripts represents a default pathway, which may have a physiological role in developmental regulation of LH receptor expression in the ovary. This hypothesis implies that the LH receptor gene is constantly turned on, but complete mRNAs encoding intact LH receptor molecules are generated not until after day 7 of neonatal life, in the rat. However, the expression of the splice variants is very low at all stages of development (Sokka *et al.*, 1992). Further information on the expression and translation of truncated LH receptor mRNAs is eagerly awaited, to try to clarify the possible role of truncated receptor proteins in the regulation of physiological responses to LH.

Receptor mRNAs encoding truncated receptor forms without a transmembrane/intracellular domain are not limited to the gonadotropin receptors. They have also been found for the TSH, interleukin-4 (IL-4), interleukin-7 (IL-7), and growth hormone receptor genes (Takeshita *et al.*, 1992; Mosley *et al.*, 1989; Goodwin *et al.*, 1990; Pleiman *et al.*, 1991; Baumbach *et al.*, 1989). Yet, the possible function of expressed splice variants remains an enigma. If secreted, soluble receptor ligand binding domains might act as inhibitors of hormone action by sequestering the ligand away from the full-length receptor. In this respect it is of interest that soluble IL-4 receptors, when transfected into cell lines, are secreted and inhibit the IL-4 effects that require the full-length receptor (Mosley *et al.*, 1989). For the growth hormone receptor gene, a circulating growth hormone binding protein has been identified in mouse and rat, that is encoded by an alternatively spliced receptor mRNA (Smith and Talamantes, 1987; Baumbach *et al.*, 1989). In contrast to such inhibitory (hormone binding) effects, a free ligand-binding domain of truncated receptors, might act to present the hormone to the receptor resulting in a more efficient signal. Other mechanisms, such as an intracellular role of non-secreted truncated receptor cannot be excluded.

Recently, two studies were reported, which point to interaction between full-length LH receptors and truncated forms. Remy *et al.* (1993) showed that co-expression of the

extracellular binding domain and the transmembrane/intracellular domain resulted in reconstitution of a high-affinity LH receptor, which was able to bind hCG and to transduce the hormonal signal to adenylyl cyclase. Although the mechanism is unclear, this observation seems to suggest that domains of the receptor can function and cooperate as separate entities. Another intriguing observation was made in a study using co-expression of full-length and truncated LH receptor cDNAs (VuHai-LuuThi *et al.*, 1992). Expression of cDNA lacking the transmembrane/intracellular domain in transfected COS cells resulted in high affinity binding, but no stimulation of cAMP production. However, co-expression of both full-length and truncated receptors resulted in enhanced stimulation of adenylyl cyclase by hCG. This enhancement was the result of an increase in the maximal hormone-induced cAMP level, and not from a decrease in the ED_{50} for hCG, invalidating a model in which hormone is presented to the intact receptor by a soluble truncated form. Similar studies with truncated forms of the FSH receptor, however, did not show effect (either stimulatory or inhibitory) on full-length receptor signal transduction (Chapter 3).

The cloning of the gonadotropin receptors provided the tools to study the molecular regulation of receptor expression. The finding of splice variants has left us with many intriguing questions, but may also open up new possibilities for the regulation of hormonal transmembrane signalling.

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Chapter 3

Alternative Splicing of Follicle-Stimulating Hormone Receptor Pre-mRNA: Cloning and Characterization of Two Alternatively Spliced mRNA Transcripts

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Abstract

Glycoprotein hormone receptors contain a large extracellular domain that is encoded by multiple exons, facilitating the possibility of expressing alternatively spliced transcripts. We have cloned two new splice variants of the rat follicle-stimulating hormone (FSH) receptor gene: FSH-R1 and FSH-R2. The splice variant FSH-R1 differs from the full-length FSH receptor mRNA by the inclusion of a small extra exon between exons 9 and 10. FSH-R2 lacks the first three base pairs of exon 4, contains an extra exon between exons 4 and 5, and has an extended 3'-untranslated region. Both mRNAs encode truncated FSH receptor proteins, consisting of the entire extracellular domain (FSH-R1) or the first half of the extracellular domain (FSH-R2), and are expressed at a low level in testes and ovaries. The expression levels of the FSH-R1 and FSH-R2 mRNAs in the gonads show a constant ratio to the expression level of the full-length FSH receptor mRNA. In contrast to comparable studies with truncated LH receptor proteins [VuHai-LuuThi, M.T., Misrahi, M., Houllier, A., Jolivet, A. and Milgrom, E. (1992) *Biochemistry*. **31**, 8377-8383.], *in vitro* co-expression of either one of the truncated proteins with the full-length FSH receptor in COS1 cells did not show interference of the truncated proteins with FSH signal transduction.

The absence of a function of the truncated FSH receptors in FSH signal transduction, and the absence of differential regulation of their transcripts, indicates that alternative splicing of the FSH receptor pre-mRNA does not have a function in the postnatal gonads.

Introduction

The gonadotropins luteinizing hormone (LH) and follicle-stimulating hormone (FSH) play key roles in the regulation of ovarian and testicular functions. In the ovary, FSH is involved in recruitment and growth of follicles, and in selection of the dominant follicle(s), whereas an LH surge induces ovulation of the mature follicles. In immature follicles, only theca cells express LH receptors, while granulosa cells are exclusively sensitive to FSH. After FSH-induced maturation of the granulosa cells, these cells will also express LH receptors (Carr, 1992). In the testis, LH stimulates the testicular Leydig cells to produce testosterone, and FSH exclusively acts on the Sertoli cells. FSH together with testosterone are considered to be the main regulators of spermatogenesis. Both hormones act, through different receptor mechanisms, on the Sertoli cells, which regulate spermatogenesis via close interactions with the developing germ cells (Griffin and Wilson, 1992).

Both the LH receptor (Loosfelt *et al.*, 1989; McFarland *et al.*, 1989) and the FSH receptor (Sprengel *et al.*, 1990) belong to the large family of GTP-binding protein (G protein)-coupled receptors. These receptors contain three domains: an amino (N)-terminal extracellular domain, a transmembrane domain, and a carboxyl (C)-terminal intracellular domain. Most characteristic for G protein-coupled receptors is the transmembrane domain consisting of seven membrane-spanning helices, which are joined together by three extracellular and three intracellular loops. The receptors for LH, FSH and thyroid-stimulating hormone (TSH) (Parmentier *et al.*, 1989) form a sub-group of glycoprotein hormone receptors. In contrast to almost all other G protein-coupled receptors, these glycoprotein hormone receptors contain a large glycosylated extracellular domain of approximately the same size as the transmembrane domain, that functions as a hormone binding domain (Braun *et al.*, 1991), and is encoded by multiple exons. The first ten exons of the LH receptor gene encode this extracellular domain, whereas the last exon encodes both the transmembrane and intracellular domains (Tsai-Morris *et al.*, 1990). In the FSH and TSH receptor genes, each consisting of ten exons (Heckert *et al.*, 1992; Gross *et al.*, 1991), the first nine exons encode the extracellular domain.

Shortly after cloning of the gonadotropin receptor cDNAs it became apparent that alternatively spliced transcripts are co-expressed with the full-length receptor mRNA transcripts (reviewed in Themmen *et al.*, 1994). Alternatively spliced transcripts encode

truncated receptors with two distinct structures: either receptor proteins containing the signal peptide and a part of the extracellular domain but no transmembrane domain, or receptor proteins that lack a part of the extracellular domain but still contain the transmembrane domain. Splice variants of the LH receptor gene, encoding different parts of the extracellular domain, have been shown to enhance human chorionic gonadotropin (hCG)-induced cAMP production, when transiently expressed in COS7 cells together with the full-length LH receptor (VuHai-LuuThi *et al.*, 1992).

In view of the possible biological importance of truncated gonadotropin receptors, we have studied two splice variants of the rat FSH receptor gene.

Materials and Methods

Cloning of FSH-R1 and FSH-R2

A rat Sertoli cell directional cDNA library (Baarends *et al.*, 1994) in lambda ZAPII (Stratagene, Westburg, Leusden, The Netherlands) was screened with two rat FSH receptor cDNA probes, corresponding to the 5'-part (positions -71 to 791) and the 3'-part (positions 793 to 2095) of the FSH receptor cDNA (Sprengel *et al.*, 1990). Nucleotide and amino acid numberings are according to this published rat FSH receptor cDNA. α -[32 P]dATP (Amersham, 's Hertogenbosch, The Netherlands)-labelled probes (Feinberg and Vogelstein, 1983) were used to screen approximately 10^6 plaque forming units (Sambrook *et al.*, 1989). Two positive plaques were purified during two subsequent rounds of rescreening. pBluescript SK plasmids containing the positive clones were isolated according to the *in vivo* excision protocol as described by the manufacturer.

Clones were subjected to restriction enzyme analysis and compared to the published FSH receptor cDNA (Sprengel *et al.*, 1990). Sequence analysis was performed on both clones in one orientation according to the dideoxy chain termination method (Sanger *et al.*, 1977), using primers derived from cDNA sequences. Clones were sequenced from the 5'-end to the BamHI site (position 2095). The 3'-untranslated regions were not sequenced completely.

Both clones were found to be splice variants of the FSH receptor gene. A full-length (wild type) FSH receptor cDNA (designated FSH-R0) was constructed by exchanging the 384 base pairs (bp) NcoI/Bsu36I fragment (positions 704 to 1088) of FSH-R2 into FSH-R1.

Expression vectors were constructed by cloning the EcoRI/BamHI fragments of FSH-R0, FSH-R1 and FSH-R2 (positions -71 to 2095) into the EcoRI/BamHI sites of pSG5 (Green *et al.*, 1988).

RNA preparation and RNase protection assay

Wistar rats, locally bred, were maintained under controlled conditions (20-23 C, lights on from 5.00-19.00 h). Animal care was performed in accordance with the NIH Guide for the Care and Use of Laboratory Animals.

Total testes were isolated from 21-day-post-coitus (p.c.) fetuses, 21-day-old immature rats and 56-day-old mature rats, and snap frozen in liquid nitrogen. Folliculogenesis and ovulation were induced in 30-day-old immature rats by injecting (s.c.) 30 IU PMSG (Folligonan; Intervet, Boxmeer, The Netherlands) followed 56 hours later by injecting (s.c.) 10 IU hCG (Pregnyl; Organon International NV, Oss, The Netherlands). Ovaries were isolated and snap frozen in liquid nitrogen 0, 24, 48, 72 and 168 hours after PMSG injection. Sertoli cells were isolated from 21-day-old rats, cultured for 4 days in the absence of FSH, and then stimulated for 4 hours with 500 ng/ml ovine FSH-S16 (NIH, Bethesda, MD, USA) in order to down-regulate FSH receptor mRNA levels, as described previously (Themmen *et al.*, 1991). Total RNA was isolated from tissues and isolated cells using the LiCl/urea method (Auffray and Rougeon, 1980).

RNase protection assays were performed using FSH-R1 and FSH-R2 specific probes. For FSH-R1, a 485 bp NcoI/EcoRV FSH-R1 cDNA fragment encompassing a part of exon 9 (from position 704), exon 9A and a part of exon 10 (to position 1068) was subcloned in pBluescript KS (Stratagene) and used to generate α -[³²P]UTP (Amersham)-labelled anti-sense transcripts *in vitro*. For FSH-R2, a 291 bp HindIII/PvuII FSH-R2 cDNA fragment was used. This fragment encompasses a part of exon 2 (from position 168), exon 3, exon 4 without the first three base pairs, exon 4A and a part of exon 5 (to position 406).

α -[³²P]UTP labelled anti-sense transcripts were generated *in vitro* (Sambrook *et al.*, 1989), using T7 RNA polymerase (Stratagene). Approximately 5×10^4 cpm of FSH-R1 or FSH-R2 probe was mixed with 25 μ g of total RNA in a total volume of 30 μ l hybridization mixture containing 40 mM Pipes (pH6.4), 1 mM EDTA, 0.4 M NaCl and 80% (v/v) formamide. Hybridizations were performed overnight at 55 C. The RNase protection assay was performed as described (Sambrook *et al.*, 1989).

Both probes also hybridize to FSH-R0 transcripts giving rise to smaller protected fragments. Protected fragments of the full FSH-R1 probe (485 bp) and the full FSH-R2 probe (291 bp) represent FSH-R1 and FSH-R2 mRNA expression levels, respectively. The protected fragment of exon 10 (215 bp) of FSH-R1 probe represents FSH-R0 mRNA expression levels.

In vitro expression in COS1 cells

COS1 cells were maintained in DMEM/HAM's F12 (1:1 v/v) (Gibco BRL, Gaithersburg, MD, USA) containing 5% (v/v) Fetal Calf Serum (SEBAK, Aidenbach, Germany), 2×10^5 IU/l penicillin (Brocades Pharma, Leiderdorp, The Netherlands) and 0.2 g/l streptomycin (Radium Farma, Milan, Italy). Cells were seeded at 10% confluence, incubated in a humidified incubator at 37 C and 5% CO₂, and split at 80-90% confluence.

One day before transfection, cells were seeded at 15% confluence in 80 cm² flasks (Nunc, Roskilde, Denmark). CaPO₄ transfections were performed as described (Chen and Okayama, 1987). Each flask received 1 ml precipitate containing 10 µg expression plasmid (5 µg expression vector with 5 µg empty vector (pSG5) for expression of the cDNAs alone, or 5 µg FSH-R0 expression vector with either 5 µg FSH-R1 or FSH-R2 expression vector for co-expression of the cDNAs), and 10 µg pTZ19R (Pharmacia, Uppsala, Sweden) as carrier DNA. In order to perform hormonal stimulations, Western blotting, and [¹²⁵I]FSH-binding assays with the same batch of transfected cells, several 80 cm² flasks were transfected with each combination of expression vectors, pooled two days after transfection by harvesting the cells with 0.02% EDTA in PBS without trypsin, and split 1:1 into different tissue culture dishes. Experiments were performed three days after transfection.

FSH-induced cAMP production was determined in quadruplicate in 12-well plates (Costar, Cambridge, MA, USA). Conditioned medium was prepared by incubation of the cells for 18 hours prior to stimulation with FSH in medium without serum, but containing 25 mM HEPES pH7.5 (Gibco) and 0.1% (w/v) BSA (Sigma, St. Louis, MO, USA). Part of this medium was removed from each well and used to prepare the stimulation medium, containing different doses of recombinant human FSH (Org32489; Organon International NV) and 0.2 mM isobutyl-methylxanthine (Sigma). After stimulation for 2 hours, cAMP production was determined in the medium (Stoof *et al.*, 1987).

For Western blotting, cells were cultured in 25 cm² flasks (Nunc), and washed three times at 4 °C with HBS (25 mM HEPES pH7.5, 150 mM NaCl, 0.9 mM CaCl₂). Cells from each flask were lysed in 300 µl lysis-buffer (20 mM Tris pH 7.5, 150 mM NaCl, 1 mM EDTA, 0.5% Triton X100, 10% glycerol, 0.1 mg/ml phenylmethylsulfonylfluoride, 1 µg/ml leupeptin, 1 µg/ml aprotinin) for 10 minutes at 4 °C on a rocking platform. Lysates were stored at -20 °C and subsequently used for Western blotting.

For human [¹²⁵I]FSH (DuPont/NEN, Du Pont de Nemours GmbH, Dreieich, Germany) binding assays, cells were cultured in 80 cm² flasks (Nunc), and subjected to FSH-binding assays on partially purified membrane preparations (Ketelslegers and Catt, 1978).

Antibodies and Western blotting

Peptide SP297, corresponding to residues 15-32 (CQDSKVTEIPTLPRNAI) of the rat FSH receptor, was synthesized (Atherton and Sheppard, 1989) and used to raise a polyclonal antiserum directed against the N-terminus of the FSH receptor. Three mg peptide was coupled to 3 mg m-maleimidobenzoyl-N-hydroxysuccinimide (Pierce, Rockford, IL, USA)-activated keyhole limpet haemocyanin (Calbiochem, La Jolla, CA, USA) according to the manufacturers' instructions. The coupled peptide (100 µg) was mixed with an equal volume of Freund's complete adjuvant (Difco, Detroit, MI, USA) and used to immunize a castrated male New Zealand White rabbit by subcutaneous injection. Six and 12 weeks after the initial injection, the rabbit was boosted with the same amount of coupled peptide mixed with an equal volume of Freund's incomplete adjuvant (Difco). Two weeks after the second boost, the rabbit was bled and serum was prepared. The resulting polyclonal antiserum was designated 1601-5. Pre-immune antiserum (1601-0) was obtained two weeks before the start of the immunization procedure.

Western blotting was performed on a Mini-Protean II gel electrophoresis and electro blotting system (Biorad, Hercules, CA, USA). The lysate (10 µl) was mixed with 2.5 µl 5 x sample-buffer (50 mM Tris pH6.8, 10 mM DTT, 10% (v/v) glycerol, 2% (w/v) SDS, 0.001% bromophenolblue), boiled for 5 minutes, separated on a 15% SDS-polyacrylamide gel and blotted onto nitrocellulose filters (Schleicher and Schuell, Dassel, Germany), according to the manufacturer's instructions. Non-specific binding sites were blocked for 1 hour with 5% (w/v) non-fat dry milk in PBS/0.1% (v/v) Tween-20, incubated with antiserum 1601-5 (1:1000) in PBS/0.1% Tween-20/1% non-fat dry milk for 1 hour, washed 4 times for

15 minutes with PBS/0.1% Tween-20, incubated with goat-anti-rabbit conjugated with horseradish peroxidase (Sigma) (1: 4000) in PBS/0.1% Tween-20/1% non-fat dry milk for 1 hour, and washed again four times with PBS/0.1% Tween-20. Chemiluminescence detection of proteins with Luminol (DuPont/NEN) was performed according to the manufacturer's instructions. Blots were exposed to MP films (Amersham) for 1 and 60 minutes.

Results

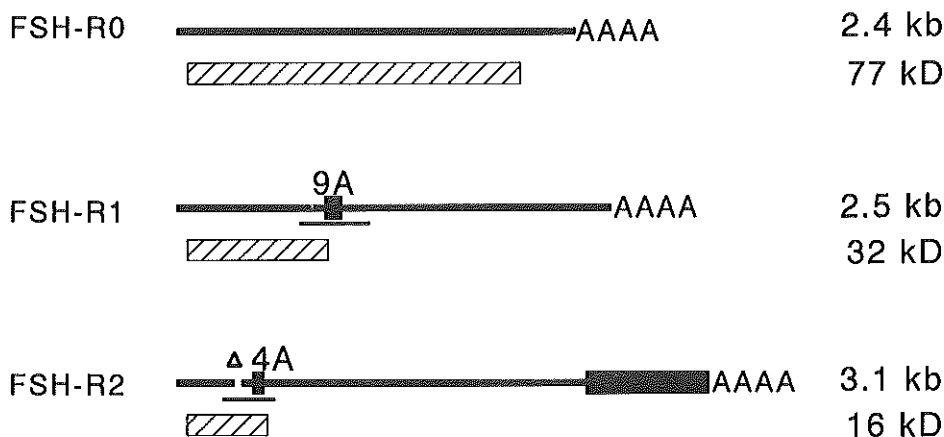
Cloning of two rat FSH receptor splice variants

Screening of the rat Sertoli cell cDNA library with FSH receptor cDNA probes resulted in two positive clones: FSH-R1 and FSH-R2, that were submitted to restriction and sequence analysis. Comparison with the original FSH receptor cDNA (Sprengel *et al.*, 1990) and the genomic organization of the FSH receptor gene (Heckert *et al.*, 1992), showed that both clones are splice variants of the FSH receptor gene.

When compared to the full-length FSH receptor, FSH-R1 contains an additional stretch of 121 bases between exons 9 and 10 that is not derived from intron sequences located directly downstream of exon 9 or upstream of exon 10 (Fig. 3.1B). Therefore, it is concluded that this stretch of 121 bases is an additional exon (exon 9A) that is alternatively spliced into FSH-R1 transcripts. The presence of exon 9A introduces a premature stop in the open reading frame, resulting in a truncated FSH receptor protein composed of almost the entire extracellular domain, with a calculated relative molecular mass of 32 kD (Fig. 3.1A).

The other splice variant, FSH-R2, is approximately 3.1 kb in size (Fig. 3.1A). This clone lacks the first three bases of exon 4, which results in a change of amino acid residues isoleucine and arginine (positions 83 and 84; nucleotide and amino acid numberings are according to the published rat FSH receptor cDNA (Sprengel *et al.*, 1990)) into one methionine residue (Fig. 3.1B). Between exons 4 and 5, an additional stretch of 56 bases is present that has no homology with the intron sequences flanking exons 4 and 5 (Fig. 3.1B), indicating that this stretch is an additional exon (exon 4A). The open reading frame is continued throughout exon 4A, but stops after transition into exon 5, since the introduction of exon 4A causes a frame shift in the open reading frame in exon 5. The FSH-R2 transcript

A



B

—exon 9—
LeuLysArgGlnIl
FSH-R0 TTGAAGCGGCAAAATgtcgag.....
FSH-R1 TTGAAGCGGCAAAATGTGAGTCTTCTATGTCAATCCCTGAAGTCTGGCTTGT
LeuLysArgGlnMet***

—exon 9A—
FSH-R0
FSH-R1 GCCGTCACACAGCATAGAATGGCTACATATTGGTGGCATGAAGAAAATCCA

—exon 10—
.....eSerGluLeuHisPro
FSH-R0ttctagCTTGAACCTTCATCCA
FSH-R1 TACATGGAGAGAGTTCCTGACTGAAGGCCTAAGCTCTGAACCTTCATCCA

—exon 3—
LeuProLysLeuHisGluIleArgIleGluLysAlaAsnAsnLeuLeuTyr
FSH-R0 CTACCCAAGTTGCATGAAATTAGGATTGAAAAGGCCAACAATCTTCTGTAC
FSH-R2 CTACCCAAGTTGCATGAAAT...GATTGAAAAGGCCAACAATCTTCTGTAC
LeuProLysLeuHisGluMe...tileGluLysAlaAsnAsnLeuLeuTyr

—exon 4—
IleAsnProGluAlaPheGlnAsnLeuProSerLeuArgTyrLe.....
FSH-R0 ATCAACCCGGAGGCCTTCCAGAATCTCCCACTCTCAGATATCTgttaaga.
FSH-R2 ATCAACCCGGAGGCCTTCCAGAATCTCCCACTCTCAGATATCTTAGCCAC
IleAsnProGluAlaPheGlnAsnLeuProSerLeuArgTyrLeuSerHis

—exon 4A—
.....uL
FSH-R0ctctagGT
FSH-R2 TTGTCAACCACAGAAGAAGTCACACTTGAGTTACATGGCTCTTAAATGAGT
LeuSerProGlnLysLysSerHisLeuSerTyrMetAlaLeuLysMetSer

—exon 5—
euIleSerAsnThrGlyIleLysHisLeuProAlaValHisLysIleGln
FSH-R0 TAATATCCAACACAGGCATTAAGCACTTGCCAGCTGTTCACAAGATCCAG
FSH-R2 TAATATCCAACACAGGCATTAAGCACTTGCCAGCTGTTCACAAGATCCAG

C

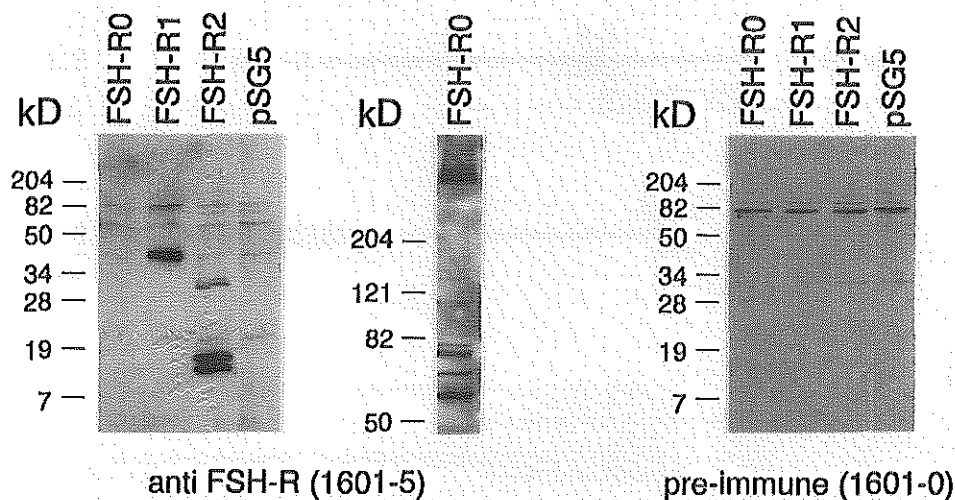


Fig. 3.1. Characterization of FSH-R1 and FSH-R2 cDNA clones.

(A) Schematic representation of FSH receptor splice variants FSH-R1 and FSH-R2, and the full-length FSH receptor (FSH-R0). Messenger RNA sequences are represented by the fat lines, additional sequences (exon 9A, exon 4A, and the extended 3'-untranslated region of FSH-R2) by black boxes, and deletion of the first three base pairs of exon 4 in FSH-R2 by a gap. The predicted open reading frames are indicated under the RNA sequences by hatched boxes, and probes which are used in the RNase protection assays as thin lines. (B) Sequence comparison of FSH-R1 and FSH-R2 with FSH-R0. Only relevant sequences are shown: position 840 to 869 for comparison with FSH-R1, and position 279 to 425 for comparison with FSH-R2. Exon sequences are shown in capital characters, intron sequences in lower case characters, and encoded amino acids in three character code above or underneath the nucleotide sequences. Sequence identity is indicated by vertical lines, and differences with FSH-R0 in bold characters. (C) Western blots of FSH-R0, FSH-R1, and FSH-R2. FSH receptor expression vectors or empty vector pSG5 (control) were transfected into COS1 cells and subjected to Western blotting, as described in Materials and Methods. Proteins were separated on a 15% SDS-PAGE gel (panels left and right). For FSH-R0 (middle panel), 30 μ l cell lysate was separated on a 7% gel. Positions of molecular mass standards are given at the left of each panel, and the first antibody that was used is indicated at the bottom.

encodes a truncated FSH receptor protein composed of the first half of the extracellular domain, with a calculated relative molecular mass of 16 kD. Furthermore, FSH-R2 transcripts use an alternative polyadenylation signal that is located approximately 600 nucleotides downstream of the polyadenylation signal that is used in the full-length FSH receptor transcript (Fig. 3.1A). The sequence of the 3'-untranslated region was not determined. A single base pair deletion (G at position 1467) was observed in FSH-R2 outside the open reading frame. This deletion may be an artefact, introduced during phage library construction or subsequent cloning procedures, since it is located within exon 10. A cDNA encoding the full-length FSH receptor (FSH-R0) was constructed from FSH-R1 and FSH-R2 cDNAs.

The polyclonal antiserum 1601-5, raised against a synthetic peptide corresponding to amino acid residues 15-32 of the rat FSH receptor, was used to verify protein translation from the FSH receptor transcripts. The cDNA clones were transiently expressed in COS1 cells, and total cell lysates were analysed on Western blots (Fig. 3.1C). FSH-R1 displayed two bands of approximately 40 and 80 kD. The 40 kD band is somewhat larger than the predicted size (32 kD), which is probably due to glycosylation of the protein. The 80 kD band possibly is a dimer of the 40 kD protein. FSH-R2 displayed a double band of the predicted size (16 kD) on Western blot, and possible dimer bands of approximately 30 kD (Fig. 3.1C). Western blotting of FSH-R0 did not result in the expected 80 kD (Quintana *et al.*, 1993) or 240 kD (Dattatreyaumurty *et al.*, 1992) bands. Instead, a high molecular mass band was observed on top of the separating gel (Fig. 3.1C), which was not present in the pre-immune control (also not upon longer exposure; data not shown). Aggregation of the full-length receptor during protein extraction or gel electrophoresis may prevent entrance of this protein into the separating gel.

FSH-R1 and FSH-R2 mRNAs are expressed in Sertoli cells and in ovaries

Differential regulation of the expression of the FSH-R1 and FSH-R2 mRNAs, might provide indications on possible functions of the truncated receptor proteins. Therefore, the mRNA expression of both splice variants in rat tissues was investigated using RNase protection assays. To monitor the expression of exon 4A (FSH-R2) and exon 9A (FSH-R1) specifically, probes were directed against these exons and parts of their flanking exons (Fig. 3.1A). Hybridization of a probe to its respective splice variant mRNA will result in pro-

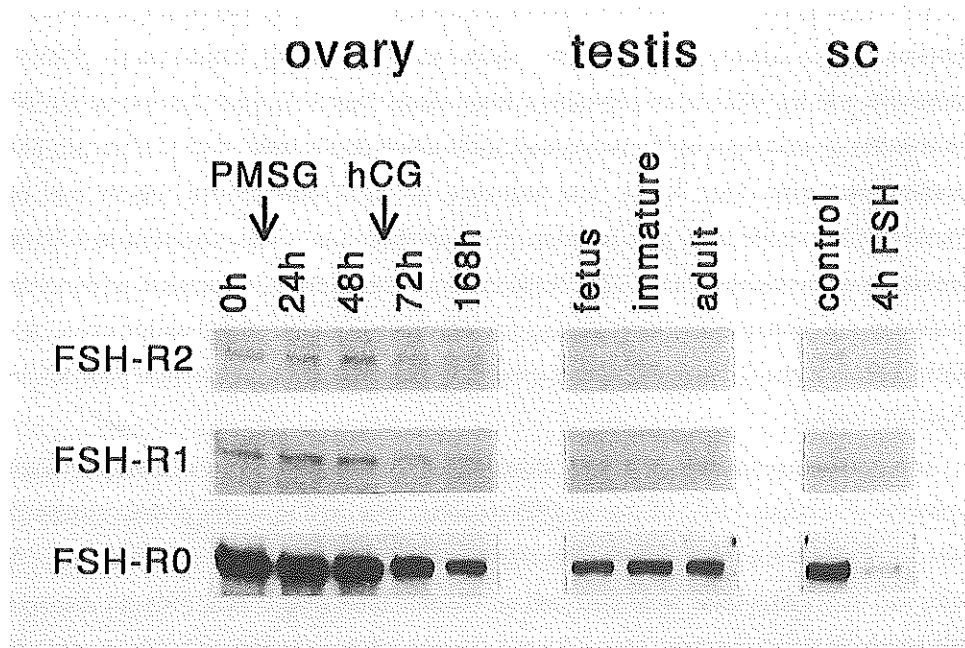


Fig. 3.2. *FSH-R0, FSH-R1, and FSH-R2 mRNA expression in ovary, testis, and Sertoli cells (SC).*

Animal treatment, tissue preparation, and RNase protection assay were performed as described in Materials and Methods. Protected fragments specific for FSH-R0, FSH-R1, and FSH-R2 are shown.

tection of the full probe without vector sequences. Furthermore, the flanking exons will also hybridize to FSH-R0 mRNA, and give rise to smaller protected fragments. Using this approach, the mRNA expression of a splice variant and the expression of FSH-R0 mRNA can be monitored in one assay, and compared directly.

It has been described, that during the ovarian cycle, full-length FSH receptor mRNA expression is down-regulated after ovulation, whereas a 7.0 kb FSH receptor mRNA transcript is up-regulated (LaPolt *et al.*, 1991). To test whether FSH-R1 and FSH-R2 mRNAs also show this differentially regulated expression pattern, their levels were investigated in ovaries during the cycle. In 30-day-old immature rat ovaries, many premature follicles are induced to grow in a synchronized manner after stimulation with PMSG. Antral follicles are present 24 and 48 hours after PMSG injection (Baarends *et al.*, 1995). During this follicular growth phase, FSH-R0 expression was relatively high (Fig. 3.2; ovary: 0h,

24h, and 48h). Antral follicles are induced to ovulate by an hCG injection 56 hours after the initial PMSG injection. Ovulated follicles, that are present 72 hours after PMSG injection, will have developed into corpora lutea at 168 hours after PMSG injection (Baarends *et al.*, 1995). After ovulation, FSH-R0 expression appeared to be down-regulated and maintained at a low level (Fig. 3.2; ovary: 72h and 168h). Compared to the expression of FSH-R0 mRNA, the FSH-R1 and FSH-R2 mRNA levels were very low, and changed in concordance with changes in the expression of FSH-R0 mRNA.

In male rats, the splice variant mRNA expression levels were investigated during testicular development. Testes were isolated from rats at three different ages, representing different stages of testicular development: fetal rats (21-day-p.c.), immature rats (21-day-old), and young-adult rats (56-day-old). During testicular development, FSH-R0 mRNA expression was found to remain constant per total amount of RNA (Fig. 3.2; testis). As was observed in the ovary, the splice variants showed a very low expression level, with a pattern similar to the expression pattern of FSH-R0 mRNA.

When Sertoli cells are isolated and cultured, FSH-R0 mRNA can clearly be demonstrated (Fig. 3.2; SC). In agreement with previous results (Themmen *et al.*, 1991), stimulation of these cells for 4 hours with FSH resulted in an almost complete down-regulation of FSH receptor mRNA. The low expression levels of the FSH-R1 and FSH-R2 splice variants were also down-regulated by incubation of Sertoli cells with FSH.

FSH-R1 and FSH-R2 do not affect FSH-R0 signal transduction

Although the mRNAs of the splice variants are expressed at a low level, and do not seem to be regulated independently from the full-length FSH receptor mRNA, the encoded truncated proteins could play a role in modulation of FSH sensitivity of Sertoli and granulosa cells. Truncated receptors might present the ligand to the full-length receptor and increase the bioavailability of FSH, or, alternatively, they might lower it by sequestering the ligand from the full-length receptor. Truncated LH receptors are able to bind hCG, and it has been demonstrated that this binding increases the effects of hCG on full-length LH receptors (VuHai-LuuThi *et al.*, 1992).

The effects of FSH-R1 and FSH-R2 on the bioavailability of FSH were tested in co-expression experiments. The splice variants were transiently expressed in COS1 cells together with FSH-R0. Two days after transfection, medium was renewed and the cells were

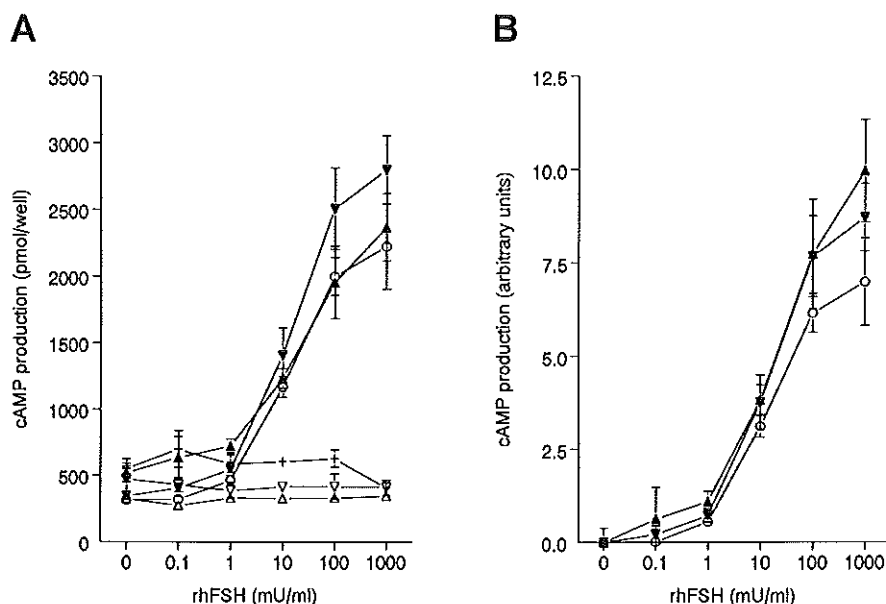


Fig. 3.3. In vitro co-expression of FSH-R0 with either FSH-R1 or FSH-R2 in COS1 cells. FSH receptor expression vectors or empty vector pSG5 (control) were transfected into COS1 cells as described in Materials and Methods: +-+; pSG5; o-o; FSH-R0 alone; Δ-Δ; FSH-R1 alone; ▽-▽; FSH-R2 alone; ▲-▲; FSH-R0 and FSH-R1; ▼-▼; FSH-R0 and FSH-R2. Cyclic AMP production was measured in the medium and expressed as pmol/well (left panel) or expressed in arbitrary units after correction for basal cAMP levels and the number of FSH-R0 binding sites (right panel).

incubated overnight, in order to allow possible secretion of the truncated receptors into the medium. Subsequently, the cells were stimulated by adding different doses of ovine FSH to this medium, and cAMP production was measured over a 2 hours period. In the same experiment, using the same batch of transfected cells, the expression of the truncated proteins was verified on Western blots, and FSH-R0 expression was determined in [¹²⁵I]FSH binding assays.

It was observed that expression of the truncated receptor proteins, in the absence of FSH-R0, did not result in FSH-induced cAMP production (Fig. 3.3A). However, basal levels of cAMP varied between cells transfected with different constructs, with the pSG5 control showing the highest basal level (Fig. 3.3A). Over-expression of proteins in COS1 cells may negatively affect the basal activity of the adenylyl cyclase enzyme. Therefore, cAMP productions for each combination of constructs were expressed as net increase.

By measuring [125 I]FSH binding, it was found that co-expression of the splice variants lowered the expression of FSH-R0 on the plasma membrane. For co-expression of FSH-R2, only a minor decrease in FSH-R0 binding sites was observed. Co-expression with FSH-R1, however, resulted in a 30% reduction of the number of FSH-R0 binding sites (data not shown). This reduction may be due to competition for translation and post-translational modification by the different proteins, although it cannot be excluded that co-expression of the splice variants affects binding of FSH to FSH-R0 in the binding assay.

Net cAMP production by cells that were transfected with FSH-R0 in the absence or presence of either FSH-R1 or FSH-R2 was corrected for the number of FSH-R0 molecules expressed, as determined in the binding assay. These calculations resulted in comparable FSH-induced cAMP production values for FSH-R0 alone, and FSH-R0 in combination with either one of the splice variants (Fig. 3.3B). Cyclic AMP production in the presence of maximal stimulating doses of FSH seemed to be slightly higher in cells co-expressing FSH-R0 and the splice variants than in cells that express FSH-R0 alone. However, this effect was not statistically significant and it is concluded that co-expression of FSH-R0 with either FSH-R1 or FSH-R2 does not affect FSH-R0 signal transduction.

Discussion

The present paper describes the cloning of two splice variants, FSH-R1 and FSH-R2, of the rat FSH receptor gene. Compared to the full-length FSH receptor cDNA, both transcripts contain extra sequences, which are located at an exon-exon boundary and are not homologous to flanking intron sequences (Heckert *et al.*, 1992), indicating that they are spliced in as separate exons and do not result from the use of cryptic splice sites. The deletion of the first three nucleotides of exon 4 in FSH-R2 transcripts is probably due to the use of an alternative splice acceptor site. The minimal sequence for a splice acceptor site is AG (Padgett *et al.*, 1986), which is present in both the original as well as the new site. The same pyrimidine stretch 5' of the splice acceptor site can be used for both sites.

The FSH-R1 protein, with a calculated relative molecular mass of 32 kD, appeared as a band of approximately 40 kD on Western blots. This difference may be explained by glycosylation of the protein, since two consensus N-linked glycosylation sites are present

(Sprengel *et al.*, 1990). FSH-R2 protein, which does not contain consensus N-linked glycosylation sites, appeared as a double band of the calculated relative molecular mass (16 kD). The double band appearance of FSH-R2 is puzzling: no internal translation initiation codons, which would result in translation of a protein still containing the N-terminal epitope, are present. An explanation might be that the double band arises from partial signal peptide cleavage, or there may be secondary structure left that is not denatured under the current Western blotting conditions. Both FSH-R1 and FSH-R2 proteins appeared probably as dimers on Western blots. Dimerization and even tetramerization have been described for an extracellular domain splice variant of the human TSH receptor (Graves *et al.*, 1995), but not for extracellular domain splice variants of the porcine LH receptor (VuHai-LuuThi *et al.*, 1992). There is no indication that G protein-coupled receptors function as multimers, and dimerization may be a result of receptor protein aggregation during separation on SDS-PAGE gels. On Western blots, FSH-R0 appeared as a very high molecular mass band. Bands at the expected sizes of 74 (Quintana *et al.*, 1993) or 240 kD (Dattatreyaumurthy *et al.*, 1992) were not detectable.

Many studies have been performed of FSH and LH receptor splice variants (Themmen *et al.*, 1994), but only one study addressed a possible functional role of truncated LH receptors (VuHai-LuuThi *et al.*, 1992). In this study it was shown that truncated LH receptor proteins enhance hCG-induced cAMP production, when transiently co-expressed with the full-length receptor in COS7 cells (VuHai-LuuThi *et al.*, 1992). In contrast, the present results indicate that co-expression of the FSH-R1 and FSH-R2 splice variants with FSH-R0 did not affect FSH signal transduction. Even in the presence of large amounts of truncated receptor protein, no or maybe a small increase in cAMP production could be observed.

A function of alternative splicing of gonadotropin receptors may be regulation of FSH receptor gene expression at the post-transcriptional level. Alternative splicing of the FSH receptor pre-mRNA could be a mechanism to prevent synthesis of functional FSH receptors. This has been described for LH receptor expression during ovarian development, where LH receptor splice variants are expressed 10 days prior to the expression of the full-length receptor (Sokka *et al.*, 1992). For LH receptor expression in testes (Zhang *et al.*, 1994) and FSH receptor expression in testes and ovaries (Rannikki *et al.*, 1995) similar events have been observed. In the present experiments, no differential regulation of the FSH-R1 and FSH-R2 splice variants was observed in the postnatal gonads. In ovaries during the cycle,

in testes of different ages, and in cultured Sertoli cells during FSH-induced down-regulation of FSH receptor mRNA, the expression levels of both alternative transcripts appeared to be very low, and showed a constant ratio to the expression level of full-length FSH receptor mRNA. If indeed alternative splicing of gonadotropin receptor mRNAs is a mechanism that is involved in developmental control of gonadotropin receptor expression, the splice variants FSH-R1 and FSH-R2 may result from incomplete inhibition of this mechanism at later developmental stages.

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Chapter 4

Follicle-Stimulating Hormone-Induced Destabilization of Follicle-Stimulating Hormone Receptor mRNA Involves a Hormone-Specific Mechanism that is Dependent on Ongoing Transcription

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Abstract

Upon stimulation with follicle-stimulating hormone (FSH), cultured Sertoli cells from immature rats show rapid and almost complete down-regulation of the FSH receptor mRNA level. This might serve as a cellular negative feedback mechanism, to control the extent of FSH stimulation of the Sertoli cells. Injection of immature rats with a high dose of FSH demonstrated that this down-regulation also functions *in vivo*, emphasizing its possible physiological importance. Other factors that are known to exert autocrine or paracrine actions on Sertoli cells (insulin, insulin-like growth factor I, transforming growth factor- β , androgens and estrogens) did not affect FSH receptor mRNA expression in cultured cells, indicating that FSH receptor mRNA down-regulation is specifically evoked by FSH. Investigations on the molecular mechanism that is involved in this mRNA down-regulation showed that, in the presence of an RNA synthesis inhibitor, the half-life of FSH receptor mRNA was not affected upon stimulation of the cells with FSH. However, when transcription was inhibited after pre-stimulation with FSH, a decreased half-life of the mRNA was observed. It is concluded that transcription (but not translation) is necessary to produce an FSH-induced factor that mediates FSH receptor mRNA breakdown. This factor is probably an RNA species, since translation of mRNA into protein is not necessary for FSH-induced down-regulation of FSH receptor mRNA.

Introduction

Follicle-stimulating hormone (FSH), a glycoprotein hormone that is produced in the anterior pituitary gland, plays an important role in the regulation of Sertoli cell function (reviewed by Sharpe, 1994). The physiological actions of FSH change depending on both the differentiation state of the Sertoli cells and the progress of spermatogenesis. In fetal rats, and during the first two weeks of postnatal life, FSH stimulates Sertoli cell proliferation. This is a critical event, since the number of Sertoli cells will eventually determine the capacity of spermatogenesis in the adult testis (Sharpe, 1994). In 21-day-old rats, Sertoli cell proliferation has stopped and the cells are maturing to become the supporting cells that completely surround the developing germ cells. Hormonal effects on spermatogenesis are mediated by the Sertoli cells. During the initial spermatogenic wave, both FSH and the Leydig cell product testosterone are required. In the adult rat, however, maintenance of full spermatogenesis is achieved by testosterone, whereas the role of FSH is less pronounced (Sharpe, 1994).

Actions of FSH on Sertoli cells are mediated by the FSH receptor, which is a transmembrane receptor that belongs to the family of GTP-binding protein (G protein)-coupled receptors (Sprengel *et al.*, 1990). These receptors contain a typical transmembrane domain, consisting of seven membrane spanning helices that are connected by three intracellular and three extracellular loops. The amino (N)-terminal domain of these receptors has an extracellular position, and the carboxyl (C)-terminus is located inside the cell. In contrast to most other G protein-coupled receptors that have a short N-terminal domain and are encoded by a single exon gene, receptors for the glycoprotein hormones FSH, luteinizing hormone/chorionic gonadotropin (LH/CG; McFarland *et al.*, 1989; Loosfelt *et al.*, 1989), and thyroid-stimulating hormone (TSH; Parmentier *et al.*, 1989) have an extended N-terminal domain that is encoded by multiple exons. This extended domain is involved in specific binding of the large hormone (Braun *et al.*, 1991). The main second messenger for glycoprotein hormone receptors is 3',5'-cyclic adenosine monophosphate (cAMP), although Ca^{2+} has also been described as second messenger (Grasso and Reichert, 1989; Gudermann *et al.*, 1992; Van Sande *et al.*, 1990).

Several mechanisms exist to control the extent of Sertoli cell stimulation by FSH. In the hypothalamus-pituitary-testis axis, the rate of LH-induced Leydig cell testosterone

production acts as a negative feedback regulator of both LH and FSH secretion (Griffin and Wilson, 1992). Specific negative feedback regulation of FSH action on Sertoli cells can be achieved by inhibin, which is an FSH-induced Sertoli cell product that inhibits FSH secretion from the pituitary gland (Griffin and Wilson, 1992). The sensitivity of Sertoli cells to FSH is regulated at the cellular level. After binding of FSH to its receptor and stimulation of adenylyl cyclase, the hormone-receptor complex is thought to be internalized and transported to an intracellular compartment, where the bound hormone is degraded (Shimizu and Kawashima, 1989). Also, increased phosphodiesterase activity has been observed in Sertoli cells after FSH stimulation (Verhoeven *et al.*, 1981; Conti *et al.*, 1982). Phosphodiesterase activity will metabolize cAMP, and contributes to the regulatory mechanism that prevents overstimulation of FSH stimulated Sertoli cells.

Regulation of the cellular level of FSH receptor mRNA expression is another possible mechanism to regulate FSH sensitivity of Sertoli cells. Incubation of cultured Sertoli cells with high doses of FSH resulted in a rapid and almost complete down-regulation of FSH receptor mRNA (Themmen *et al.*, 1991). This was caused by cAMP-mediated stimulation of the breakdown of FSH receptor mRNA. Down-regulation of receptor encoding mRNA upon stimulation with the respective hormone has also been described for the LH receptor in MA-10 mouse tumor Leydig cells (Wang *et al.*, 1991b), and for the TSH receptor in FRTL-5 cells (Akamizu *et al.*, 1990). However, these down-regulation processes are achieved through inhibition of transcription, and not through enhanced breakdown of the mRNAs (Nelson and Ascoli, 1992; Saji *et al.*, 1992). In contrast to the mechanism observed in MA-10 cells, LH/hCG-induced down-regulation of LH receptor mRNA in cultured porcine Leydig cells involves a receptor mRNA destabilizing mechanism (Chuzel *et al.*, 1995). After 12 hours of stimulation with hCG, the LH receptor mRNA level is down-regulated to approximately 10% of the initial level. This was caused by reduction of the receptor mRNA half-life from approximately 22 hours to approximately 2 hours (Chuzel *et al.*, 1995).

In view of the possible physiological significance of FSH-induced receptor mRNA down-regulation, this process was studied in more detail.

Materials and Methods

Animal handling and tissue culture

Wistar rats, locally bred, were maintained under controlled conditions (20-23 C, lights on from 5.00-19.00 h). Animal care was performed in accordance with the Guidelines on the Handling and Training of Laboratory Animals (Universities Federation of Animal Welfare). Immature 21-day-old rats were injected intra-peritoneally with 1 µg/g body weight ovine FSH-S16 (NIH, Bethesda, MD, USA). Total testes were isolated at different time points after injection. The methods for isolation and culture of Sertoli cells from 21-day-old rats have been described before (Themmen *et al.*, 1991). Cells were incubated in the presence of 500 ng/ml ovine FSH-S16, 0.5 mM dibutyryl cAMP (dbcAMP; Sigma, St. Louis, MO, USA), transforming growth factor β (TGF β), insulin-like growth factor-1 (IGF-1) (both were a kind gift of Dr. J.A. Foekens, Rotterdam), insulin, 1 µM testosterone, 1 µM estradiol, 1 µM dihydrotestosterone, or 1 µM R1881 (New England Nuclear, Boston, MA, USA). For half-life determination of FSH receptor mRNA, transcription was inhibited by treatment of the cells with 5 µg/ml actinomycin D (ActD; Boehringer Mannheim, Mannheim, Germany) or 30 µg/ml 5,6-dichlorobenzimidazole riboside (DRB; Sigma).

The mouse Sertoli cell-1 (MSC-1; Peschon *et al.*, 1992) cell line was maintained in DMEM/HAM's F12 (1:1 v/v) (Gibco BRL, Gaithersburg, MD, USA) containing 10% v/v Fetal Calf Serum (SEBAK, Aidenbach, Germany), 2×10^5 IU/l penicillin (Brocades Pharma, Leiderdorp, The Netherlands) and 0.2 g/l streptomycin (Radium Farma, Milan, Italy). Cells were seeded at 20% confluence, incubated in a humidified incubator at 37 C and 5% CO₂ in air, and split at 80% confluence.

One day before transfection, the MSC-1 cells were seeded in 25 or 80 cm² flasks. Calcium phosphate precipitate transfections were performed as described (Chen and Okayama, 1987). Each precipitate contained 10 µg FSH receptor expression plasmid, 1 µg pSV2CAT as an internal control, and 9 µg pTZ19R (Pharmacia, Uppsala, Sweden) as carrier DNA. The FSH receptor expression plasmid was constructed by replacing the chloramphenicol transferase (CAT) gene from pSV2CAT with the complete FSH receptor cDNA clone (Chapter 3). Forty-eight hours after transfection, cells were stimulated for 3 or 6 hours with 0.5 mM dbcAMP.

RNase protection assay

Total RNA was isolated using the LiCl/ureum method (Auffray and Rougeon, 1980). RNase protection assays were performed using FSH receptor, glyceraldehyde 3-phosphate dehydrogenase (GAPDH), and CAT cDNA probes. A HindIII/PvuII fragment of the FSH receptor cDNA (positions 240-480) (Sprengel *et al.*, 1990), was subcloned in pBluescript KS (Stratagene; Westburg, Leusden, The Netherlands), and used to generate α -[32 P]UTP-labelled (Amersham, 's Hertogenbosch, The Netherlands) anti-sense transcripts *in vitro*. In some experiments, an anti-sense probe directed against the FSH receptor splice variant FSH-R1 (Chapter 3) was used. This probe is directed against exon 9 (from position 704) and exon 10 (to position 1068), which are separated by an alternative exon (exon 9A). Hybridization of this probe to full-length FSH receptor mRNA results in two bands that originate from hybridization to exon 9 and to exon 10. The GAPDH control probe was synthesized from a pBluescript construct containing the PstI/Sau3A fragment (positions 1-310) from rat GAPDH cDNA (Fort *et al.*, 1985). The CAT probe was synthesized from a pBluescript construct containing the 250 base pairs HindIII/EcoRI fragment of the 5'-end of the CAT gene from pSV2CAT. α -[32 P]UTP-labelled anti-sense transcripts were generated *in vitro* (Sambrook *et al.*, 1989) using T7 RNA polymerase (Stratagene). Approximately 5×10^4 cpm of FSH receptor probe alone, or together with either 5×10^4 cpm GAPDH probe or 5×10^4 cpm CAT probe were mixed with 10 μ g of total RNA, in a total volume of 30 μ l hybridization mixture containing 40 mM PIPES (pH6.4), 1 mM EDTA, 0.4 M NaCl, and 80% (v/v) formamide. Hybridizations were performed overnight at 55 C. The RNase protection assay was performed as described (Sambrook *et al.*, 1989).

Results

FSH receptor mRNA is down-regulated specifically by FSH, both in vitro and in vivo

Destabilization of FSH receptor mRNA might be involved in the regulation of FSH action on Sertoli cells. To investigate whether this destabilization also occurs *in vivo*, we studied the effects of FSH on FSH receptor mRNA expression testis tissue. Immature 21-day-old rats were injected with FSH, and testes were isolated after different time periods. The FSH receptor mRNA expression level was determined using the sensitive and

quantitative RNase protection assay technique (Fig. 4.1). It was found that FSH-induced FSH receptor mRNA down-regulation also is observed in intact animals (Fig. 4.1; left panel), showing similar kinetics as FSH-induced FSH receptor mRNA down-regulation in cultured Sertoli cells (Fig. 4.1; right panel).

Down-regulation of LH receptor mRNA expression in MA-10 cells is evoked by LH/CG or dbcAMP, but can also be achieved by incubation of these cells with epidermal growth factor (EGF) and phorbol esters (Wang *et al.*, 1991a; Nelson and Ascoli, 1992). We investigated whether factors, that have been shown to affect Sertoli cell function, were able to affect FSH receptor mRNA expression in cultured Sertoli cells. Cells were incubated for 4 hours in the presence of different concentrations of insulin or IGF-1, or for 24 hours with different concentrations of TGF β (Fig. 4.2A). These factors did not affect FSH receptor mRNA expression during these time periods. Also, incubation of Sertoli cells with high concentrations of insulin or IGF-1 for 24 hours, and a maximal dose of TGF β for 4 hours had no effect on FSH receptor mRNA expression (not shown). FSH receptor mRNA expression was also not affected by stimulation of protein kinase C with the phorbol ester phorbol 12-myristate 13-acetate (PMA) (not shown).

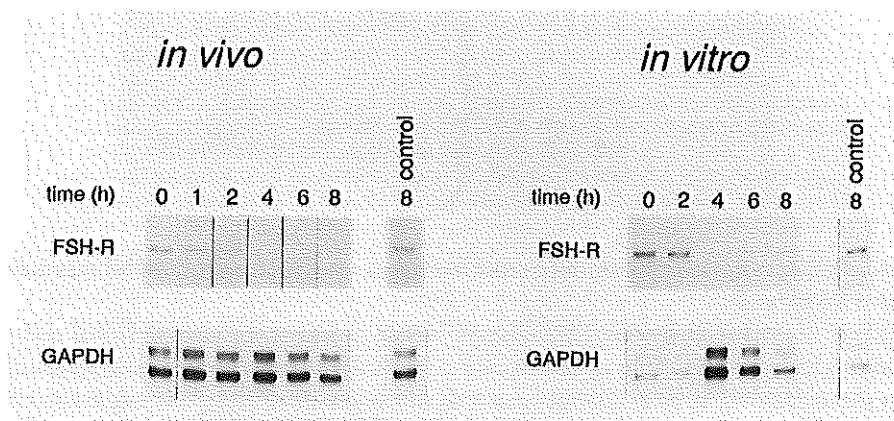
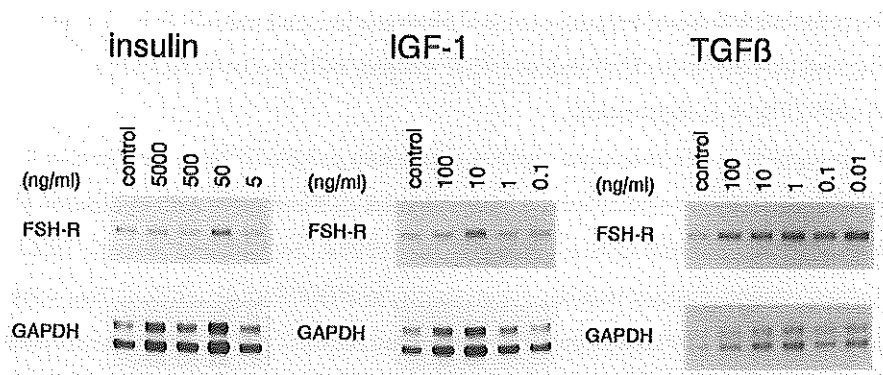


Fig. 4.1. FSH-induced destabilization of FSH receptor mRNA in vitro and in vivo. Immature rats (*in vivo*; left panel) were injected with 1 μ g/g body weight FSH, and cultured Sertoli cells (*in vitro*; right panel) were treated with 500 ng/ml FSH. Total testes were isolated and Sertoli cells were harvested after different times of treatment, indicated above the lanes. Total RNA was isolated and 10 μ g was subjected to RNase protection assay with FSH receptor (FSH-R) and GAPDH anti-sense probes. Results were similar in two different experiments.

A



B



Fig. 4.2. Effects of growth factors and steroid hormones on FSH receptor mRNA expression in cultured Sertoli cells.

(A) Effects of growth factors on FSH receptor mRNA expression. Cultured Sertoli cells were incubated with increasing doses of insulin, IGF-1, or TGFβ as indicated above the lanes. After 4 h. (insulin and IGF-1), or 24 h. (TGFβ), total RNA was isolated and 10 μg was subjected to RNase protection assay with FSH receptor (FSH-R) and GAPDH probes. Results were similar in two different experiments. (B) Effects of steroid hormones on FSH receptor mRNA expression and FSH-induced down-regulation of FSH receptor mRNA. Cultured Sertoli cells were incubated for 4 hours with 500 ng/ml FSH, or with 1 μM testosterone (T), 1 μM estradiol (E₂), 1 μM dihydrotestosterone (DHT), or 1 μM R1881 in the absence or presence of 500 ng/ml FSH. Total RNA was isolated and 10 μg was subjected to RNase protection assay with FSH receptor (FSH-R) and GAPDH anti-sense probes. Results were similar in two different experiments.

In addition to growth factors, steroid hormones play an important role in regulation of Sertoli cell function. The effects of androgens and estrogens on FSH receptor mRNA expression, and on FSH-induced FSH receptor mRNA down-regulation, were investigated. Sertoli cells were incubated for 4 (Fig. 4.2B) or 24 (not shown) hours with testosterone, estradiol, dihydrotestosterone, or the stable synthetic androgen R1881, in the absence or presence of FSH. It was observed that the steroid hormones did not affect FSH receptor mRNA expression or FSH-induced FSH receptor mRNA down-regulation. It is concluded that FSH receptor mRNA down-regulation represents a hormone-specific phenomenon.

FSH receptor mRNA down-regulation is dependent on ongoing transcription

To investigate the mechanism of FSH-induced FSH receptor mRNA breakdown, the half-life of the mRNA in Sertoli cells was determined in the absence or presence of FSH or dbcAMP. Transcription was inhibited by incubating the cells with ActD starting 30 minutes prior to FSH stimulation. The decay of FSH receptor mRNA was determined with RNase protection assay (Fig. 4.3). Surprisingly, the half-life of FSH receptor mRNA was influenced neither by FSH stimulation nor by stimulation with the potent cAMP analogue dbcAMP (Fig. 4.3). When transcription was inhibited, the half-life of FSH receptor mRNA was approximately 2 hours, irrespective of the presence of FSH or dbcAMP.

We hypothesized that FSH receptor mRNA breakdown is dependent on ongoing transcription, which is needed to transcribe an FSH-induced factor that is involved in the process of receptor mRNA breakdown. Therefore, an experiment was designed that would allow synthesis of this factor under control of FSH, followed by FSH receptor mRNA half-life determination in the presence of a transcription inhibitor. Cultured Sertoli cells were incubated for 1 hour with FSH, and, subsequently, transcription was inhibited using DRB, an RNA polymerase inhibitor that in contrast to ActD does not interact with RNA. The decay of FSH receptor mRNA was determined (Fig. 4.4), and it was observed that pre-stimulation with FSH resulted in a shortening of FSH receptor mRNA half-life (approximately 1.5 hours vs approximately 2 hours in the DRB control). Thus, FSH induces the transcription of a factor that mediates breakdown of FSH receptor mRNA. Previous results have shown that receptor mRNA down-regulation is not dependent on ongoing translation (Themmen *et al.*, 1991), which indicates that the FSH-induced factor is probably an RNA species.

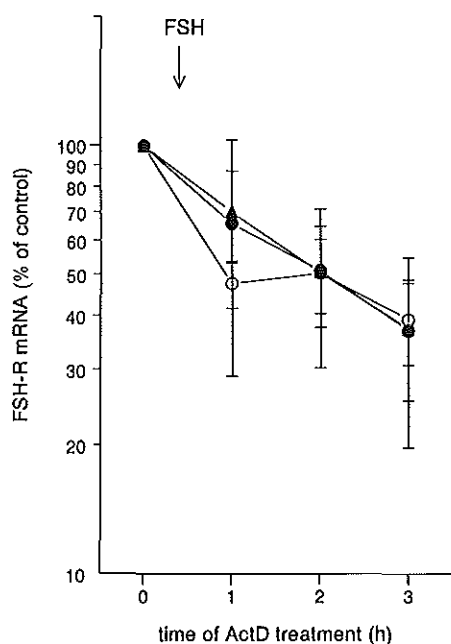


Fig. 4.3. Half-life determination of FSH receptor mRNA in the presence or absence of FSH.

Cultured Sertoli cells were incubated for 30 min with 5 $\mu\text{g/ml}$ ActD to inhibit transcription, and subsequently cultured for different time periods in the absence (open circles) or presence (closed circles) of 500 ng/ml FSH, in the continuous presence of ActD. Total RNA was isolated and 10 μg was subjected to RNase protection assay with FSH receptor (FSH-R) anti-sense probe. The autoradiographs were scanned, and the relative optical density of FSH receptor mRNA was plotted against the time after the start of ActD treatment. In some experiments, Northern blotting (Sambrook et al., 1989) was used to determine the FSH receptor mRNA level. Each point represents the mean \pm SD of four different experiments.

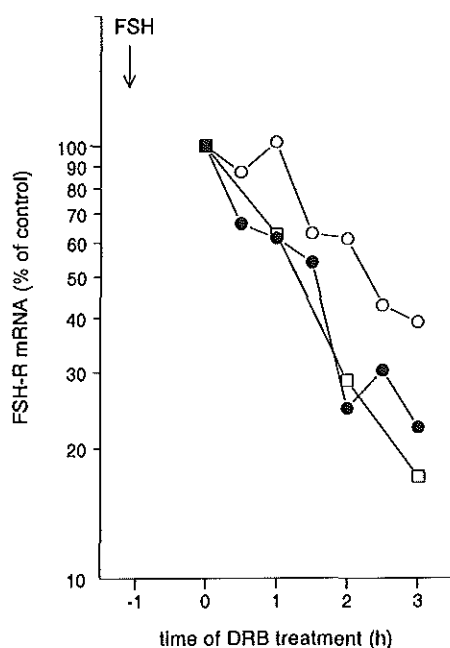


Fig 4.4. Half-life determination of FSH receptor mRNA after FSH stimulation.

Sertoli cells were pre-incubated for 1 hour in the absence (open circles) or presence (closed circles) of 500 ng/ml FSH, and subsequently the incubation was continued (without or with FSH) in the presence of 30 $\mu\text{g/ml}$ DRB to inhibit transcription. As a control, Sertoli cells were cultured in the presence of 500 ng/ml FSH only (open squares). After different time periods, total RNA was isolated and 10 μg was subjected to RNase protection assay with FSH receptor anti-sense probe. The amount of radioactivity in the FSH receptor fragment was determined using a phospho-imager, and plotted against the time after the start of DRB treatment. Results were similar for two different experiments.

FSH receptor mRNA down-regulation does not occur in MSC-1 cells

Specific breakdown of FSH receptor mRNA may be mediated through specific sequences within this mRNA. Identification of such sequences is a valuable first step in the identification of factors that are involved in the process of FSH-induced down-regulation of the respective receptor mRNA. To identify these sequences, hybrid mRNAs can be used, which consist of the full-length FSH receptor mRNA from which parts are exchanged with parts of another mRNA that is insensitive to FSH. The hybrid mRNAs can be expressed in cultured Sertoli cells, and tested for FSH-induced down-regulation. Unfortunately, the transfection efficiency of cultured Sertoli cells was found to be very low, and this approach therefore failed. Another possibility is to use MSC-1 cells, a cell line derived from a mouse testicular tumor that expresses Sertoli cell associated markers (Peschon *et al.*, 1992). However, these cells do not express functional FSH receptors, and it is uncertain whether this mouse cell line has similar characteristics as cultured 21-day-old rat Sertoli cells, with respect to FSH-induced down-regulation of rat FSH receptor mRNA. To test the suitability of the MSC-1 cell line, the complete FSH receptor cDNA, including the 5', and 3'-untranslated regions, was transiently expressed in MSC-1 cells, together with a CAT expression vector as an internal control for transfection efficiency. Furthermore, both the CAT expression vector (pSV2CAT) and the FSH receptor expression vector contained the

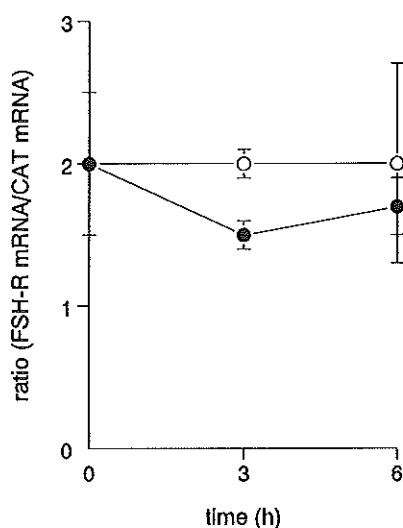


Fig. 4.5. Down-regulation of transiently expressed FSH receptor mRNA in MSC-1 cells.

MSC-1 cells were transfected with FSH receptor expression vector DNA, and, as an internal control, with CAT expression vector DNA. Two days after transfection, the cells were treated with dbcAMP for 3 or 6 hours, and FSH receptor and CAT mRNA levels were determined using RNase protection assays. The amount of radioactivity in each fragment was determined using a phosphorimager. The amount of FSH receptor mRNA, normalized using the amount of CAT mRNA, was plotted against the incubation time. Results were similar in two different experiments.

same promoter (SV40 early promoter), which allows for correction of possible cAMP effects on the promoter. Transfected MSC-1 cells were treated for 3 and 6 hours with dbcAMP, and FSH receptor and CAT mRNA expression levels were determined using RNase protection assays (Fig. 4.5). Treatment with dbcAMP did not result in significant down-regulation of transiently expressed FSH receptor mRNA. Therefore, it is concluded that the MSC-1 cell line is not suitable to test down-regulation of hybrid FSH receptor mRNAs. In order to obtain a suitable model system, an efficient transfection protocol for cultured Sertoli cells should be developed.

Discussion

FSH and androgens are the major hormones that regulate Sertoli cell function. FSH regulates protein phosphorylation and the synthesis of many proteins, such as aromatase, androgen binding protein, plasminogen activator, and transferrin (reviewed by Sharpe, 1994). The expression of several genes is regulated very rapidly, such as the primary response gene *LRPR1* (Slegtenhorst-Eegdemann *et al.*, 1995), and the α -inhibin gene (Klaaij *et al.*, 1990). At the cellular level, processes function that attenuate the action of FSH, including receptor internalization (Shimizu and Kawashima, 1989) and receptor mRNA down-regulation (Themmen *et al.*, 1991). FSH-induced FSH receptor mRNA down-regulation involves enhanced FSH receptor mRNA breakdown, but not decreased gene transcription. The expression level of FSH receptor mRNA is down-regulated to approximately 10% of the initial level within 4 hours (Themmen *et al.*, 1991). The present paper describes that this process also functions in intact animals, emphasizing its possible physiological importance. Hormones and growth factors other than FSH, that are known to regulate Sertoli cell function, did not affect the FSH receptor mRNA expression level in cultured Sertoli cells. Although many possible factors could have been tested, insulin, IGF-1, TGF β , and steroid hormones were selected. Insulin and IGF-1 were included because of their stimulatory effect on lactate production by Sertoli cells (Oonk *et al.*, 1989). Furthermore, the suppressive effect of FSH and hCG on apoptosis in cultured preovulatory follicles is thought to be mediated via an IGF-1 autocrine loop (Chun *et al.*, 1994), which may also function in Sertoli cells. TGF β attenuates FSH-induced aromatase activity and lactate production, a

process that is achieved through elevation of phosphodiesterase activity (Morera *et al.*, 1992). Androgens were included, since both FSH and testosterone are important for the initiation of spermatogenesis. Furthermore, FSH-induced aromatase activity results in increased estrogen production that might exert an autocrine loop on Sertoli cells. The absence of any effect of these factors on FSH receptor mRNA expression, in spite of their obvious role in the physiological regulation of Sertoli cell function, indicates the specificity of FSH to down-regulate its receptor mRNA.

The half-life of FSH receptor mRNA after FSH stimulation was found to be approximately 1.5 hours, whereas the unstimulated half-life is estimated at 2 hours. However, it was found that treatment with FSH alone (thus without subsequent DRB treatment) resulted in a mRNA decay curve similar to the curve observed after treatment with FSH and DRB (see Fig. 4.4). One would expect that the half-life of FSH receptor mRNA during FSH and DRB treatment would be reduced, compared to the half-life observed during treatment with FSH alone, since DRB inhibits *de novo* synthesis of FSH receptor mRNA. However, the results of the present experiments suggest that DRB treatment not only inhibits FSH receptor mRNA synthesis, but also inhibits synthesis of an FSH-induced RNA species that mediates the mRNA breakdown. Furthermore, it can be concluded that this RNA species must have a high turnover rate, and that constant FSH stimulation is required to obtain full down-regulation of FSH receptor mRNA. Similarly, for phorbol ester-induced destabilization of estrogen receptor mRNA in MCF-7 cells, induction of a labile RNA factor that mediates mRNA breakdown has been postulated (Ree *et al.*, 1992). Both for the FSH receptor and the estrogen receptor, these regulatory RNA species have not yet been identified.

To identify the RNA species that are involved in the process of FSH receptor mRNA destabilization, sequences in the mRNA that are involved in this process should be identified first. For this purpose, down-regulation of FSH receptor mRNA, that was transiently expressed in MSC-1 mouse tumor Sertoli cells, was tested. However, no down-regulation could be observed upon stimulation with dbcAMP, which may be due to specific properties of this cell line. MSC-1 cells are morphologically similar to cultured Sertoli cells, and express Sertoli cell associated markers, such as androgen binding protein, sulfated glycoproteins 1 and 2, transferrin, and the β -subunit of activin/inhibin (Peschon *et al.*, 1992; McGuinness *et al.*, 1994). Furthermore, expression of the RII_β regulatory subunit of cAMP-

dependent protein kinase is up-regulated after exposure of MSC-1 cells to forskolin, which indicates that these cells are still responsive to cAMP (Peschon *et al.*, 1992). However, the absence of FSH receptor expression (McGuinness *et al.*, 1994) suggests partial dedifferentiation of the cell line. Furthermore, MSC-1 cells were probably derived from very young Sertoli cells, in which the down-regulatory process may not be functional yet. Therefore, Sertoli cells in primary culture would probably be the best model system, although a protocol for efficient transfection of these non-dividing cells still needs to be developed. Another possibility would be the use of an *in vitro* model, where *in vitro* transcribed FSH receptor mRNA is incubated with extracts of Sertoli cells that have been cultured in the absence or presence of FSH.

The experiments described herein address short-term responses of Sertoli cells to exposure to a high doses of FSH. In intact animals, however, Sertoli cells show a long-term response to a sub-maximal FSH level. When cultured Sertoli cells were incubated for 24 hours with different doses of FSH, a dose-dependent decrease in FSH receptor mRNA level was observed (data not shown). Thus, after prolonged FSH stimulation, the expression level of FSH receptor mRNA is adjusted to the level of FSH stimulation, which is the net effect of the various processes that regulate FSH action on Sertoli cells. In these long-term experiments, the transcriptional activity of the FSH receptor gene promoter was not determined, and the observed effects may be caused by decreased transcription of the FSH receptor gene as well. Although an effect of FSH on FSH receptor gene transcription has not yet been described, this may be another process regulating FSH receptor expression.

In conclusion, FSH-induced FSH receptor mRNA destabilization is dependent on *de novo* synthesis of a factor that is probably an RNA species. This process is observed both *in vitro* and *in vivo*, and is specific for the FSH/cAMP pathway.

Acknowledgments

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Chapter 5

A Missense Mutation in the Second Transmembrane Segment of the Luteinizing Hormone Receptor Causes Familial Male-Limited Precocious Puberty

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Abstract

Patients with familial male-limited precocious puberty (FMPP) present with a very early onset of puberty. Several missense mutations in the luteinizing hormone (LH) receptor gene that cause amino acid substitutions in the sixth transmembrane segment of the receptor protein have been shown to be a cause of the disorder. We have identified a novel LH receptor gene mutation in an FMPP patient, which results in a threonine for methionine substitution at position 398 in the second transmembrane segment of the receptor protein. *In vitro* expression in human embryonic kidney 293 cells of this LH receptor mutant and two previously described LH receptor mutants, showed that cAMP production in the absence of hormone was elevated up to 25-fold as compared to the basal level observed for the wild type receptor. The ED_{50} values of hormone-induced cAMP production were within the same range for wild type and mutant receptors, but maximal hormone-induced cAMP production was relatively low for mutant receptors. We also produced receptors containing amino acid substitutions in both the second and the sixth transmembrane segments. For these double mutants, basal receptor activities were similar to the basal activities observed in single mutants, while hormone-induced receptor activation was almost completely abolished.

Introduction

Receptors for luteinizing hormone (LH; McFarland *et al.*, 1989; Loosfelt *et al.*, 1989), thyroid-stimulating hormone (TSH; Parmentier *et al.*, 1989), and follicle-stimulating hormone (FSH; Sprengel *et al.*, 1990) form a subgroup of glycoprotein hormone receptors within the family of GTP-binding protein (G protein)-coupled receptors. Receptors of this family are characterized by a transmembrane domain, an N-terminal extracellular domain, and a C-terminal intracellular domain. Most G protein-coupled receptors contain a short extracellular domain, which does not seem to have a specific function. In contrast, glycoprotein hormone receptors contain a large extracellular domain, of approximately the same size as the transmembrane domain, which is involved in high affinity hormone binding (Braun *et al.*, 1991).

The transmembrane domain of G protein-coupled receptors consists of seven transmembrane segments, which are connected by three intra- and three extracellular loops. This domain anchors the receptor in the plasma membrane and is involved in ligand binding and receptor activation. Construction and expression of chimeric adrenergic receptors, in which domains of α_2 - and β_2 -adrenergic receptors were exchanged, showed that most of the seven transmembrane segments are involved in specific agonist ligand binding, whereas the seventh segment is the major determinant for antagonist specificity (Kobilka *et al.*, 1988). More subtle swapping experiments, in which small parts of the third intracellular loop were exchanged between different adrenergic receptors, have shown that this loop is involved in specific G protein coupling and activation [reviewed by Dohlman *et al.* (1991)]. Substitution of the alanine 293 residue located in this third intracellular loop of the α_{1B} -adrenergic receptor by any other amino acid residue resulted in elevated basal receptor activities (Kjelsberg *et al.*, 1992). These results indicate that the receptor resides in an inactive conformation, which can be released after conformational changes induced by either interaction of the ligand with the transmembrane domain or by amino acid substitutions in the transmembrane domain. For glycoprotein hormone receptors, the transmembrane domain by itself is not an important high affinity hormone binding domain, and hormone-induced conformational changes may result from interactions of the large extracellular domain and/or the bound hormone with the transmembrane domain (Braun *et al.*, 1991).

Mutagenesis is an important tool to investigate structure-function relationship of G protein-coupled receptors. Another approach to characterize domains important for receptor function, is to study diseases that are caused by receptor gene mutations. Patients with the syndrome of familial male-limited precocious puberty (FMPP) have LH receptor gene mutations (Kremer *et al.*, 1993; Shenker *et al.*, 1993; Yano *et al.*, 1994; Kosugi *et al.*, 1995). FMPP is an autosomal dominant condition. Affected boys present with a very early onset of puberty, which is caused by a gonadotropin independent stimulation of testicular androgen production (Egli *et al.*, 1985). The LH receptor gene mutations identified in FMPP patients result in amino acid substitutions in the sixth transmembrane domain of the receptor protein. *In vitro* expression of these mutant receptors in COS cells, revealed constitutively activated receptors in terms of increased basal cAMP production (Shenker *et al.*, 1993), whereas basal inositol phosphate production and hormone-binding affinity were comparable with the wild type receptor (Yano *et al.*, 1994; Kosugi *et al.*, 1995). This elevated basal receptor activity to stimulate cAMP production is most likely the cause of autonomous steroid production in these FMPP patients. The location of the amino acid substitutions near the third intracellular loop, suggests mutational induction of conformational changes similar to those induced in the mutated α_{1B} -adrenergic receptor, which partially release the LH receptor from its inactive conformation.

In the present paper, we report a new FMPP family, which carries an LH receptor gene mutation that, in contrast to the thus far identified mutations, results in a receptor activating amino acid substitution in the second transmembrane segment of the receptor protein.

Materials and methods

Patients

The patient was referred at the age of 2 years and 9 months, because of progressive sexual development. Serum LH and FSH levels were in the prepubertal range and did not rise after stimulation with gonadotropin-releasing hormone (GnRH). Serum testosterone was elevated to pubertal levels (22.5 nmol/l; normal prepubertal levels: <0.1 nmol/l). The patient's mother and sister were normal, however the father had also developed pubertal signs at a very early age. Informed consent was obtained from each subject or his parents.

Patients in which the Met571Ile and Asp578Gly substitutions were identified have been described by Kremer *et al.* (1993).

DNA analysis

Isolation of genomic DNA, PCR DNA amplification, single strand conformational polymorphism (SSCP), and sequence analysis, were performed as described before (Kremer *et al.*, 1993), with the exception that for the SSCP the PCR amplification was performed with one labelled primer. Subjects were screened using several primer sets amplifying overlapping parts of the transmembrane domain. Primer set 2 [forward primer 5'-ATGACTGTTCTTTTGTCTCCTG-3' and reverse primer 5'-GGTCCAGG-TGAATAGCATAGG-3', amplifying fragment 1132-1426; numbering according to Minegishi *et al.* (1990)] gave rise to an abnormally migrating band. This fragment was subjected to sequence analysis.

Construction of mutant LH receptor expression plasmids

Standard molecular biology techniques were performed as described by Sambrook *et al.* (1989). A wild type human LH receptor expression plasmid (pSG5-hLHR) was constructed by cloning the human LH receptor cDNA (nucleotides -3 to 2374 relative to the translation start site) into the EcoRI site of pSG5 (Green *et al.*, 1988). The protein encoded by this cDNA differs from the published human LH receptor (Minegishi *et al.*, 1990) by the insertion of two amino acids (Leu and Gln) at positions 21 and 22 (not shown). Mutations were introduced into this plasmid by site-directed mutagenesis (Higuchi *et al.*, 1988), using PCR DNA amplification techniques. For substitutions Met571Ile and Asp578Gly, a 300 base pairs (bp) fragment (1512-1812) was amplified using primers LHR1512FOR (5'-GTCGGTGT-CAGCAATTAC-3') and LHR1812REV (5'-GGTTACTGTGATAAGAGG-3'). Mutations were introduced during two rounds of amplification using primers LHR571MIFOR (5'-GCTAAGAAAATAGCAATCC-3') and LHR571MIREV (5'-GGATTGCTATTTT-CTTAGC-3') for the Met571Ile substitution, and LHR578DGFOR (5'-CTTCACCGGTTTC-ACCTG-3') and LHR578DGREV (5'-CAGGTGAAACCGGTGAAG-3') for the Asp578Gly substitution. The amplified DNA was digested with BstXI and BspMI (positions 1550 and 1744) and the resulting fragments were exchanged into the wild type LH receptor expression plasmid yielding pSG5-LHR1 and pSG5-LHR2, respectively. For the

Met398Thr substitution a 400 bp fragment (1030-1430) was amplified using primers LHR1030FOR (5'-CCGATGTGCTCCTGAACC-3') and LHR1430REV (5'-GTCCAGGTGAATAGCATAG-3'). The mutation was introduced using primers LHR398MTFOR (5'-CGTTTTCTCACG-TGCAATC-3') and LHR398MTREV (5'-GATTGCACGTGAGAAAACG-3'). The amplified DNA was digested with Bsu36I and XbaI (positions 1090 and 1390), and the resulting fragment was exchanged into the wild type LH receptor expression vector, yielding pSG5-LHR3. Primers were purchased from Eurogentec S.A. (Seraing, Belgium). Mutagenesis was verified by sequence analysis of the exchanged fragments. Double mutants were constructed by exchanging the 1173 bp XbaI fragment from pSG5-LHR1 and pSG5-LHR2 in pSG5-LHR3, yielding pSG5-LHR31 and pSG5-LHR32, respectively. For transfection purposes, plasmids were isolated using a Plasmid Maxi Kit (Qiagen, Düsseldorf, Germany).

In vitro expression of mutant LH receptors in HEK293 cells

Human Embryonic Kidney 293 cells (ATTC designation CRL 1573) were maintained in DMEM/HAM's F12 (1:1 v/v) (Gibco BRL, Gaithersburg, MD, USA) containing 10% v/v Fetal Calf Serum (SEBAK, Aidenbach, Germany), 2×10^5 IU/l Penicillin (Brocades Pharma, Leiderdorp, The Netherlands) and 0.2 g/l streptomycin (Radium Farma, Milan, Italy). Cells were seeded at 10% confluence, incubated in a humidified incubator at 37 C and 5% CO₂, and split at 50-70% confluence.

One day before transfection, cells were seeded at 15-20% confluence in 12-well plates (Costar, Cambridge, MA, USA). Calciumphosphate-precipitate transfections were performed as described (Chen and Okayama, 1987). Each well received 50 µl precipitate, containing 0.5 µg expression plasmid, 0.1 µg pRSVluc (Van Dijk *et al.*, 1991) as an internal control, and 0.4 µg pTZ19R (Pharmacia, Uppsala, Sweden) as carrier DNA. Forty-eight hours after transfection, cells were washed with medium without serum, but containing 25 mM HEPES (Gibco), 0.1% (w/v) BSA and 0.2 mM isobutyl-methylxanthine (Sigma, St. Louis, MO, USA), and subsequently incubated for two hours in the same medium containing different doses of hCG (urinary hCG; Organon International, Oss, The Netherlands). Medium was collected for cAMP determination (Stoof *et al.*, 1987) and cells were lysed for luciferase activity determination (Blok *et al.*, 1992). Values for cAMP were corrected by subtracting control cAMP levels, as determined in cells transfected with empty expression vector

(pSG5). Control cAMP levels were approximately 50% of basal levels in wild type LH receptor-transfected cells. The cAMP values were corrected for transfection efficiency using the luciferase activity in the same well, yielding cAMP production in pmol/relative light unit.

Results

Identification of a new LH receptor missense mutation in an FMPP family

A family, in which both father and son showed clinical symptoms of the FMPP syndrome, was screened for mutations in the LH receptor gene. Overlapping DNA fragments encoding parts of the transmembrane domain were amplified and subjected to SSCP analysis. A primer set amplifying the second transmembrane segment gave rise to abnormally migrating bands for the father and the son, but not for the mother and four control subjects (Fig. 5.1A). DNA sequence analysis of this fragment revealed a heterozygous T to C conversion at position 1193 relative to the translation start site in both the father and the son (Fig. 5.1B). This mutation causes a threonine for methionine substitution at position 398 (Met398Thr), which is located in the second transmembrane segment of the receptor protein.

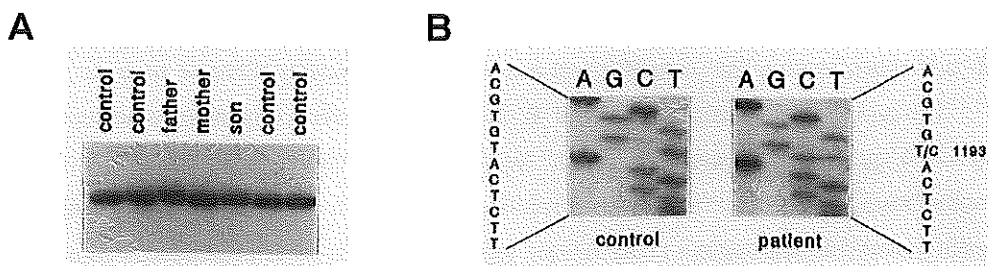


Fig. 5.1. Identification of a missense mutation in the LH receptor gene.

A: Single strand conformational polymorphism for the LH receptor gene in an FMPP family (father, mother, and son). For comparison, SSCP patterns for normal individuals (control) are shown on either side. Both father and son show a slight mobility shift in this fragment. B: Sequence analysis of the LH receptor gene (from position 1187 to 1198, relative to the translation start) in a normal individual (control) and the son from the FMPP family. A heterozygous T to C conversion is detected at position 1193. The father contained the same mutation.

Mutant receptors constitutively stimulate cAMP production

Previously, we have identified two other LH receptor gene mutations in FMPP patients, that result in amino acid substitutions in the sixth transmembrane segment (Met571Ile and Asp578Gly) (Kremer *et al.*, 1993). For these substitutions, and for the newly identified Met398Thr substitution, mutant receptor-expression plasmids were constructed, and transiently expressed in HEK293 cells, to test the effects of the substitutions on receptor function. Forty-eight hours after transfection, the cells were incubated in the absence or presence of different doses of hCG for two hours. It was observed that stimulation of the mutant receptors with a high hCG concentration resulted in cAMP production at a level of approximately 40% of the level obtained with the wild type LH receptor, while the ED₅₀ values were within the same range (3 ng/ml) (Fig 5.2A). However, basal levels of cAMP production in the absence of hCG were increased approximately 15-25-fold for cells expressing the mutant receptors as compared to cells expressing the wild type receptor.

Elevated basal cAMP production should result in elevated androgen production by Leydig cells from FMPP patients in the absence of LH. To test whether the mutant receptors are able to induce steroid production in the absence of gonadotropins, they were transiently expressed in mouse tumor Leydig cells (MA10). This indeed resulted in a 2-6-fold increase in pregnenolone production in the absence of hCG (data not shown). Expression of the wild type human LH receptor in these cells resulted only in a 1.5-fold increase in the absence of hCG.

Double mutants have the same effect on basal cAMP production as single mutants

The amino acid substitutions found in the second and sixth transmembrane segments resulted in similar effects on receptor function. Substitutions in both transmembrane segments might act in an independent fashion, i.e. induction of conformational changes in each transmembrane segment may release separate domains for G protein activation from their inactive conformation. LH receptors containing substitutions in both segments then might exhibit higher or even additive basal activities.

To test this hypothesis, double mutant receptor expression plasmids were constructed containing both the substitutions Met398Thr and Met571Ile, or both the substitutions Met398Thr and Asp578Gly. These double mutants were transiently expressed in HEK293 cells, and basal and hCG-induced cAMP productions were determined. It was found that

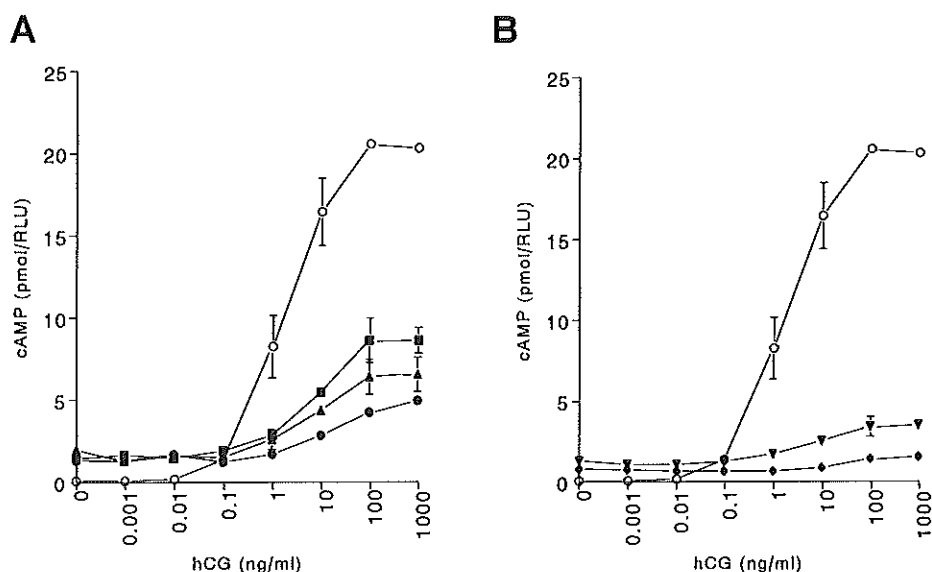


Fig. 5.2. Dose-response relationships of hCG-induced cAMP production by wild type and mutant LH receptors.

Receptors were transiently expressed in HEK293 cells, and cAMP production was determined as described in Materials and Methods. Each point represents the mean \pm s.e.m. for three replicate wells from a single experiment. Results were similar in three different experiments. A: Single amino acid substitutions. Wild type: ○; Met571Ile: ●; Asp578Gly: ▲; Met398Thr: ■. B: Double amino acid substitutions. Wild type: ○; Met398Thr & Met571Ile: ◆; Met398Thr & Asp578Gly: ▼.

these double mutants show only very little hCG-induced cAMP production, and the basal levels of cAMP production in the absence of hCG were increased up to 10-15-fold the level observed in cells expressing the wild type receptor (Fig 5.2B). These results indicate that substitutions in the second and sixth transmembrane segments do not act independently.

Discussion

The present paper describes a new LH receptor gene mutation that causes an amino acid substitution in the second transmembrane segment, in contrast to the previously reported LH receptor mutations that change the sixth transmembrane segment (Kremer *et al.*, 1993; Shenker *et al.*, 1993; Yano *et al.*, 1994). *In vitro* expression studies of this LH receptor

mutant and two previously described mutants, show elevated cAMP levels in the absence of hormone, and approximately 60% reduction of their activities at maximal doses of hCG. *In vitro* expression of the mutant receptors in MA10 mouse tumor Leydig cells, which also express the wild type LH receptor, resulted in a 2-6-fold increase in basal steroid production. The phenotypes of the mutant receptors in HEK293 and MA10 cells, and the genetic analysis (Kremer *et al.*, 1993), indicate that these mutations are most likely the cause of precocious puberty in FMPP boys.

The low response of the mutant receptors to high doses of hCG is different from results described by other investigators (Shenker *et al.*, 1993; Yano *et al.*, 1994; Kosugi *et al.*, 1995), who reported *in vitro* expression studies on Asp578Gly, Thr577Ile, and Met571Ile mutant LH receptors. They found elevated cAMP production levels in the absence of hormone for the mutant receptors, but a similar response to high hormone concentrations as compared to the wild type receptor. In one study, maximal [¹²⁵I]-hCG binding to cells expressing mutant receptors reached only up to 66% of the level found in cells expressing the wild type receptor, whereas maximal hCG-induced cAMP production levels were identical for mutant and wild type receptors (Kosugi *et al.*, 1995). A reason for this discrepancy may be found in the very high expression levels which can be reached in the COS-7 cells used in these studies. Duprez *et al.* (1994) have shown that transfection of increasing amounts of TSH receptor expression plasmid into COS-7 cells leads to saturating expression of TSH receptors in the plasma membrane. Full stimulation of such cells may result in a plateau level of the maximal cAMP response because the number of G proteins and/or adenylyl cyclase proteins are limited, thus not all occupied receptors can interact in an efficient manner with the signal transduction cascade. In the present experiments, the expression levels of wild type LH receptors in HEK293 cells ranged from 3,000 to 30,000 hCG binding sites per transfected cell (data not shown), which is within the physiological range of rat Leydig cells (Warren *et al.*, 1984). Correction for transfection efficiency of the different expression plasmids by means of co-transfection with a luciferase expression plasmid does not result in equal numbers of binding sites on the plasma membrane. This implicates that the amino acid substitutions not only change basal receptor activity, but also post-translational modifications and transport.

All activating LH and TSH receptor mutations identified thus far, cause changes in the transmembrane domain, with the sixth transmembrane segment and the third intracellular

loop as apparent mutational hot spots (Kremer *et al.*, 1993; Shenker *et al.*, 1993; Parma *et al.*, 1993; Duprez *et al.*, 1994; Porcellini *et al.*, 1994; Yano *et al.*, 1994; Kosugi *et al.*, 1994; Kosugi *et al.*, 1995). Because these regions have been implicated to play an important role in G protein coupling (Kobilka *et al.*, 1988), it may be suggested that the amino acid changes mimic the hormone-induced conformational shift to a certain extent. The location of substitutions in the second transmembrane segment of the LH receptor (this report), and the third and seventh transmembrane segments of the TSH receptor (Duprez *et al.*, 1994), indicate that more segments of the transmembrane domain are involved in retaining the receptor in an inactive conformation. Therefore, it is likely that amino acid substitutions in several of the seven transmembrane segments may be identified in new cases of syndromes involving activated G protein-coupled receptors. The structural alterations caused by the mutations are diverse: polar and charged amino acid residues are replaced by non-polar amino acid residues, and *vice versa*. The positions of the side chains relative to the pocket of the transmembrane domain or to the other transmembrane segments are also diverse. It would be interesting to study whether amino acid substitutions affect the overall inactive conformation of the receptor in a non-specific fashion, or whether specific substitutions are required to activate the receptor.

Amino acid substitutions leading to more drastic conformational changes, will probably result in inactivation of the receptor. LH receptor double mutants, containing substitutions in both the second and the sixth transmembrane segments, do hardly show any hCG-induced cAMP production, although the basal receptor activities are comparable to that of the single mutants. Alterations in the double mutant receptors are probably more pronounced, and may have a drastic effect on post-translational modifications and intracellular transport of the protein or they may disrupt the protein structure in such a way that hormone-induced receptor activation is abolished.

Recently we have identified an inactivating LH receptor mutation in patients with Leydig cell hypoplasia (Kremer *et al.*, 1995). These 46,XY individuals present with a female external phenotype, with abdominal testes and without female internal sex organs. Testes from these patients contain very few Leydig cells and testosterone levels are very low. The disorder appeared to be caused by an LH receptor gene mutation causing a proline for alanine substitution in the sixth transmembrane segment. This Ala593Pro substitution renders the receptor, which is expressed on the plasma membrane, completely inactive even

at high hCG concentrations. Another receptor inactivating mutation has been described for the TSH receptor in *hyt/hyt* mice (Stein *et al.*, 1994). In this case a conserved proline residue in the fourth transmembrane segment was substituted by a leucine residue. These drastic changes (both involving a proline residue) in the LH and TSH receptors may have comparable effects on protein structure, post-translational modifications and intracellular transport as the double amino acid substitutions.

In the subfamily of glycoprotein hormone receptors, hormone binding and receptor activation are probably sequential events. It is not likely to find activating substitutions in the large N-terminal extracellular domain, since this domain is involved mainly in hormone binding and probably in proper positioning of the hormone relative to the transmembrane domain. Such positioning may enhance the interaction of the hormone with the transmembrane domain. In fact, it has been reported that hCG can activate a truncated LH receptor lacking the extracellular domain (Ji and Ji, 1991), suggesting an interaction site for the hormone with the transmembrane domain. However, high concentrations of hCG had to be used, and similar experiments with the TSH receptor did not result in receptor activation (Paschke *et al.*, 1994). The functional separation of hormone binding and receptor activation is in agreement with the observation that the mutant LH receptors described herein do not show a shift in ED_{50} of hCG-stimulated cAMP production. In contrast, activating substitutions of the α_{1B} -adrenergic receptor do cause a shift in the ED_{50} to lower concentrations. This shift is proportional to the degree of receptor activation by the amino acid change, which correlates very well with the fact that ligand-activated α_1 -adrenergic receptors show a higher affinity for the ligand (Kjelsberg *et al.*, 1992; Cotecchia *et al.*, 1990).

In conclusion, an amino acid substitution in the second transmembrane segment of the LH receptor has been identified in FMPP patients. The effect of this substitution on basal receptor activity is identical to the effects of amino acid substitutions in the sixth transmembrane segment. This suggests an important role for several segments of the transmembrane domain in receptor function.

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Chapter 6

Male Pseudohermaphroditism Due to a Homozygous Missense Mutation of the Luteinizing Hormone Receptor Gene

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Abstract

Leydig cell hypoplasia is a rare autosomal recessive condition that interferes with normal development of male external genitalia in 46,XY individuals. We have studied two Leydig cell hypoplasia patients (siblings born to consanguineous parents), and found these patients to be homozygous for a missense mutation (Ala593Pro) in the 6th transmembrane segment of the LH receptor gene. *In vitro* expression studies showed that this mutated receptor binds hCG with a normal K_d, but the ligand binding did not result in increased production of cAMP. We conclude that a homozygous LH receptor gene mutation underlies the syndrome of autosomal recessive congenital Leydig cell hypoplasia in this family. These results have implications for the understanding of the development of the male internal and external genitalia.

Introduction

The process that leads to the development of male sex characteristics is generally accepted to consist of three sequential steps (Jost, 1970). The first step is the establishment of male genetic sex by the XY chromosome pattern at conception. The second step involves the induction of male gonadal sex, that is testis formation, through the action of SRY (sex determining region of the Y chromosome). The third step consists of the development of internal and external male genitalia, a process which is basically controlled by two factors, testosterone and anti-Müllerian hormone (AMH), both produced by the fetal testis. The Sertoli cell product AMH prevents the formation of the Müllerian duct derivatives (fallopian tubes, uterus and the upper part of the vagina), whereas the development of the male internal and external genitalia is dependent on the Leydig cell product testosterone, which is converted to dihydrotestosterone in certain target tissues. Various genetic defects in this pathway have been reported, supporting the concept of sequential determination of male genetic, gonadal, and phenotypic sex (Nordqvist and Lovell-Badge, 1994). An important step in this differentiation cascade is the development of Leydig cells, which starts, apparently independent of stimulation by gonadotropins, at approximately the same time that the formation of the testicular cords occurs (Huhtaniemi, 1994), although the process still occurs in cases where testicular cords do not develop (Patsavoudi *et al.*, 1985). Leydig cell differentiation is crucial for sufficient production of androgens by the testis. Absence of Leydig cells or insufficient Leydig cell differentiation can occur as an autosomal recessive condition (Schwartz *et al.*, 1981; Martinez-Mora, 1991; Perez-Palacios *et al.*, 1981; Lee *et al.*, 1982; Toledo *et al.*, 1985; Toledo, 1992; El-Awady *et al.*, 1987; Saldanha *et al.*, 1987; Arnhold *et al.*, 1987). The phenotype of inherited Leydig cell hypoplasia ranges from extreme forms presenting as 46,XY females to milder forms in which males present with hypergonadotropic hypogonadism and a micropenis (Toledo *et al.*, 1985; Toledo, 1992). Testicular LH binding was decreased or absent in some studies, which could be either the cause or the consequence of hypoplasia of Leydig cells (Schwartz *et al.*, 1981; Martinez-Mora, 1991). Here, we report studies of the LH receptor gene in siblings born to consanguineous parents who have male pseudohermaphroditism due to Leydig cell hypoplasia.

Materials and methods

Patients

The patients are 2 siblings born to consanguineous first cousin parents. The patients originate from a small village in rural north/northeast Brazil (Piauí State). They were referred at ages 37 and 42, because of primary amenorrhea and lack of breast development. Both cases had a eunuchoid habitus, absence of breast tissue, and sparse axillary hair. The external genitalia were female with distinct urethral and vaginal orifices, and triangle-shaped pubic hair. Pelvic ultrasound examination showed no internal genitalia, except for the testes which were located in the inguinal region. A genitogram demonstrated only the vertical component of the urethra and a short blind ending vaginal pouch. The karyotype was 46,XY. Baseline testosterone levels were low (0.69 and 0.53 nmol/l respectively; normal 8.3-35.8 nmol/l). Testosterone precursors such as DHEA, 17 α -hydroxyprogesterone, progesterone, androstenedione, and estradiol were low compared to normal males. LH levels were elevated to 23.3 and 19.0 IU/l; normal 1-8.4 IU/l). FSH and inhibin levels were normal. A stimulation test with hCG (6000 IU i.m.) elicited no increase in testosterone levels. The LHRH test (100 μ g i.v.) elicited a LH hyper response, and normal FSH increase. An acute adrenal cortex stimulation test using 1-24 synthetic ACTH (Cortrosyn, Organon, Oss, the Netherlands) did not reveal defects in enzymatic steps leading to adrenal androgen synthesis. The patients underwent bilateral gonadectomy. Testes were removed with normal vasa deferens and epididymides. Histopathological examination revealed seminiferous tubules with thickened membranes, few germ cells and a normal number of Sertoli cells. The interstitial space contained vascular conjunctive stroma and fibroblasts with absence of Leydig cells.

Mutation analysis

Genomic DNA was isolated from peripheral blood as described (Miller *et al.*, 1988). Because other family members live approximately 4000 kilometres from São Paulo, only the index cases were studied.

DNA fragments were amplified with the polymerase chain reaction (PCR) and analysed for single strand conformation polymorphisms (SSCP) according to Kremer (1993) with the following adaptations for primer set 1 (see below): 1mM MgCl₂ in the PCR and an annealing

temperature of 58 °C. Two different sets of primers were used in the PCR: (1a) 5'-CGATTTCACCTGCATGGC-3' (nucleotides 1731-1748), (1b) 5'-CCCGACGTTT-ACAGCAGCC-3' (nucleotides 1924-1942), (2a) 5'-TATCCCATCAATTCTTGTGCC-3' (nucleotides 1834-1854), (2b) 5'-GGATTGAGAAGGCTTATTTGATCC-3' (nucleotides 2005-2028). Nucleotide numbers are according to Minegishi *et al.* (1990).

For sequencing, the DNA fragments were separated on a 1.5% agarose gel (Low melting Point, BRL Life technologies, Inc) and purified with the gelase system (Epicentre Technologies) according to the "fast protocol" given by the manufacturer. About 50 fmol of the purified DNA fragments and the 1a and 1b primers were used for sequencing with the cycle sequencing kit of BRL (BRL Life technologies).

In vitro expression

The wild type human LH receptor expression plasmid (pSG5-hLHR) was constructed by cloning the human LH receptor cDNA (nucleotides -3 to 2374 relative to the translation start site) into the EcoRI site of pSG5, an expression vector that contains the SV40 large T early promoter, intron II of the rabbit β -globin gene, and an SV40 polyadenylation signal (Green *et al.*, 1988). The protein encoded by this cDNA differs from the published human LH receptor (Minegishi *et al.*, 1993) by the insertion of two amino acids (Leu and Gln) at positions 20 and 21 (E. Milgrom, unpublished observations). The Ala593Pro mutation was constructed in pSG5-hLHR by site-directed mutagenesis, using PCR DNA amplification techniques (Higuchi *et al.*, 1988), yielding pSG5-hLHR4. Mutagenesis was confirmed by sequence analysis, and revealed an additional nucleotide change (C to A) at position 1983 in the pSG5-hLHR4 vector. This change does not alter the amino acid encoded by this codon. Human embryonic kidney 293 (HEK293) cells were transiently transfected as described before (Chen and Okayama, 1987). Forty-eight hours after transfection [125 I]-hCG (Dupont, Wilmington, DE, USA) binding to partially purified membranes was determined according to Ketelslegers and Catt (1978). For the estimation of hCG-dependent cAMP production, HEK293 cells were co-transfected in 12 well tissue culture plates (Costar, Cambridge, MA, USA) with a luciferase expression plasmid pRSVluc (Van Dijk *et al.*, 1991) and pSG5, pSG5-hLHR or pSG5-hLHR4. Forty-eight hours after transfection cells were incubated for two hours with culture medium containing 0.1% BSA and 0.2 mM isobutylmethylxanthine (Sigma, St Louis, MO, USA) and different concentrations of hCG (urinary

hCG; Organon International, Oss, The Netherlands). Medium was collected and cAMP production determined as described before (Themmen *et al.*, 1991; Stoof *et al.*, 1987). The remaining cells were lysed, and luciferase activity was determined (Blok *et al.*, 1992). The final cAMP production in the transfected cells was determined by subtraction by the basal cAMP production in pSG5-transfected cells, and subsequent correction for transfection efficiency using the luciferase activities of the same wells.

Results

Case report

Our study examined two 46,XY siblings who presented with female external genitalia, primary amenorrhea, and lack of breast development. Their parents are first cousins, and there are 14 additional siblings (Fig. 6.1). Both cases had a short blind ending vagina, without uterus or fallopian tubes. Serum levels of testosterone and testosterone precursors were abnormally low, and did not respond to stimulation by hCG. Basal levels of LH were markedly increased, but follicle-stimulating hormone (FSH) was within the normal range. The gonads were removed, and upon histological examination found to be testes with normal Sertoli cells, but no mature Leydig cells (Fig. 6.2).

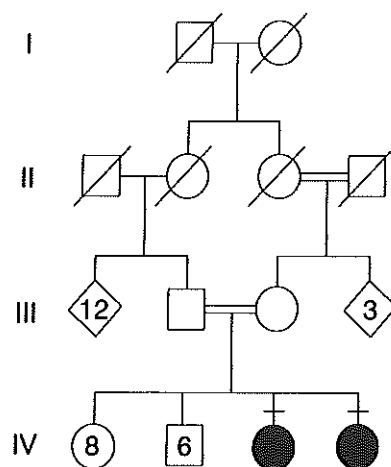


Fig. 6.1. Pedigree of two siblings with male pseudohermaphroditism and Leydig cell hypoplasia.

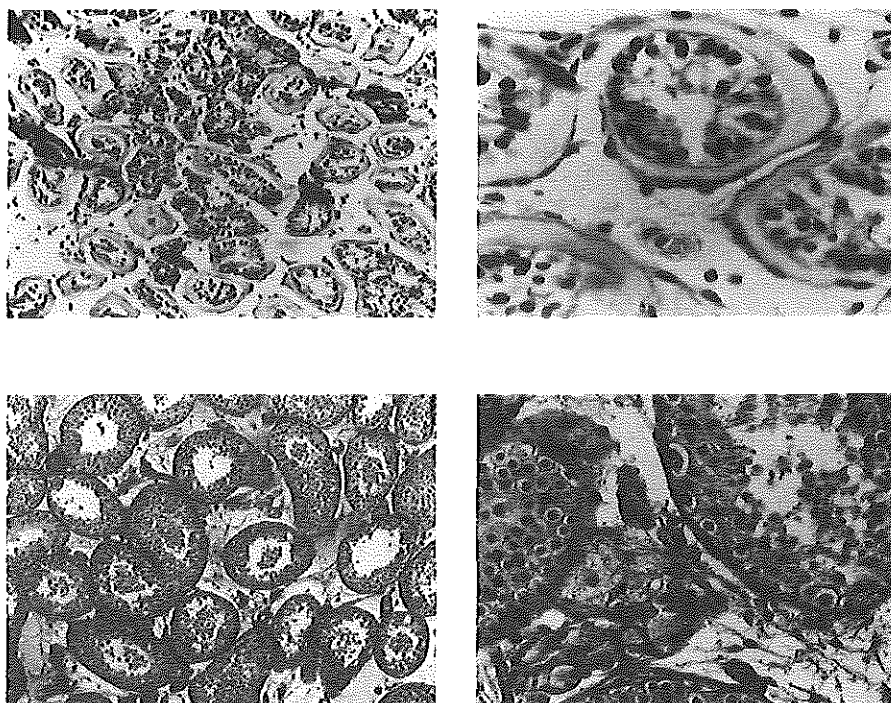


Fig. 6.2. Light photomicrographs of seminiferous tubules and interstitium.

Top left and right: seminiferous tubules and interstitium of one of the patients show hyalinization of the basal membrane and normal peritubular myoid cells. In the interstitium few Leydig cells, with immature characteristics, are found. The seminiferous epithelium contains Sertoli cells, and almost no germ cells. Bottom left and right: seminiferous tubules and interstitium of control testis with fully developed interstitium and spermatogenic epithelium. (Left: 100X; Right: 400X; H/E staining).

Detection of a homozygous mutation in the LH receptor gene

The LH receptor gene of the two patients with male pseudohermaphroditism was studied for the presence of mutations by SSCP analysis. Initially, exon 11 which contains the transmembrane and cytoplasmic domains was studied with two sets of primers (see Materials and Methods). The fragment that was amplified with primer set 1 showed abnormal migration. This fragment comprises the third extracellular loop and the seventh transmembrane segment, as well as parts of the sixth transmembrane segment and the intracellular tail. This part of the LH receptor gene was then sequenced in the two affected siblings and in two unaffected controls with primers 1a and 1b as sequencing primers. A

transversion of G to C was detected at position 1787 in the patients (Fig. 6.3A), changing codon 593 from GCC for alanine to CCC for proline in the sixth transmembrane segment (Fig. 6.3B). The normal sequence could not be detected, indicating homozygosity of the mutated allele. The presence of two copies of the LH receptor gene was confirmed by dosage analysis (data not shown).

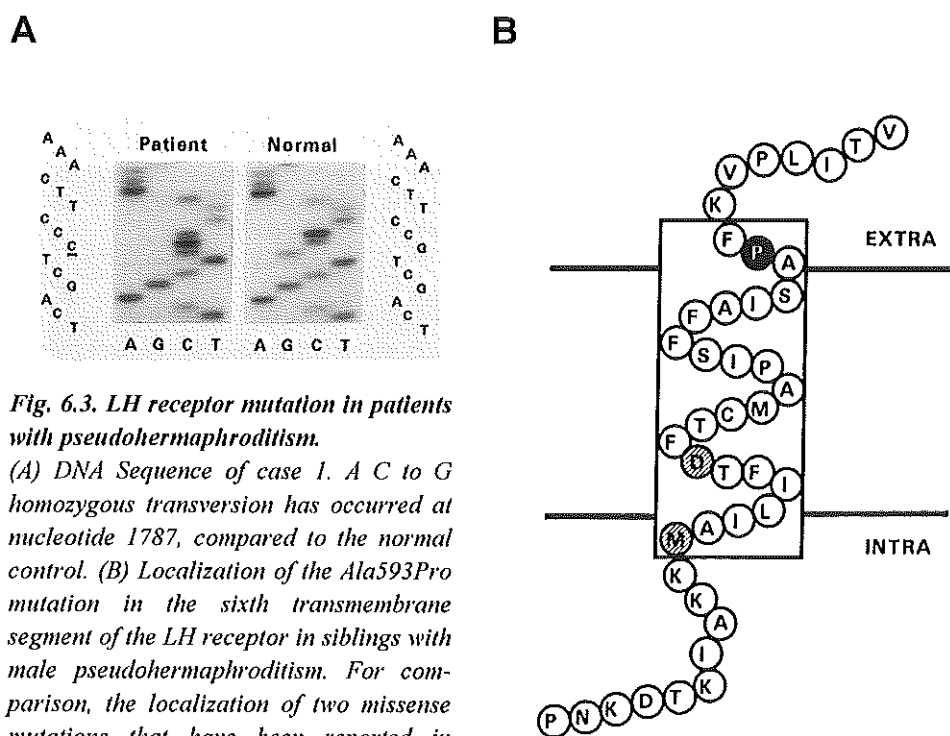


Fig. 6.3. LH receptor mutation in patients with pseudohermaphroditism.

(A) DNA Sequence of case 1. A C to G homozygous transversion has occurred at nucleotide 1787, compared to the normal control. (B) Localization of the Ala593Pro mutation in the sixth transmembrane segment of the LH receptor in siblings with male pseudohermaphroditism. For comparison, the localization of two missense mutations that have been reported in families with male-limited precocious puberty (Kremer et al., 1993; Shenker et al., 1993) are indicated.

The Ala593Pro mutation inactivates signal transduction

The identified Ala593Pro mutation was constructed *in vitro*, and the resulting mutant LH receptor cDNA was transiently expressed in human embryonic kidney 293 cells. High affinity (mean $K_d = 0.8 \pm 0.15$ nM; $n=2$) binding of [125 I]-hCG to partially purified membrane preparations was not different from binding to membrane preparations from cells

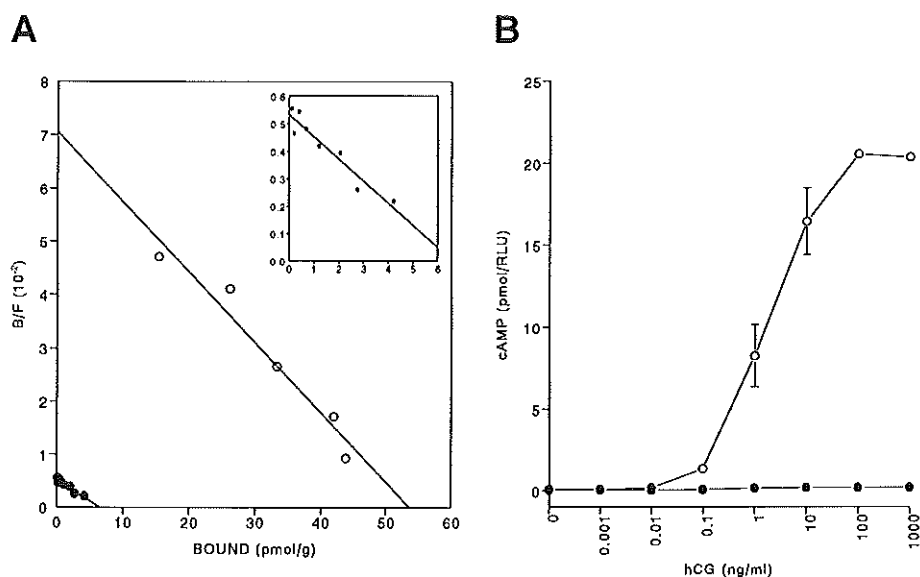


Fig. 6.4. In vitro studies of mutant and wild-type LH receptor gene constructs.

(A) Scatchard plot of the mutant receptor (filled circles; enlarged in inset) shows reduced maximal hormone binding but normal affinity, compared to the wild-type receptor (open circles). (B) Human CG-dependent cAMP production in wild-type receptor (open circles) and mutant receptor (closed circles) expressing HEK293 cells. The Ala593Pro mutation (LCH) completely abolishes hCG-dependent cAMP production.

transfected with the wild type LH receptor cDNA (mean $K_d = 0.5 \pm 0.4$ nM; $n=2$) (Fig. 6.4). The difference in the values of B_{max} of the different membrane preparations is partly due to experimental variation in transfection efficiency (five-fold less for the mutant LH receptor construct), and partly to reduced expression of the mutant receptor at the cell membrane. The number of mutant or wild-type receptors expressed per transfected cell varied between 1000 and 3000, which is similar to the figure found *in vivo* (Warren *et al.*, 1984). In contrast, when hCG-induced cAMP production was determined, no hormonal effect was detected for the mutated LH receptor, even at very high (1000 ng/ml) hCG concentrations (Fig. 6.4). These results indicate that the missense Ala593Pro mutation completely abolishes signal transduction, probably at the level of coupling to the stimulatory GTP-binding protein G_s , although changes in post-synthesis receptor transport to the plasma membrane cannot be excluded. Ligand binding is less likely to be affected because this function resides in the N-terminal half of the LH receptor molecule (Braun *et al.*, 1991).

Discussion

A homozygous missense mutation in the LH receptor gene underlies the syndrome of male pseudohermaphroditism due to Leydig cell hypoplasia in this family. This mutation substitutes a proline for an alanine residue in the 6th transmembrane segment of the receptor. When the mutant LH receptor gene was transiently expressed in HEK293 cells, hormone binding to the receptor was present albeit at lower maximal capacity. No increase in cAMP was observed on stimulation with hCG, even at very high hCG concentrations. Therefore, this mutation precludes the normal increase in cAMP in response to LH/hCG, which renders the mutant receptor non-functional.

The finding of an LH receptor gene defect as a cause of inherited Leydig cell hypoplasia is consistent with previous studies of other patients which have indicated reduced LH binding capacity of testicular tissue (Schwartz *et al.*, 1981; Martinez-Mora, 1991) and absence of a testosterone response to LH (Schwartz *et al.*, 1981; Martinez-Mora, 1991, Perez-Palacios *et al.*, 1981; Lee *et al.*, 1982; Toledo *et al.*, 1985; Toledo, 1992; El-Awady *et al.*, 1987; Saldanha *et al.*, 1987; Arnhold *et al.*, 1987). It may be anticipated that in other families with autosomal recessive forms of male pseudohermaphroditism a wide array of mutations of the LH receptor will be found. Such mutations could conceivably affect LH binding, G protein activation, post-translational modification or post-synthesis transport, or a combination of these processes. In addition, large LH receptor gene deletions without any LH receptor mRNA and protein expression cannot be excluded.

It is likely that milder forms of LH receptor defects exist, in which sufficient residual activity is present to allow partial masculinization of external genitalia. In fact, patients have already been described in whom Leydig cell hypoplasia was associated with hypergonadotropic hypogonadism, and micropenis, but not hypospadias (Toledo *et al.*, 1985; Toledo, 1992).

The alanine to proline mutation of codon 593 that is reported here abolishes LH receptor function in vitro (Fig. 6.4). This is very different from the effect of missense mutations of the LH receptor gene that have been found in patients with autosomal dominant LH independent male precocious puberty (Fig 6.3B; Kremer *et al.*, 1993; Shenker *et al.*, 1993; Chapter 5), although they also are located near the sixth transmembrane segment. These latter mutations act in a dominant fashion by constitutively activating cAMP production

(Shenker *et al.*, 1993; Chapter 5), and appear to be subtle (Met571Ile and Asp578Gly). Possibly, they result in a conformational change that facilitates ligand-independent coupling to the G-protein. The Ala593Pro mutation described here in patients with Leydig cell hypoplasia possibly introduces a more drastic conformational change in the receptor molecule caused by the constrained angle conferred by the proline residue. Such mutations are more likely to render the receptor inactive.

Many studies indicate that undifferentiated Leydig cells require LH for proliferation and differentiation. In humans, fetal Leydig cell number and testosterone production correlate with plasma hCG levels (Clements *et al.*, 1976; Huhtaniemi *et al.*, 1977; Huhtaniemi and Pelliniemi, 1992). This is consistent with experimental studies in rats that have shown that full-length LH receptor mRNA is expressed in testis from day 16.5 of gestation onwards, suggesting an active role for LH/hCG-dependent testosterone production in the determination of male external genitalia (Zhang *et al.*, 1994). After specific removal of differentiated Leydig cells from adult rat testes, immature Leydig cells reappear more quickly if LH is present (Molenaar *et al.*, 1986). Furthermore, LH can induce morphological differentiation of immature to mature Leydig cells in neonatal rats (Kuopio *et al.*, 1989), and this differentiation is paralleled by an increase in the activity of the enzymes that are responsible for testicular steroidogenesis (Murono *et al.*, 1994).

The initiation of androgen synthesis by Leydig cells early in fetal life may be independent of LH or hCG as indicated by data from rats and rabbits (Huhtaniemi, 1994; George *et al.*, 1978). Our findings may support this notion, because testes with associated epididymis and vas deferens were found in both patients. These Wolffian duct-derived structures can only have been formed in the presence of Leydig cell androgens at some point in fetal development. Our findings further demonstrate that at a later fetal stage the absence of a functional LH receptor interferes with Leydig cell proliferation and maturation. In patients with Leydig cell hypoplasia, the testes contain normal Sertoli cells, with no mature Leydig cells. Sometimes, small clusters of immature Leydig cells are present (Perez-Palacios *et al.*, 1981). The histological findings in Leydig cell hypoplasia can now be interpreted as reflecting lack of stimulation through the LH receptor.

No abnormal female sex characteristics have been noted in sisters of patients with this form of male pseudohermaphroditism (Saldanha *et al.*, 1987), which is consistent with experimental results that indicate absence of a functional ovarian LH receptor until after

birth (Zhang *et al.*, 1994). However, LH receptor function is required for a normal female menstrual cycle. Hence, the presence of primary amenorrhea in a 46,XX sister of the patients described above should be of particular interest. As this patient lives approximately 4000 kilometres from our center, we have not yet been able to contact the patient and obtain a blood sample. Further studies should reveal if she is indeed homozygous for the same LH receptor missense mutation. Primary amenorrhea with ovarian resistance to luteinizing hormone might conceivably be the female counterpart to the pattern of male pseudohermaphroditism studied here. Amenorrhea has been noted in another sister of a patient with pseudohermaphroditism due to Leydig cell hypoplasia (Arnhold *et al.*, 1987).

In conclusion, the finding of an LH receptor defect in male pseudohermaphroditism with testicular Leydig cell hypoplasia provides a biological basis for this disorder. These data support a crucial role for the LH receptor in Leydig cell differentiation, and hence in the regulation of embryonal production of testosterone by the male gonad.

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Chapter 7

General Discussion

Introduction

The cloning of the gonadotropin receptors some seven years ago (Loosfelt *et al.*, 1989; McFarland *et al.*, 1989; Parmentier *et al.*, 1989; Sprengel *et al.*, 1990) resulted in a major advance of research on gonadotropin action at the molecular level. In this thesis, molecular studies on gonadotropin function and expression are described in relation to regulation of gonadal function and clinical data. Alternative splicing of FSH receptor pre-mRNA (Chapter 3), and homologous down-regulation of FSH receptor mRNA expression (Chapter 4) are regulatory processes at the mRNA level. Mutational analysis of the LH receptor gene (Chapters 5 and 6) provides insight into the mechanism of LH receptor function. Furthermore, these studies offer the opportunity to learn more about the role of LH/hCG in male and female sex differentiation. This chapter aims to integrate the results that have been described in the previous chapters with data from the literature, and to evaluate the importance of the regulation of gonadotropin receptor mRNA expression and other aspects of gonadotropin receptor function. Furthermore, discussion of the phenotypes of patients with FSH or LH insensitivity gives new information about the key role of the gonadotropins in sex differentiation, puberty, and adult gonadal functions.

Regulation of gonadotropin receptor expression

Alternative splicing of gonadotropin receptor pre-mRNAs

In search for possible functions of alternative splicing of gonadotropin receptor pre-mRNAs, many studies addressed regulation of alternative splicing (e.g. LaPolt *et al.*, 1991; Vihko *et al.*, 1992; LaPolt *et al.*, 1992; Sokka *et al.*, 1992; O'Shaughnessy and Dudley, 1993; Zhang *et al.*, 1994; O'Shaughnessy *et al.*, 1994; Veldhuizen-Tsoerkan *et al.*, 1994; Tena-Sempere *et al.*, 1994; Rannikki *et al.*, 1995; Chapter 3). For the FSH receptor, one situation has been described, where alternative transcripts and full-length receptor mRNA transcript are differentially expressed. In both ovary and testis, the expression of full-length receptor mRNA transcript is preceded, during gonadal development, by expression of alternative transcripts (O'Shaughnessy *et al.*, 1994; Rannikki *et al.*, 1995). In the fetal ovary, full-length receptor mRNA expression is observed approximately 2 days after the onset of FSH receptor

gene expression. This suggests a developmental switch in FSH receptor gene expression, which is not achieved at the level of the FSH receptor gene promoter, but by an on/off switch of alternative splicing. For expression of full-length LH receptor mRNA in the perinatal testis, a similar delay of 2 days is observed (Zhang *et al.*, 1994), but in the ovary, expression of full-length LH receptor mRNA is delayed for at least 12 days after the first appearance of mRNA species encoding truncated receptors (Sokka *et al.*, 1992). Ovarian expression of the splice-variants starts 4 days before birth, whereas expression of the full-length LH receptor mRNA is first detected 7 days after birth.

LH receptor gene regulation can also be studied in a regenerating Leydig cell model. In this model, adult rats are treated with the alkylating agent ethane dimethyl sulphonate (EDS) that quite specifically destroys Leydig cells. The decrease in Leydig cell number is accompanied by a decrease in LH receptor mRNA expression, with the exception of one transcript that encodes a truncated LH receptor protein (Veldhuizen-Tsoerkan *et al.*, 1994; Tena-Sempere *et al.*, 1994). The cells that remain present after EDS treatment are precursor Leydig cells, indicating that these cells may constitutively express a truncated form of the LH receptor, and that Leydig cell maturation may be preceded or followed by a switch to expression of functional, full-length LH receptor molecules. However, differentiation of Leydig cell precursors into mature Leydig cells is dependent on the presence of LH (Teerds *et al.*, 1989), which implies that full-length LH receptor expression must be present in the precursor Leydig cells, albeit at a very low level (Veldhuizen-Tsoerkan *et al.*, 1994; Tena-Sempere *et al.*, 1994).

Although these studies strongly suggest that alternative splicing of gonadotropin receptor pre-mRNAs is a mechanism involved in regulation of full-length receptor expression, it is interesting to note that in adult tissues there is a constant, very low expression of splice variants together with the full-length receptor encoding transcript (Chapter 3). This may suggest a physiological role for truncated receptor proteins encoded by alternative transcripts. Effects of the truncated receptor proteins on signal transduction are marginal (VuHai-LuuThi *et al.*, 1992) or absent (Chapter 3), but a possible role in transport of the hormone from the blood to the interstitial space may be proposed. Ghinea *et al.* (1994) showed, using immuno-gold labelling, that transport of hCG from the circulation to Leydig cells occurs across the endothelial cells of the blood vessels. Specific uptake of hCG from the blood might be conferred by truncated and/or full-length LH receptors, which are present

in the plasma membrane of the endothelial cells. After binding, hormone-receptor complexes are internalized in coated vesicles and trafficked to the interstitial side of the endothelial cells, where the vesicles merge with the plasma membrane and the hormone is released into the interstitial space of the testis. Truncated LH receptor proteins may be involved in this transport process, although the plasma membrane location of the transporter protein suggests that specific uptake and transport of LH/hCG is achieved by the full-length LH receptor molecule, and not by truncated receptor proteins (Ghinea *et al.*, 1994; Ghinea and Milgrom, 1995).

It seems probable that developmental switches in full-length gonadotropin receptor mRNA expression, as described above, may represent a mechanism to regulate developmental expression of the gonadotropin receptors. There may be no functional role for the truncated gonadotropin receptors as such, and the low and non-regulated expression of splice variants in adult tissues may be a result of incomplete inhibition of alternative splicing at later developmental stages.

Homologous down-regulation of gonadotropin receptor mRNA

In granulosa cells, the expression levels of both LH and FSH receptor mRNAs are down-regulated after the pre-ovulatory LH peak (LaPolt *et al.*, 1990; LaPolt *et al.*, 1992; Camp *et al.*, 1991). Within 24 hours after this peak, the mRNA levels gradually decrease, which involves, at least for the LH receptor, enhanced breakdown of mRNA (Lu *et al.*, 1993). Also, in primary Leydig cell cultures LH-induced LH receptor mRNA down-regulation does involve enhanced receptor mRNA breakdown (Chuzel *et al.*, 1995). However, in the MA-10 mouse tumor Leydig cell line homologous LH receptor mRNA down-regulation does not involve mRNA destabilization (Nelson and Ascoli, 1992). The absence of such a down-regulation mechanism in MA-10 cells may be due to altered characteristics of these tumor cells.

Regulation of gene expression by means of regulation of mRNA stability is not unique for G protein-coupled receptors. Well studied examples of regulated mRNA stability are vitellogenin mRNA stabilization by estrogens (Brock and Shapiro, 1983), iron-dependent destabilization of transferrin receptor mRNA (Harford and Klausner, 1990), and granulocyte-monocyte colony-stimulating factor (GM-CSF) mRNA destabilization in lectin-stimulated T cells (Shaw and Kamen, 1986). In GM-CSF mRNA an A/U-rich sequence has been

identified in the 3'-untranslated region, which is also present in the 3'-untranslated regions of other mRNAs, such as *c-fos* and *c-myc* (Kabnick and Housman, 1988; Jones and Cole, 1987). An AUUUA motif within this sequence binds to a 32 kD protein that appeared to be involved in the destabilization of these mRNAs (Vakalopoulou *et al.*, 1991). Also for the β_2 -adrenergic receptor, a protein that binds to an AUUUA motif in the receptor encoding mRNA is involved in homologous down-regulation of this mRNA (Port *et al.*, 1992; Huang *et al.*, 1993; see Chapter 2).

The mechanism of homologous down-regulation of LH receptor mRNA in cultured Leydig cells is dependent on both transcription and translation (Chuzel *et al.*, 1995), whereas homologous FSH receptor mRNA down-regulation in Sertoli cells is only dependent on transcription (Themmen *et al.*, 1991; Chapter 4). Furthermore, FSH receptor down-regulation in Sertoli cells is a very rapid process. Within 4 hours, the mRNA level is decreased to approximately 10% of the initial level (Themmen *et al.*, 1991), whereas for the LH receptor in Leydig cells this process takes approximately 12 hours (Chuzel *et al.*, 1995). Also for the β_2 -adrenergic receptor, homologous down-regulation is a relatively slow process, where mRNA levels are reduced to approximately 50% of the initial level after 18 hours of exposure of DDT₁ MF-2 cells to a maximal dose of agonist (Haddock and Malbon, 1988). As for LH receptor mRNA, this down-regulation process is dependent on both transcription and translation. Therefore, the mechanism of down-regulation of FSH receptor mRNA in Sertoli cells seems to be quite unique, in that it is a rapid mechanism that involves an RNA factor (RNA-RNA interaction) rather than an RNA-binding protein. In Sertoli cells, this process acts in symphony with receptor internalization (Shimizu and Kawashima, 1989; Themmen *et al.*, 1991), and possibly, the processes of receptor G protein-uncoupling and inhibition of receptor gene transcription also take part in the cellular feedback system that prevents over-stimulation.

It has been observed in spermatogenic stage-synchronized rats that the FSH receptor mRNA level is down-regulated at Stages IV-X of the spermatogenic cycle (Heckert and Griswold, 1991), but this cyclic pattern represents relatively slow changes, which occur in a time-frame of several days rather than hours (Sharpe, 1994). Therefore, it is unlikely that rapid down-regulation processes cooperate in Sertoli cells, to obtain the cyclic pattern of the spermatogenic epithelium. The possible physiological role of the conspicuous and fast mechanism of FSH receptor mRNA destabilization in Sertoli cells remains intriguing.

Gonadotropin receptor function

Gonadotropin receptor activation

Constitutively activating mutations have been found not only for the LH receptor, but also for other G protein-coupled receptors. Activating germ-line mutations in the TSH receptor lead to congenital hyperthyroidism (Duprez *et al.*, 1994; Kopp *et al.*, 1995), whereas somatic mutations have been found in autonomously hyperfunctioning thyroid adenomas (Parma *et al.*, 1993; Parma *et al.*, 1995; Paschke *et al.*, 1994; Porcellini *et al.*, 1994; Russo *et al.*, 1995). Activating germ-line mutations in opsin/rhodopsin lead to either night blindness or retinitis pigmentosa (Robinson *et al.*, 1992; Dryja *et al.*, 1993). Similarly, a constitutively activated receptor for parathyroid hormone-parathyroid hormone related peptide (PTH-PTHrP) causes Jansen-type metaphyseal chondrodysplasia (Schipani *et al.*, 1995). An interesting activating mutation has been described for the Ca^{2+} -sensing receptor in association with autosomal dominant hypocalcaemia (Pollak *et al.*, 1994). The mutation in this receptor leads to increased receptor activity at low Ca^{2+} concentrations, whereas the receptor activity in the absence of Ca^{2+} was normal. The mutation appears to confer a ligand-hypersensitive phenotype to the receptor, probably by increasing the affinity of the receptor for Ca^{2+} (Pollak *et al.*, 1994). Furthermore, in mice, constitutive activation of the melanocyte-stimulating hormone (MSH) receptor leads to increased coat pigmentation (Robbins *et al.*, 1993).

Focussing on the glycoprotein hormone receptors, a compilation of all activating amino acid substitutions in the receptors for LH and TSH described so far (Table 7.1) shows a clustering of substitutions in the sixth transmembrane segment and the third intracellular loop. *In vitro* mutagenesis studies with adrenergic receptors have shown the importance of this region for G protein coupling and activation (see Chapter 2). Clustering of mutations in this region support the hypothesis that these mutations induce conformational changes, which result in the release of the third intracellular loop from an inactive conformation. Such an inactive conformation was already postulated on basis of G protein activation experiments, using synthetic peptides that were derived from this loop (Cheung *et al.*, 1991; Okamoto *et al.*, 1991). These peptides basically have a free conformation and were found to be able to activate G proteins in a very efficient manner. This indicated that unstimulated receptors are constrained in a conformation, such that the G protein activation region of the

Table 7.1. Activating mutations in the receptors for LH (left panel) and TSH (right panel), detected so far. TM: transmembrane segment; ICL: intracellular loop; ECL: extracellular loop.

LH receptor			TSH receptor		
Mutation	Location	Ref.	Mutation	Location	Ref.
Met398Thr	TM II	<i>a</i>	ILe486Met/Phe	ECL 1	<i>k</i>
Ile542Leu	TM V	<i>b</i>	Ile568Thr	ECL 2	<i>l</i>
Asp564Gly	ICL 3	<i>c</i>	Val509Ala	TM III	<i>m</i>
Ala568Val	ICL 3	<i>d</i>	Asp619Gly	ICL 3	<i>n</i>
Met571Ile	TM VI	<i>e</i>	Ala623Ile/Ser/Val	ICL 3	<i>o</i>
Ala572Val	TM VI	<i>f</i>	Phe631Leu/Cys	TM VI	<i>p</i>
Ile575Leu	TM VI	<i>g</i>	Thr632Ile	TM VI	<i>q</i>
Thr577Ile	TM VI	<i>h</i>	Asp633Glu/Tyr	TM VI	<i>r</i>
Asp578Gly/Tyr	TM VI	<i>i</i>	Cys672Tyr	TM VII	<i>s</i>
Cys581Arg	TM VI	<i>j</i>			

a: Chapter 5; b: Laue et al., 1995; c: Laue et al., 1995; d: Latronico et al., 1995; e: Kremer et al., 1993; Kosugi et al., 1995; f: Yano et al., 1995; g: H. Kremer, unpublished observation; h: Kosugi et al., 1995; i: Shenker et al., 1993; Yano et al., 1994; Laue et al., 1995; Mueller et al., 1995; j: Laue et al., 1995; k: Parma et al., 1995; l: Parma et al., 1995; m: Duprez et al., 1994; n: Parma et al., 1993; o: Parma et al., 1993; Paschke et al., 1994; Russo et al., 1995; p: Porcellini et al., 1994; Kopp et al., 1995; q: Paschke et al., 1994; Porcellini et al., 1994; r: Porcellini et al., 1994; s: Duprez et al., 1994.

third intracellular loop is hidden from the G protein. Probably the entire transmembrane domain with the associated intra- and extracellular loops is involved in this constraint. This suggestion is supported by the observation that activating LH and TSH receptor mutations have been found in transmembrane domain regions that are not directly connected to the third intracellular loop. For the LH receptor these mutations were located in the second and

fifth transmembrane segments, whereas for the TSH receptor mutations were found in the third and seventh transmembrane segments, and in the first and second extracellular loops (Table 7.1). In the absence of a molecular model of a receptor-G protein complex, it is difficult to speculate on the nature of the molecular consequences of the activating amino acid substitutions. Examination of the amino acid changes found in FMPP patients, reveals that some alterations involve a major change in amino acid type, such as D578G, D578Y, or C581R. However, other amino acid changes, such as A568V or I575L, do not result in a major change in the properties of the amino acid residues. It seems that any change of one of several amino acid residues may result in a constitutively activated receptor. The amino acid substitutions in FMPP patients are obvious candidates in future investigations on the structure-function relationship of the LH receptor during activation by LH or hCG.

Basal receptor activity and B_{max}

In Chapter 5, the basal receptor activity of wild type and mutant LH receptors were determined in transfected HEK293 cells. A major problem in such transfection studies is that basal cAMP production is corrected for the expression level of an internal control, i.e. co-transfection with a pRSV-Luc luciferase expression plasmid. However, the ensuing luciferase activity only corrects for differences in transfection efficiency. A better correction for the determination of receptor activities would be the number of receptors expressed in the plasma membrane (B_{max}). Therefore, basal and maximal cAMP production levels of the wild type and mutant receptors were determined and corrected for B_{max} values (Fig. 7.1). Scatchard analyses showed that the mutations tested did not affect binding affinity, which was comparable for wild type and mutant LH receptors (data not shown; K_d values ranged from 0.2 to 0.9 nM). Maximally stimulated receptor activities of the receptors carrying a single mutation (LHR1-3) were found to be equal to the maximal activity of the wild type receptor (Fig. 7.1), which indicates that the function of the mutant receptors is not impaired with respect to hormone-induced receptor activation. Furthermore, basal receptor activity of the mutant receptors was more clearly elevated when the data were corrected for the measured number of binding sites, and this activity ranges from 40 to 80-fold higher than the basal level of the wild type receptor (Fig. 7.1).

Interestingly, correction of receptor activity for the number of receptor molecules expressed at the cell surface, resulted in receptor activity of the double mutated receptors

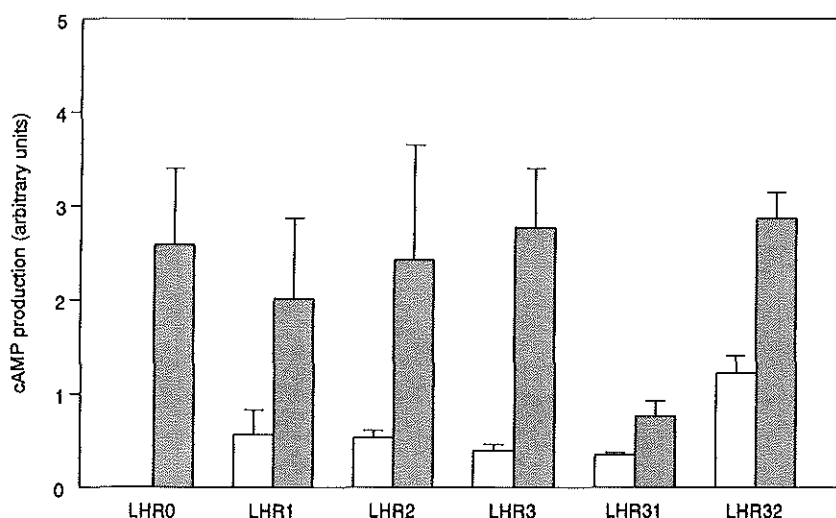


Fig. 7.1. Determination of basal (open bars) and maximally stimulated (closed bars) LH receptor activities.

HEK293 cells were transfected with LH receptor expression plasmids, as described in Chapter 5. In separate experiments, basal and maximal (in the presence of 1000 ng/ml hCG) cAMP production, luciferase activity of the internal control, and Scatchard data (K_d and B_{max}) were determined. Cyclic AMP production was corrected for the B_{max} value. K_d values were in the same range (0.2-0.9 nM). Single amino acid substitutions: wild type (LHR0); Met571Ile (LHR1); Asp578Gly (LHR2) Met398Thr (LHR3). Double amino acid substitutions: Met398Thr & Met571Ile (LHR31); Met398Thr & Asp578Gly (LHR32).

(LHR31 and LHR32) different from that presented in Fig. 5.2 B (Chapter 5). Hence, such a correction also leads to a different conclusion. Double mutant LHR32 showed the same maximal activity as the wild type and single mutant receptors, whereas the basal activity of LHR32 was higher than that of the single mutants. In the LHR32 receptor, the two amino acid substitutions might exert additive effects, resulting in a 125-fold elevation of basal activity. Furthermore, the hormone was still able to induce an additional change in the receptor, producing a maximal activity level that was equal to the maximal level observed for the wild type receptor. In contrast, double mutant LHR31 had a basal activity level that was equal to the single mutants, and the maximally stimulated activity remained lower than the maximal activity of the wild type receptor. The net effect of the amino acid substitutions in LHR31 may result in such a conformation, that a partially elevated basal receptor activity is obtained, whereas the hormone is unable to induce any additional conformational change.

Determination of Bmax values revealed another interesting phenomenon. The elevated basal receptor activity of the single mutated receptors showed an inverse correlation with the number of binding sites. The Bmax value of the wild type receptor (80 pM) is at least 2-fold higher than the values for the mutant receptors (15-40 pM). Transfection efficiency, however, was not different (data not shown). The same inverse correlation has been observed for mutant TSH receptors (G. Vassart, pers. commun.). An explanation of this phenomenon may be found in receptor internalization. For the β_2 -adrenergic receptor, a specific kinase (β -ARK) phosphorylates the activated receptor, which is then bound by β -arrestin (Hausdorff *et al.*, 1990). Although the primary function of β -arrestin seems to be uncoupling of the receptor and G protein (Lohse *et al.*, 1992), there are indications that the signal for receptor internalization is also mediated by β -arrestin (Ferguson *et al.*, 1996). The constitutive active LH and TSH receptors may be recognized as (partially) activated receptors by a β -ARK/ β -arrestin-like mechanism, that operates in a non-specific manner and facilitates internalization of these receptors. Another possibility is that the amino acid substitutions affect transport of the receptor molecule to the plasma membrane. This possibility is supported by the inactive LH receptor found in Leydig cell hypoplasia patients (Chapter 6). No coupling to adenylyl cyclase was observed for this receptor, whereas the Bmax value was 300-fold lower than the Bmax value observed for the wild type receptor. Possibly, a drastically altered conformation of this receptor caused by substitution of a valine residue for a proline residue in the sixth transmembrane segment may result in an inefficient transport to the plasma membrane. With regard to this inactive LH receptor mutant, the question can be raised whether the mutation only affects transport to the membrane, and whether the absent cAMP production is caused by the low receptor number. To address this question, very low numbers of wild type LH receptor were transiently expressed in HEK293 cells, and it was investigated whether these low receptor numbers were able to induce production of a detectable amount of cAMP. Ten-fold dilutions of wild type LH receptor expression plasmid were transfected, which resulted in a linear decrease of receptor number expressed at the plasma membrane (data not shown). However, transfection of cells with 1000-fold less wild type LH receptor expression plasmid still resulted in measurable cAMP production (data not shown). It is concluded that the LH receptor mutation found in Leydig cell hypoplasia patients (Chapter 6) renders the receptor inactive.

The physiological importance of gonadotropins: new data

Recently, an inactivating amino acid substitution of the FSH receptor has been identified in patients with primary amenorrhea (Aittomaki *et al.*, 1995). The substitution is located in the extracellular domain of the receptor molecule, in a region of five amino acids that is identical in the three glycoprotein hormone receptors and contains a consensus asparagine-linked glycosylation site. Although binding affinity is not affected, signal transduction by this receptor appeared to be completely abolished (Aittomaki *et al.*, 1995). Primary amenorrhea was also observed in 46,XX females that are homozygous for the inactivating LH receptor mutation found in Leydig cell hypoplasia patients (Toledo *et al.*, 1996). In both cases, the insensitivity for the respective gonadotropins appeared to be the cause of ovarian failure. These observations emphasize the requirement of both LH and FSH in female reproduction, whereas the gonadotropins are not important for the development of primary female sex organs. In the case of the patient with LH receptor deficiency, although a small uterus and decreased bone mass were found, the development of secondary sex characteristics was complete. The low estradiol level may have been produced through peripheral and ovarian conversion of adrenal androgens. This low estrogen level is the cause of the small uterus and the decreased bone mass, but is sufficient to induce full development of secondary sex characteristics (Toledo *et al.*, 1996). On the other hand, in females with FSH-insensitivity, partial development of secondary sex characteristics was observed (Aittomaki *et al.*, 1995). In this case, conversion of adrenal androgens can only occur in the periphery, which is not sufficient to induce full development of secondary sex organs.

Another interesting observation in the study on the inactivating FSH receptor gene mutation was, that two out of three homozygous males appeared to be fertile (Aittomaki *et al.*, 1995). Although no complete examination of these men was presented, the available data seems to suggest that FSH might not be obligatory for either initiation or maintenance of spermatogenesis. However, the mutant receptor may still have some FSH-inducible activity, which might be sufficient to cooperate with testosterone in the initiation and maintenance of spermatogenesis, although the *in vitro* studies presented by Aittomaki *et al.* do not reveal such residual activity. Testosterone, and thus LH, probably is the most important hormone in regulation of spermatogenesis (see Chapter 1). Patients with familial male-limited precocious puberty show enlargement of the testes (Egli *et al.*, 1985), which indicates that

spermatogenesis is initiated through LH action before a pubertal rise in FSH occurs.

Furthermore, LH and/or hCG action is indispensable for the development of male sex organs (Chapter 6). The presence of androgen-dependent epididymides and vasa deferentia in Leydig cell hypoplasia patients indicates that there is autonomous (LH/hCG-independent) androgen production from the fetal testis. But this autonomous production is probably low, since more distantly located androgen-dependent organs, such as prostate and seminal vesicles, had not developed in these Leydig cell hypoplasia patients.

Although no studies of female carriers of an activated LH receptor gene have been reported, expression of such a receptor in preovulatory follicles might lead to premature ovulation. Possibly, at the time of ovulation the LH receptor needs to be fully activated to induce ovulation. Dose-response curves of the activated LH receptor studied herein (LHR1-3; Fig. 5.2; Chapter 5) show that full stimulation with hCG results in a more elevated cAMP production, which reaches the same maximum as observed for the wild type receptor (Fig. 7.1). Therefore, the elevated basal cAMP production may not be sufficient to induce ovulation, whereas the mutant receptor contributes, together with the wild type receptor expressed from the other allele, to obtain a normal ovulatory response.

Thus far, one activating FSH receptor mutation has been reported in the human (Gromoll *et al.*, 1996). In this mutant receptor, an aspartic acid to glycine substitution is identified at position 567 in the third intracellular loop. The mutation was isolated from a hypophysectomized man, who appeared to be fertile under testosterone substitution alone. When compared to the wild type FSH receptor, the mutant receptor showed a 1.5-fold increase in basal cAMP production. This marginal increase seems to be sufficient to overcome the absence of FSH in this man, and this observation suggests that low FSH action is sufficient to maintain spermatogenesis.

In female carriers of constitutively activated FSH receptors, one would expect prepubertal growth of follicles. Although these follicles will probably not ovulate due to the absence of LH peaks, early recruitment of many follicles to grow and subsequent atresia might affect fertility in adult life. A recent study on the circulating FSH level during the menstrual cycle, showed that the FSH plasma concentration varies considerably between individuals (Van Santbrink *et al.*, 1995). Although this variation can have many causes, it will be of interest to investigate whether a relatively low level of FSH during the cycle might in some cases be associated with a mutated (constitutive active) FSH receptor. If such a

mutation results in a partial constitutive activation of the FSH receptor, there may be no major effect on fertility. However, such an investigation may point to several aspects of a less prominent phenotype. Moreover, a search for the occurrence of gonadotropin receptor mutations that have a minor effect on receptor activity may provide detailed structure-function information.

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SUMMARY

The gonadotropins, LH (luteinizing hormone) and FSH (follicle-stimulating hormone), are the molecular messengers between the central nervous system (hypothalamus/pituitary) and the gonads. These hormones are considered to be the main regulators of gonadal function, playing key roles during puberty and in adult reproductive functions. In the gonads, these hormones stimulate distinct cell types. LH stimulates the testicular Leydig cells to produce testosterone, while the Sertoli cells, the cells which support germ cell development, are regulated by FSH. In the ovary, the theca cells are sensitive to LH, whereas granulosa cells respond to both LH and FSH. Gonadotropin signal transduction is mediated by gonadotropin receptors (LH receptor and FSH receptor), which are transmembrane receptors that belong to the family of G protein-coupled receptors. Members of this receptor family contain a characteristic transmembrane domain that consists of seven membrane spanning helices that are connected by three extracellular and three intracellular loops. The N-terminal part of the receptor molecule is located at the extracellular side, and the C-terminal part at the intracellular side.

In this thesis, studies addressing different molecular aspects of gonadotropin receptors are described. Expression studies have been performed for the FSH receptor, focusing on the processes of alternative splicing of pre-mRNA, and homologous down-regulation of mature mRNA. For the LH receptor, receptor activation has been investigated using LH receptor mutants that were identified in patients with familial male-limited precocious puberty (FMPP) and in patients with Leydig cell hypoplasia.

Alternative splicing of pre-mRNAs transcribed from the genes encoding gonadotropin receptors leads to expression of transcripts that encode truncated receptor proteins. In Chapter 3, the cloning and characterization of two FSH receptor splice variants are described. Both transcripts encode a part of the extracellular domain of the receptor protein, a domain specific for the gonadotropin receptors, that is essential for hormone binding. Investigations on the level of expression of these transcripts, using transcript-specific RNase protection assays, showed that in testes of rats of different ages and in ovaries at different stages of the cycle these transcripts are expressed at a low level, and that these transcripts are not differentially expressed relative to expression of full-length receptor mRNA. The low and non-regulated expression level of these transcripts indicate that the

truncated receptor proteins do not have a function independent from the full-length receptor. A constant expression of truncated proteins might be involved in signal transduction by the full-length receptor molecule. Therefore, the full-length receptor was expressed in COS cells together with either one of the splice variants, and it was investigated whether co-expression of the splice variants could stimulate or inhibit full-length receptor signal transduction. It was observed that the truncated proteins did not affect full-length receptor signal transduction, and it was concluded that alternative splicing of FSH receptor pre-mRNA may not have a function in postnatal gonads.

Stimulation of cultured Sertoli cells with FSH results in a rapid and almost complete down-regulation of FSH receptor mRNA. This is caused by an FSH-induced decrease in the half-life of the mRNA. The process may serve as a cellular negative feedback mechanism to prevent over-stimulation of the cell. Investigations on this process (Chapter 4) showed that it also functions in intact testes of immature animals. Furthermore, it was found that the mechanism is dependent on active transcription, but not on ongoing translation, which indicates that an FSH-induced RNA species may be involved in the process of homologous down-regulation of FSH receptor mRNA.

Chapters 5 and 6 are concerned with the identification of mutant LH receptors in patients with FMPP and patients with Leydig cell hypoplasia. FMPP is an autosomal dominant inherited condition, that causes precocious puberty in boys. The cause of this condition was found to be mutation of the LH receptor gene. The mutations that have been described in the literature result in amino acid substitutions in the LH receptor molecule, in particular in the third intracellular loop and the sixth transmembrane segment. The amino acid substitutions result in activation of the receptor in the absence of hormone (constitutive activity). In FMPP patients, this causes prepubertal testosterone production and, subsequently, the onset of puberty at a very young age. The mutations give insight into the mechanism of receptor activation. Clustering of the amino acid substitutions in the third intracellular loop and the sixth transmembrane segment, a region of the receptor that is involved in G protein coupling, supports the hypothesis that conformational changes in this part of the receptor molecule are crucial steps in receptor activation. In Chapter 5, a new LH receptor mutation is described, which results in an amino acid substitution in the second transmembrane segment of the receptor protein. The effect of this substitution on receptor activity was found to be similar to the effects of substitutions in the third

intracellular loop and the sixth transmembrane segment, suggesting that also this transmembrane segment is involved in the process of receptor activation.

Besides activating LH receptor gene mutations, an inactivating mutation also is described in this thesis (Chapter 6). *In vitro* expression of this mutant receptor in HEK293 cells does not result in measurable signal transduction, not even at high hormone concentrations, although the mutant receptor molecule binds hCG with the same affinity as the wild-type LH receptor. The mutation was identified in patients with Leydig cell hypoplasia. These patients have a male (46,XY) genotype, and testes, but present with a female external phenotype. The insensitivity to LH (and to maternal hCG) causes the absence of Leydig cell proliferation and the absence of testosterone production, which led to incomplete male sex differentiation.

SAMENVATTING

De gonadotropines, LH (luteïniserend hormoon) en FSH (follikel-stimulerend hormoon), zijn de moleculaire boodschappers tussen de hersenen (hypothalamus/hypofyse) en de gonaden. Deze hormonen worden gezien als de belangrijkste regulatoren van gonadale functies. In de testis stimuleert LH de productie van testosteron door Leydig cellen, terwijl FSH de Sertoli cellen stimuleert, die de ontwikkeling van germinale cellen ondersteunen. In het ovarium worden zowel theca als granulosa cellen gereguleerd door LH, terwijl FSH werking meer specifiek gericht is op granulosa cellen. Signaaltransductie van de gonadotropines verloopt via gonadotropine receptoren (LH receptor en FSH receptor). Deze receptoren zijn membraan-gebonden en behoren tot de familie van G eiwit-gekoppelde receptoren. Karakteristiek voor deze receptorfamilie is het transmembraan domein, dat bestaat uit zeven transmembraan segmenten die zijn verbonden door drie extracellulaire en drie intracellulaire lussen. Verder heeft het N-terminale deel van het receptor eiwit een extracellulaire positie en het C-terminale deel een intracellulaire positie.

In dit proefschrift zijn studies over verschillende moleculaire aspecten van de gonadotropine receptoren beschreven. Voor de FSH receptor zijn expressie studies uitgevoerd, die betrekking hebben op het proces van *alternative splicing* van het pre-mRNA en op homologe *down*-regulatie van het mature mRNA. Voor de LH receptor is receptor activering onderzocht aan de hand van LH receptor mutanten, die zijn geïdentificeerd in patiënten met familiale pubertas precox (FMPP) en in patiënten met Leydig cel hypoplasie.

Alternative splicing van het FSH receptor pre-mRNA, maar ook van het LH receptor pre-mRNA, leidt tot expressie van mRNA transcripten (*splice* varianten), die coderen voor de *full-length* receptor, maar voor verkorte (getrunceerde) receptor eiwitten. In Hoofdstuk 3 worden de clonering en karakterisering van twee FSH receptor *splice* varianten beschreven. Beide transcripten coderen voor een gedeelte van het extracellulaire domein, dat in gonadotropine receptoren betrokken is bij de binding van het hormoon. Onderzoek naar regulatie van mRNA expressie van deze transcripten, door middel van transcript-specifieke RNase protectie assays, leerde dat in testes van ratten van verschillende leeftijden en in ovaria van ratten tijdens verschillende stadia van de cyclus, de transcripten op een laag niveau tot expressie komen, en dat deze expressie niet differentiëel is

gereguleerd ten opzichte van de expressie van het *full-length* FSH receptor mRNA transcript. Gezien de lage en niet-differentiële gereguleerde expressie van deze transcripten, lijkt het niet waarschijnlijk dat getrunceerde FSH receptor eiwitten een rol spelen onafhankelijk van de *full-length* receptor. Wel zouden de getrunceerde receptor eiwitten betrokken kunnen zijn bij signaal transductie door de *full-length* FSH receptor. Om dit te onderzoeken werd de *full-length* receptor, samen met elk van de *splice* varianten, tot expressie gebracht in COS cellen. Vervolgens werd de FSH-afhankelijke signaaltransductie door de *full-length* receptor bepaald. De getrunceerde receptor eiwitten bleken hierop geen effect te hebben. Er werd geconcludeerd, dat geen functie kan worden toegekend aan *alternative splicing* van het FSH receptor pre-mRNA in postnatale gonaden.

Na stimulatie van gekweekte Sertoli cellen met FSH wordt het mRNA niveau van de *full-length* FSH receptor zeer snel en bijna volledig *down*-gereguleerd. Dit wordt bewerkstelligd door een FSH-geïnduceerde verlaging van de halfwaarde-tijd van dit mRNA. Dit proces zou kunnen dienen als een cellulair negatief *feedback* mechanisme, dat overstimulatie van de cel voorkomt. Onderzoek naar dit proces (Hoofdstuk 4) leerde dat dit mechanisme niet alleen optreedt in gekweekte cellen, maar ook in intacte testes van immature ratten. Verder werd gevonden dat dit proces alleen door FSH en niet door andere hormonen en groeifactoren wordt gestimuleerd, en dat het mechanisme van deze stimulatie afhankelijk is van actieve transcriptie, maar niet van het optreden van translatie. Een FSH-geïnduceerde RNA factor lijkt betrokken te zijn bij het proces van homologe *down*-regulatie van FSH receptor mRNA.

In de Hoofdstukken 5 en 6 is de identificatie beschreven van LH receptor mutanten in patiënten met FMPP en patiënten met Leydig cel hypoplasie. FMPP is een autosomaal dominant overerfbare aandoening, waarbij jongens een vervroegde puberteit vertonen. De oorzaak van deze aandoening bleek een mutatie in het LH receptor gen te zijn. De in de literatuur beschreven mutaties resulteren in aminozuurveranderingen in met name het zesde transmembraan segment en de derde intracellulaire lus van het receptor molecuul. De aminozuurveranderingen hebben tot gevolg dat de receptor actief wordt in de afwezigheid van hormoon (constitutief actief). In FMPP patiënten resulteert dit in een vroegtijdige testosteron productie en het begin van puberteit op een zeer jonge leeftijd. De mutaties geven inzicht in het mechanisme van receptor activering. De clustering van de gevonden aminozuurveranderingen in het zesde transmembraan segment en de derde intracellulaire

lus, een gedeelte van het receptor molecuul dat betrokken is bij G eiwit koppeling, duidt erop dat conformatieveranderingen in dit deel van de receptor betrokken zijn bij receptor activering. In Hoofdstuk 5 wordt een nieuwe LH receptor mutant beschreven, waarvan de aminozuurverandering is gelegen in het tweede transmembraan segment van de receptor, maar die verder dezelfde effecten tot gevolg heeft als mutaties in het zesde transmembraan segment en de derde intracellulaire lus. Daarom mag worden aangenomen, ook dit deel van het transmembraan domein betrokken is bij receptor activering.

Naast de constitutief actieve LH receptor mutant, is in dit proefschrift ook een LH receptor mutant beschreven waarbij de receptor wordt geïnactiveerd (Hoofdstuk 6). Expressie van deze gemuteerde receptor in de HEK293 cellijn resulteert niet in meetbare signaaltransductie, zelfs niet bij hoge hormoon concentraties. De mutatie is geïdentificeerd in patiënten met Leydig cel hypoplasie, waarbij personen met een mannelijk genotype (46,XY) en testes, die homozygoot zijn voor deze mutatie, extern een vrouwelijk fenotype vertonen. Door de ongevoeligheid voor LH (en voor het maternale hCG) is er tijdens de foetale ontwikkeling van de testes van deze mannen geen Leydig cel proliferatie opgetreden, en dus is er weinig of geen productie van testosteron, hetgeen resulteert in onvolledige mannelijke geslachtsontwikkeling.

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CURRICULUM VITAE

De schrijver van dit proefschrift werd op 7 december 1966 geboren te Capelle aan den IJssel. In 1985 behaalde hij het VWO diploma aan de Openbare Scholengemeenschap J.C. de Gloppe te Capelle aan den IJssel, waarna hij in datzelfde jaar begon met de studie Biologie aan de Rijksuniversiteit Leiden. Tijdens deze studie werd een bijvakstage uitgevoerd bij de Vakgroep Moleculaire Plantkunde van de Rijksuniversiteit Leiden, waarbij regulatie van tryptofaan decarboxylase mRNA expressie in *Catharantus roseus* werd onderzocht (Dr. O.J.M. Goddijn). Een hoofdvakstage werd uitgevoerd bij de Vakgroep Celbiologie & Genetica van de Erasmus Universiteit Rotterdam. Tijdens deze stage werd het Oct6 gen geïsoleerd en de promotor van dit gen functioneel gekarakteriseerd (Dr. Ir. D.N. Meijer). In augustus 1990 studeerde hij af en bleef nog enige tijd werkzaam als gastmedewerker bij de Vakgroep Celbiologie & Genetica, waarna in juli 1991 het in dit proefschrift beschreven AIO-project werd aangevangen bij de Vakgroep Endocrinologie & Voortplanting van de Erasmus Universiteit Rotterdam.

Momenteel is hij werkzaam als wetenschappelijk medewerker bij de Vakgroep Urologie van de Erasmus Universiteit Rotterdam, en onderzoekt moleculaire aspecten van meta-stasering van prostaatkanker.

