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Gonadal Peptides as Mediators of Development and Functional Control of the Testis: An Integrated System with Hormones and Local Environment*

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I. Introduction

THE TESTIS is a complex organ that serves two crucial functions: synthesis and secretion of testosterone and production of a sufficient number of competent spermatozoa to attain fertility. To accomplish these objectives, the tissue is organized into two compartments: the tubular compartment and the interstitium. The seminiferous tubules are formed by the Sertoli cells, which provide structural support for the germinal cells, and by the peritubular myoid cells (PMC), which surround the tubules. The interstitium is composed of Leydig cells, macrophages, fibroblasts, and blood vessels. These structures are embedded in the extracellular matrix, which is immediately adjacent to the seminiferous epithelium, between the PMC and the basal surfaces of Sertoli cells and spermatogonia, and acquires the specialized form of the basement membrane.

Due to the cyclic course of spermatogenesis, any given function of the spermatogenic cells overlaps with an earlier or later generation to create a constant combination of cells known as cell associations or stages. The complete sequence of stages or cell associations constitutes one cycle of the seminiferous epithelium, whose duration appears to be specific for each species. This continuous progression of spermatogenic lineages causes profound reciprocal changes of the environment to which each cell is exposed (Fig. 1).

The adult organization of all the testis components results from complex processes of cellular proliferation and progressive acquisition of a specialized phenotype that show a remarkable degree of coordination. It is well known that the essential prerequisite for normal testicular development and maintenance of spermatogenesis is the controlled secretion of LH, FSH, and testosterone during fetal and postnatal life. As a matter of fact, the deficiency of these hormones leads to hypogonadism and sterility, a condition that can be treated with specific replacement therapies (1). However, in the majority of cases of male infertility resulting from a reduced or even absent production of spermatozoa, the levels of circulating reproductive hormones are within the normal range, thereby eliminating the possible application of any reliable therapy with the exception of *in vitro* fertilization procedures (2, 3).

These data have reinforced the assumption that the testicular physiology is not fully accounted for by traditional

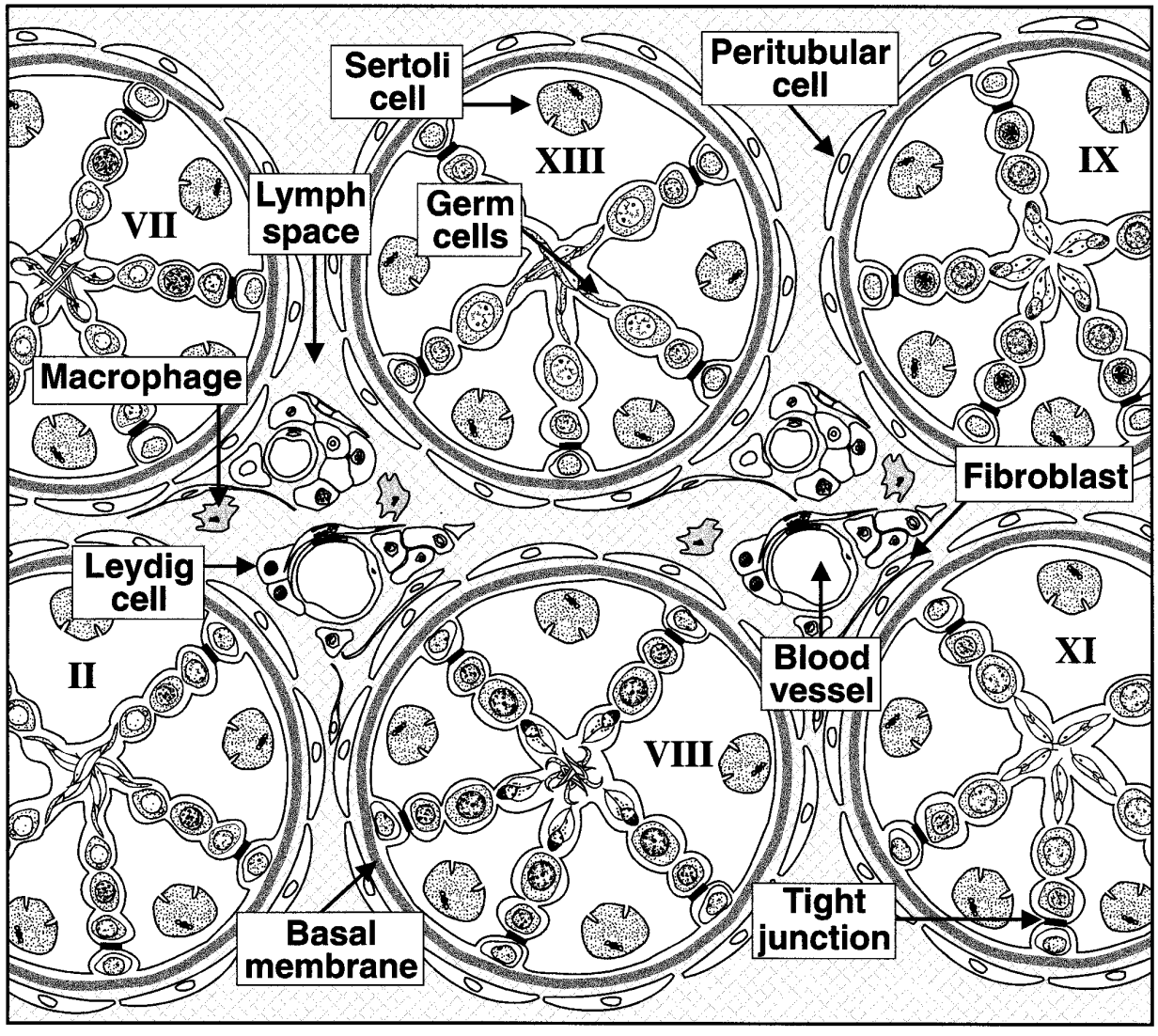

FIG. 1. Schematic representation of the anatomical arrangement of the adult rat testis. Cell associations forming some of the XIV stages of the seminiferous cycle are shown. The unsteady cellular composition of the seminiferous epithelium and the consequent spatiotemporal variation in the reciprocal interactions between all the testicular cellular components cause the cyclic microenvironmental changes typical of the mammalian testis.

endocrine paradigms. Presumably, then, there should be an intratesticular network of regulators, the exquisitely timed and highly regionalized expression of which might participate first in the development of the male gonad and later in the initiation and maintenance of testicular function. This involves intercellular, intracellular, and cellular-environmental communication rather than total reliance on intracellular programming and classic hormonal control. Thus, testicular development can be influenced by a number of variables, including physical parameters, nutrients, extracellular matrix components, cell adhesion molecules, soluble factors, and membrane junctional complexes between apposing cells. In addition to development and differentiation, which are readily evident during embryogenesis, these variables are involved in the fully developed organ in tissue maintenance, cellular renewal, and local control mechanisms. These general rules appear to be particularly important in the testis since in the seminiferous tubules, as in the hemopoietic system, a limited number of multipotent stem cells give rise to a much larger population of functionally mature cells, and thus the processes of proliferation and differentiation must be finely tuned throughout life.

In the last two decades, these considerations have produced a shift from endocrine to paracrine research, generating a large body of studies that have been subject to debates and attempts to fit the available information into hypothetical models (4–16). Among the substances employed as symbols in the language of intercellular communication are the polypeptidic factors. At present, we refer to the polypeptidic factors as regulatory substances released by practically any type of cells. They can also be referred to with a broader term such as cytokines (from the ancient greek κυτοσ, cell, κινεω, to stimulate). More than 50 of these substances have been described and the majority have been cloned. Most of them have pleiotropic effects, their actions are markedly influenced by the context in which they operate, and some act

synergistically or are capable of inducing or inhibiting the production of other cytokines. They form the fourth major class of soluble intercellular signaling molecules, alongside neurotransmitters, endocrine hormones, and autacoids. Many of these factors are produced by the various cellular components of the testis.

This article will attempt to provide an analysis of the available information on the functional interactions between the cells of the male gonad mediated by locally produced regulatory peptides. When dealing with the literature on this topic, there are some focal points that must be kept firmly in mind. The vast majority of the results are generated from *in vitro* studies, and thus to extrapolate the products *in vivo* is to force the evidence. It should never be forgotten that *in vitro* we are out of context, and cellular behavior can be markedly affected by artificial experimental conditions. For example, Sertoli cells or PMC isolated from their normal environment and placed in culture look different and function differently (17, 18). Furthermore, the effect of a regulatory substance can be profoundly influenced by reciprocal interactions of the substance itself and the responding cells with the extracellular matrix and the normal cellular complement (19). Modern techniques now allow us to measure very small quantities, perhaps even below the limit of biological significance. What regulates the testicular cell components in adult life may not be the same as during pre- and early postnatal development or during puberty. Although comparable regulatory interactions exist in mammals, the relative importance of distinct local control systems may vary from species to species.

Crucially important in the identification and validation of peptide factors such as local modulators is the demonstration of local production, expression of specific receptors, and evidence for a biological action on the producing cell or on neighboring cells. The agreement of different methodological approaches gives additional strength to the information. Another important criterion that should be met to define a factor as a local regulator is that local production and action is controlled under physiological conditions or altered in pathological circumstances.

The general characteristics of the substances that will form the focus of this review are listed in Table 1.

The review will encompass a detailed analysis of the literature. We will discuss the evidence for local production, receptor expression, and *in vitro* and *in vivo* actions for each of the gonadal peptides so far identified in the mammalian testis, preceded by a brief summary of their general properties. Our scope is to furnish the most complete picture as possible, trying to place the data in an integrated perspective with hormones and local environment.

The advent of transgenic and gene-targeting techniques has allowed us the unique opportunity to test the functions of specific genes directly *in vivo*. Many of the functions of the regulatory peptides within the context of a complex organism have been elucidated through the use of such experimental models. Thus, we have reported the results generated by these powerful technologies, which may help to clarify the complex functions of the regulatory peptides in the testis.

Due to the enormous amount of information available, in some instances contradictory and difficult to interpret, the experimental evidence has been summarized in tabular form to provide an easily understood format, enabling quick comparison of data. Tables 2 and 3 summarize the reports on the presence of regulatory peptides and their receptors in the mammalian testicular tissue and in isolated cells, including cellular source, detection systems, and modulation of expression/production. Tables 4 and 5 provide a synopsis of the effects of the gonadal peptides in *in vitro* and *in vivo* models. The pattern of expression of these substances and their receptors during development and in relation to the stages of the seminiferous epithelium in the rat is provided in Tables 6 and 7. Finally, Table 8 summarizes the characteristics of transgenic mice models with recognizable effects on male reproduction that are thought to be exerted through direct testicular action.

II. Neurohormones/Neuropeptides

A. GH-releasing hormone (GHRH)

GHRH is the hypothalamic neurohormone that stimulates the synthesis and secretion of GH from the somatotrophs of the anterior pituitary (20, 21). The mature hormone contains 44 amino acid (aa) residues. Additional proteolysis *in vivo* can yield peptides of 37 and 40 aa that possess full biological activity. Molecular cloning of the GHRH receptor confirmed that the receptor belongs to the family of the G protein-coupled receptors containing seven potential membrane-spanning domains (21, 22).

1. Expression, localization, and production. High levels of a GHRH-like substance in mature rat testis are present both at the level of protein product and gene transcript with the mRNA substantially larger than the GHRH transcript from the hypothalamus (23). The partially purified testicular GHRH is capable of stimulating GH secretion from cultured anterior pituitary cells in a dose-dependent manner, and GHRH-like immunoreactivity has been localized to mature sperm forms in rat testis (24).

Testicular GHRH mRNA and peptide are developmentally regulated. No GHRH mRNA is detected in testes from day 19 fetal rats, but it is present in low amounts on day 2 of life, increasing gradually to day 21 (25). The GHRH gene expression increases more dramatically beginning on day 21 and reaches adult levels by day 40 (25). Immunohistochemical studies have confirmed these findings in that the presence of GHRH-like immunoreactivity has been found in the interstitial cells of the testis from postnatal day 4 and the positively stained cells increase with age (26). Immunoreactive GHRH was also present in the acrosomal region of early and intermediate spermatids at stages III-VI of the seminiferous epithelium cycle (26). Accordingly, GHRH mRNA has been localized in developing spermatogenic cells by *in situ* hybridization and Northern blot analysis (27).

Transgenic mice bearing the fusion gene encoding the promoter region of the mouse metallothionein-1 (MT-1) gene and the coding region of the human GHRH gene have been used as a model for studying the tissue-specific expression and processing of GHRH (28, 29). In the testis of these animals the GHRH gene was clearly expressed (29), and the

TABLE 1. General properties of the regulatory peptides identified in the mammalian testis

Substance	Description	Main localization/sources	Receptors	Targets	Major functions
ACT/INH	INH, dimer of α + βA or βB subunits. ACT, dimer of the β-subunits	Gonads	ActRII and ActRIIB, serine/threonine kinases. Bind ACT and with low affinity INH	Anterior pituitary	ACT stimulates, INH suppresses FSH secretion
ANP	22–35 aa	Heart and specialized areas of CNS	Two types of receptors. Two guanylate cyclase-linked and a clearance receptor	Blood vessels, kidney	Vasodilatation, natriuresis
AT-II	8 aa	Endothelial cells, lung	ATI and AT2. Seven-transmembrane G protein coupled	Cardiovascular system, kidney, adrenal cortex, CNS	Vasoconstrictor, regulates aldosterone secretion, water and electrolyte balance
AVP	9 aa	Hypothalamus: supraoptic and paraventricular nuclei	Two classes of receptors: V1 calcium channel; V2 adenylate cyclase coupled	Vascular smooth muscle cells, liver	Stimulates the renal transport of sodium, increases the water permeability of the collecting tubules, constriction of arteries and arterioles, glycogenolytic
CRH	41 aa	Hypothalamus, paraventricular nucleus.	Glycoprotein, G protein, and adenylate cyclase coupled.	Anterior pituitary corticotropes, CNS	Stimulates the release of ACTH and related peptides
ET	21-aa isopeptides. ET-1, -2, -3	ET-1: endothelial cells and vascular smooth muscle cells, CNS, endometrial cells, hepatocytes, mesangial cells, breast epithelial cells ET-2: kidney, intestine ET-3: source not known	Two receptors (A and B), G-proteins coupled. From 45 to 50 kDa A receptor 10 times higher binding affinity for ET-1 than ET-3. B receptor binds ET-1 and ET-3 with similar affinity	Endothelial cells, vascular smooth muscle cells, neurons and astrocytes in the early stages of fetal development	Induces vasoconstriction, cell proliferation, and hormone production
EGF/TGFα	EGF 53 aa; TGFα 50 aa; 40% sequence homology	EGF: submaxillary gland, Brunners gland, mRNA in a variety of newborn animal tissues. TGFα: embryos, placenta, many transformed cells	175 kDa protein tyrosine kinase. Product of the c-erbB proto-oncogene	Epithelial, mesenchymal, glial cells	Mitogens for epithelial and mesodermal tissues
aFGF/bFGF	Acidic and basic FGF. 16–17 kDa. 55% sequence identical. Lack of consensus signal peptide. Related to other FGFs and IL-1 family	Low mRNA levels in wide range of normal and transformed cells. Associated with extracellular matrix	FGFR-1 (150 kDa), FGFR-2 (130 kDa) both tyrosine kinases. Bind with low affinity to cell surface proteoglycans	Endothelial, epithelial, mesenchyme, and neuronal cell types	Mitogens for mesodermal and endodermal cell types. Inhibits differentiation in muscle. Cause angiogenesis
GnRH	10 aa	Hypothalamus	327-aa protein, seven transmembrane regions; G-protein coupled	Pituitary gonadotropes	Stimulates the release of LH and FSH
GHRH	44 aa and 40 aa	Median eminence	Seven-transmembrane, G-protein coupled	Pituitary somatotropes	Stimulates the release of GH
GRP	27 aa, belongs to the family of BN-like peptides, GRP-subfamily	Neuroendocrine cells of the lung, brain, gut, pancreas	Three types of receptors with high degree of aa identity Seven-transmembrane, G-protein coupled	Nervous system, gastrointestinal tract, lung	Stimulates secretion of gastrointestinal hormones and smooth muscle contraction; modulates neuronal firing rate Mitogen
IGF-I/IGF-II	7 kDa. Related to each other, 62% sequence omology	IGF-I mainly produced in the liver. IGF-II mRNA in variety of cells. Both present in plasma in association with specific binding proteins	IGF-I R: two extracellular α-subunits, and two transmembrane β-subunits linked to form the mature $\alpha 2\beta 2$-holoreceptor, protein tyrosine kinase, binds IGF-I and IGF-II IGF-II R: 250 kDa, monomer with short cytoplasmatic tail, no tyrosine kinase activity	Wide variety of cell types	Mitogen and differentiating agent for cells derived from all primitive germ layers
IL-1α/IL-1β	Single-chain glycosylated 17-kDa proteins with extensive homology and similar bioactivities	Activated T cells or macrophages	Two receptors: IL-1RI and IL-1RII, transmembrane glycoproteins of the Ig superfamily	T cells, B cells, fibroblasts, monocytes, PMN leukocytes	Differentiation and function of cells involved in inflammatory and immune responses
IL-2	Glycosylated 133-aa polypeptide	Activated helper T cells	Subunit complexes of α-, β-, and γ-chains or β- and γ-chains. Tyrosine kinases Jak1 and Jak3 bound to β- and γ-chains	T cells, B cells	Promotes T cell proliferation and differentiation, NK activity, proliferation of B cells
IL-4	Polypeptide with potential N-glycosylation sites. Mouse, 140 aa; human, 153 aa	Activated T cells or macrophages	Receptor complex: IL-4-binding receptor chain (IL-4R) and associated proteins	B cells, T cells, macrophages, fibroblasts, endothelial cells	Induces differentiation into T helper cells, proliferation and differentiation of B cells
IL-6	Polypeptide. Mouse 211 aa. Human 212 aa	T cells, macrophages, fibroblasts	Two integral membrane glycoproteins: a ligand binding protein (IL-6R), and a non-ligand binding signal transducer (gp 130)	Hematopoietic cells, stem cells, T cells, B cells	Induces growth and differentiation of T cells and B cells; activates hematopoietic progenitor cells

TABLE 1. Continued

Substance	Description	Main localization/sources	Receptors	Targets	Major functions
NGF	Dimer of two 118-aa subunits	Glial cells and oligodendrocytes	Two receptors: trkA, protein tyrosine kinase, product of the trk protooncogene. LNGFR, G-protein coupled	Neurons. Nonneurons cell types: keratinocytes and smooth muscle cells	Development and maintenance of central neurons and of sensory and sympathetic peripheral neurons
NPY	36 aa	Widely distributed in CNS and PNS	Four types of receptors, Y1–Y3 and an atypical Y1. G-protein coupled	CNS	Stimulates feeding behavior
Opioids	ENKs, END, DYNs. Derived from three precursors: pENK-A, POMC, pDYN	Widely distributed throughout CNS and PNS	Three classes: δ, κ and μ. Seven-transmembrane G protein coupled. δ highest affinity for ENKs and ENDs, κ for DYN	CNS, endocrine and immune systems	Control on pain perception, modulation of endocrine, cardiovascular, respiratory, gastrointestinal, immune functions
OT	9 aa	Hypothalamus: supraoptic and paraventricular nuclei	Seven transmembrane, G-protein linked	Uterus, myoepithelial cells of the mammary gland	Stimulates contraction
PACAP	Two forms: PACAP38 (38 aa) PACAP27 (27 aa)	Hypothalamus	Three forms: PVR1 binds P38 $\approx$ P27 $\gg$ VIP; PVR2, P38 $\approx$ P27 $\approx$ VIP; PVR3, P38 $\approx$ P27 $\approx$ VIP. G-protein coupled	Anterior pituitary cells	Modulates the responses to hypophysiotropic factors
PDGF AA, AB, and BB	Dimers of A (17 kDa) and B (16 kDa) chains, B chain is product of c-*sis* protooncogene	Platelets, placenta, preimplantation embryos Endothelial cells	Two species of glycoprotein. Both tyrosine kinases. Type α (170 kDa) binds all PDGF dimers. Type β (180 kDa) binds PDGF BB and AB	Mesenchymal cells, glial cells. Smooth muscle. Placental trophoblasts	Mitogen for mesenchymal cell types. Chemotactic for fibroblasts, smooth muscle cells, monocytes and neutrophils
PModS	Two forms: PModSA (54 kDa), PModSB (56 kDa)	Testicular pentubular myoid cells	Not characterized	Sertoli cells	Stimulates transferrin, ABP and INH secretion of Sertoli cells
SRIF	14 aa	CNS hypothalamic and extrahypothalamic areas, gastrointestinal system, pancreas, placenta, retina, thymus, adrenal medulla	Five receptor subtypes, G-protein coupled	Pituitary gland, pancreas, gastrointestinal tract	Inhibits GH, insulin, gastrin release Inhibits gastric acid secretion, intestinal motility
SLF	273 aa, both soluble and membrane-bound forms, result from alternative splicing	Fibroblasts and endothelial-like cells of the bone marrow, Sertoli cells, granulosa cells, skin keratinocytes and fibroblasts	Product of the c-*kit* gene, family of tyrosine kinases	Germ cells, melanocytes, hematopoietic stem cells	Influences growth and differentiation of melanocytes, germ cells, hematopoietic stem cells
SP	11 aa	CNS, PNS	Seven-transmembrane, G-protein coupled	Central and peripheral neurons	Neurotransmitter, neuromodulator, involved with the senses of pain and touch
TGFβ	β1, β2, β3, 25 kDa homodimers, secreted as latent complexes	Preimplantation mouse embryos, later embryos Widespread throughout adult tissues and cultured cells	Type I, 53 kDa. Type II, 70–85 kDa. Type III, 200–400 kDa. Types I and II involved in signaling, type III regulates access to the signaling receptors. Type I and II are serine/threonine kinase receptors	Wide variety of cell types	Inhibitor of epithelial cell proliferation but biphasic action on mesodermal cell types. Inhibits differentiation in muscle Chemotactic for monocytes and fibroblasts. Promotes fibronectin and collagen synthesis
TNFα	157-aa polypeptide. There is an unprocessed membrane-bound form of 26 kDa	Activated macrophages, monocytes	55-kDa TNF-R1 and 75-kDa TNF-R2. Absence of homology in the intracellular domains of the receptors	Wide variety of cell types	Immunostimulant and mediator of inflammatory, proliferative, and antiviral responses. Key mediator against malignant neoplasm
TRH	3 aa	Hypothalamus, median eminence	Seven-transmembrane-spanning G-protein coupled	Pituitary thyrotropes	Stimulates synthesis and secretion of TSH

Abbreviations: ABP, Androgen binding protein; ACT, activin; AT-II, angiotensin-II; ANP; atrial natriuretic peptide; AVP, arginine vasopressin; BN, bombesin; CNS, central nervous system; DYN, dynorphin; βEND, β-endorphin; ENK, enkephalin; ET, endothelin; EGF, epidermal growth factor; TGFα, transforming growth factor α; aFGF, acidic fibroblast growth factor; bFGF, basic FGF; GHRH, growth hormone releasing hormone; GRP, gastrin-releasing peptide; IGF, insulin-like growth factor; IL, interleukin; INH, inhibin; NGF, nerve growth factor; NK, natural killer cells; NPY, neuropeptide Y; OT, oxitocin; PACAP, pituitary adenylate cyclase-activating polypeptide; PDGF, platelet-derived growth factor; PModS, peritubular cells factor that modulates Sertoli cells; PNS, peripheral nervous system; POMC, proopiomelanocortin; SLF, steel factor; SRIF, somatostatin; SP, substance P; TGFβ, transforming growth factor β; TNFα, tumor necrosis factor α. References describing the general properties of the cited regulatory substances are reported in the text.

TABLE 2. Regulatory peptides in the mammalian testis

Substance	Species	Source/localization	Detected by	Modulation	Ref.
ACT	Rat	Leydig cells	Bioassay		344
ACT	Rat	PMC	Western, Bioassay		346
ACT	Rat	Sertoli cells	Bioassay		345
ACT	Pig	Leydig cells	Bioassay		344
INH	Rat	Sertoli cells	Bioassay, RIA, IHC	FSH, EGF ⇑	342, 347
INH	Rat	Testis extract	RIA		347
INH	Rat	Leydig cells	RIA, Bioassay	LH ⇑	341
INH	Pig	Sertoli cells	Bioassay		344
INH-α	Rat	Testis extract	S1-NPA, Northern	FSH ⇑	321, 348
INH-α	Rat	Leydig cells	IHC, ISH, Northern	hCG ⇑	340, 341
INH-α	Rat	Sertoli cells	IHC, ISH, Northern	FSH ⇑	340, 348
INH-α	Rat	ST extract	Northern		349
INH-α	Human	Sertoli cells	IHC		355–357
INH-α	Human	Leydig cells	IHC		355–357
INH-α	Human	Testis culture	RIA	FSH, hCG ⇑	353
INH-βA	Rat	Testis extract	S1-NPA		321
INH-βA	Rat	Sertoli cells	IHC, ISH		340, 350
INH-βA	Rat	PMC	Northern		346
INH-βA	Rat	SpC	IHC		343
INH-βA	Human	Sertoli cells	IHC		357
INH-βA	Human	Leydig cells	IHC		357
INH-βB	Rat	Testis extract	S1-NPA		321
INH-βB	Rat	Leydig cells	IHC		340
INH-βB	Rat	Sertoli cells	IHC, ISH, Northern		340, 343, 350
INH-βB	Rat	ST extract	Northern		349
ANP	Rat	Testis homogenate	RIA (proANP), RIA/HPLC		804, 807
ANP	Rat	SpT, eSpZ	IHC		807
ANP	Rat	Testis extract	Northern, PCR		805, 806
ANP	Mouse	Testis homogenate	RIA/HPLC		807
ANP	Mouse	SpT, eSpZ	IHC		807
AT-II	Rat	Leydig cells	RIA/HPLC		762
AT-II	Rat	Germ cells	RIA/HPLC		762
AT-II	Mouse	Leydig tumor cells	RIA/HPLC	LH/hCG ⇑	760
AVP	Rat	Testis extract	RIA/C, RRA, PCR, Northern		164, 185
AVP	Rat	TF	RIA	Disruption spermatogenesis ⇓	189
AVP	Rat	Leydig cells	RIA		185
AVP	Rat	Leydig tumor cells	PCR		195
AVP	Mouse	Testis extract	PCR		153
AVP	Mouse	Leydig cells	PCR		195
AVP	Mouse	Leydig tumor cells	PCR		195
AVP	Pig	Testis extract	RIA/HPLC		186
CRH	Rat	Testis extract	Northern, RIA	Immobilization stress ⇑	126, 130, 134
CRH	Rat	Leydig cells	IHC, RIA/HPLC	LH/hCG, 5HT, cAMP ⇑	128, 131–133
CRH	Sheep	Testis extract	RIA/HPLC		128
CRH	Human	Leydig cells	IHC		129, 132
DYN	Rat	Testis extract	Northern, RIA/GC, RIA/HPLC		298–301
DYN	Rat	Leydig cells	IHC		299
DYN	Rat	Leydig tumor cells	Northern, RIA	cAMP ⇑	300
DYN	Rat	Sertoli cells	Northern, ISH, RIA	cAMP ⇑	302
DYN	Guinea pig	Testis extract	RIA/HPLC		301
EGF	Rat	Testis extract	RIA		408
EGF	Mouse	Testis extract	RIA, RRA		497, 499
EGF	Mouse	Sertoli cells	IHC		499
EGF	Mouse	SpC, SpT	IHC		499
EGF	Human	Testis extract	RRA		498
ET-1	Rat	Sertoli cells	RIA/HPLC, IHC	FSH ⇓	733, 734
ET-1	Rat	Testis extract	PCR, Northern, EIA, RIA	hCG ⇑	729–731, 734
ET-1	Rat	Leydig cells	IHC		734
ET-1	Rat	Endothelial cells	IHC		734
ET-1	Human	Sertoli cells	IHC		736
ET-3	Rat	Testis extract	PCR, EIA		732
POMC	Rat	Testis extract	Northern, S1-NPA, RT-PCR	hCG ⇑	231, 232, 238, 243, 255
POMC	Rat	Leydig cells	ISH		231, 234
POMC	Rat	Macrophages	ISH		234
POMC	Mouse	Testis extract	Northern, RT-PCR		232, 243
POMC	Mouse	Leydig cells line	Northern, RT-PCR		232, 243
POMC	Mouse	Leydig cells	ISH, RT-PCR		235, 243
POMC	Mouse	SpC/SpT	Northern		237
POMC	Hamster	Testis extract	Northern		232
POMC	Hamster	SpC/SpT	Northern		237
POMC	Human	Testis extract	S1-NPA		240

TABLE 2. Continued

Substance	Species	Source/localization	Detected by	Modulation	Ref.
βEND	Rat	Leydig cells	IHC, RIA/HPLC	hCG ⇑, CRH ⇑, T ⇓, GnRH ⇓, Dex ⇓	142, 145, 245, 252, 253
βEND	Rat (Hypox)	IF	RIA	hCG ⇑, GnRH ⇓	254
βEND	Rat	IF	RIA	Restraint stress ⇑	275
βEND	Mouse	Leydig cells	IHC	hCG ⇑	250
βEND	Human	Leydig cells	IHC		246
ppENK	Rat	Testis extract	Northern		277, 279, 280
ppENK	Rat	Sertoli cells	Northern	FSH, cAMP ⇑	281, 282
ppENK	Rat	Leydig cells	Northern	hCG, cAMP ⇑	291
ppENK	Rat	PMC	Northern	TPA, cAMP ⇑	292
ppENK	Rat	SpT	Northern		280
ppENK	Mouse	SpC, SpT	Northern	237, 278	
ppENK	Hamster	Testis extract	Northern		279
ppENK	Human	Testis extract	Northern		285
Met-ENK	Rat	Testis extract	RIA/HPLC		277
Met-ENK	Rat	Sertoli cells	IHC, ICC	hCG, cAMP ⇑	293, 294
b-FGF	Rat	Testis extract	Western	S1-NPA	554
b-FGF	Rat	Germ cells	Western, immunoprecipitation		555
b-FGF	Rat	Sertoli cells	S1-NPA, Western	FSH ⇑	552
b-FGF	Rat	PMC	S1-NPA, Western		552
b-FGF	Rat	SpC	IHC		554
b-FGF	Rat	Leydig cells	IHC, S1-NPA		555
b-FGF	Mouse	Testis extract	Western		554
b-FGF	Mouse	SpC	IHC, S1-NPA		554
b-FGF	Bovine	Testis extract	RIA, bioassay		557, 558
b-FGF	Human	Testis extract	Bioassay		558
FGF-6	Mouse	Testis extract	Northern		559
GnRH	Rat	Testis extract	RRA, Bioassay, RIA		67, 68–70
GnRH	Rat	IF	RIA, Bioassay	hCG ⇑	63
GnRH	Rat	ST	RRA, Bioassay		64
GnRH	Rat	Sertoli cells	RRA, Bioassay, ISH		72
GnRH	Rat	Germ cells	IHC, ISH		66, 72
GnRH	Monkey	ST	RRA, Bioassay		65
GnRH	Human	Germ cells	ISH		72
GHRH	Rat	Testis extract	ELISA, Northern, Bioassay		23
GHRH	Rat	Sperm cells	IHC		24
GHRH	Rat	Leydig cells	IHC, RIA	hCG ⇑	26, 28, 30
GHRH	Rat	SpC	ISH, Northern		27
GHRH	Rat	Round SpT	ISH, Northern		27
GHRH	Mouse	Testis extract	Northern		28
GHRH	Mouse	Leydig cells	IHC		28
GRP	Rat	Leydig cells	RIA/HPLC, IHC	hCG ⇔	643
GRP	Human	Leydig cells	IHC		644
IGF-I	Rat	Testis extract	RIA, Northern, Dot-blot	LH, FSH, GH ⇑	25, 398–409, 411
IGF-I	Rat	PMC	RIA, immunofluorescence		399, 404
IGF-I	Rat	Sertoli cells	RIA, RRA, bioassay	FSH ⇑	396, 399, 400
IGF-I	Rat	Leydig cells	RIA	LH ⇑, GH ⇑, IL-1 ⇓	399, 412, 690
IGF-I	Rat	SpC, SpT	IHC		406–408
IGF-I	Mouse	Interstitium, SpT	ISH		414
IGF-I	Pig	Testis extract	Northern, Dot-blot		413
IGF-I	Pig	Leydig cells	RIA	LH, FGF ⇑	415
IGF-I	Pig	Sertoli cell	RIA, RRA	FSH, FGF, EGF ⇑	401–403, 415
IGF-I	Human	Sertoli cells	IHC		420
IGF-I	Human	SpC	IHC		419, 420
IGF-I	Human	Leydig cells	IHC		419, 420
IGF-II	Rat	Testis extract	Northern		25
IGF-II	Pig	Testis extract	Northern		413
IGF-II	Human	PMC, bv	ISH		421
IL-1	Rat	Testis homogenate	Bioassay/HPLC		653
IL-1	Rat	ST, ST cm	Bioassay/HPLC		653, 654
IL-1	Rat	ST cm	Bioassay		655
IL-1	Rat	IF	Bioassay/HPLC		657
IL-1	Rat	Sertoli cells	Bioassay	LPS, RB/CeSpT ⇑	658, 661
IL-1	Rat	Leydig cells	Northern	IL-1β, LPS, hCG ⇑	438, 659
IL-1	Rat	Macrophages	Bioassay		662
IL-1	Human	Testis cytosol	Bioassay		656
IL-6	Rat	Sertoli cells	Bioassay	LPS, RB/CeSpT, IL-1α, FSH ⇑	663
IL-6	Rat	Leydig cells	Bioassay, Northern	hCG, IL-1β ⇑	664
IL-6	Mouse	Sertoli cells	Bioassay	IL-β ⇑, IL-1α ⇑, TNF-α ⇑, LPS ⇑, IFN-γ ⇓	665
β-NGF	Mice	Testis extract	Northern, bioassay		604

TABLE 2. Continued

Substance	Species	Source/localization	Detected by	Modulation	Ref.
β-NGF	Mice	SpC	IHC, ISH		603, 604
β-NGF	Mice	SpT	ISH, IHC		603
β-NGF	Rat	Testis extract	Northern, bioassay		604
β-NGF	Rat	Germ cells	IHC, ISH		604
β-NGF	Human	Testis extract	EIA		606
β-NGF	Human	Germ cells	Immunofluorescence		608
NPY	Rat	Leydig cells	RT-PCR, Northern, ICC	LH, IL-1α, IL-1β ⇑	318
NPY	Rat	Sertoli cells	RT-PCR, Northern, ICC	FSH ⇑	318
OT	Rat	Testis extract	RIA, PCR		152, 164, 172
OT	Rat	Leydig cells	IHC, ICC, RIA	LH ⇑, T ⇑	156, 159, 160, 162
OT	Mouse	Testis extract	RIA		153
OT	Dog	Testis extract	RIA		153
OT	Cat	Testis extract	RIA		153
OT	Bovine	Testis extract	RIA, Northern		154
OT	Bovine	Sertoli cells	ISH		165, 167
OT	Bovine	Leydig cells	IHC		167
OT	Rabbit	Testis extract	RIA		153
OT	Guinea pig	Leydig cells	RIA		161
OT	Human	Testis extract	RIA, PCR		157, 168
PACAP	Rat	Testis extract	RIA, Northern		47, 48, 51
PACAP	Rat	SpG, SpC, SpT	IHC ISH		49, 50
PACAP	Human	cDNA	cDNA cloning		53
PRP	Rat	Testis extract	Northern		52
PRP	Mice	Testis extract	Northern		52
PRP	Human	Testis extract	Northern		52
PRP	Bovine	Testis extract	Northern		52
PDGF	Rat	Testis extract	Northern		591
PDGF	Rat	Leydig cells	RRA, Northern, IHC	hCG ⇑	591, 595
PDGF	Rat	Sertoli cells	RRA, Northern, bioassay	FSH ⇓	591
PModS	Rat	PMC	RP-HPLC	T ⇑	387
SRIF	Rat	Testis extract	RIA/C	Hypophysectomy ⇓	224
SRIF	Human	Testis extract	RIA/C		225
SLF	Mouse	Sertoli cells	Northern, PCR	cAMP, FSH ⇑	621, 623
SLF	Mouse	Sertoli cells	Bioassay	cAMP ⇑, FSH ⇔	622, 624
SLF	Rat	Sertoli cells	Northern	GHRH ⇑, GHRH-RP ⇑	32
SLF	Human	Sertoli cells, Leydig	IHC		625
SP	Human	Testis extract	PCR		312
SP	Human	Leydig cells	IHC, ICC		306–308
SP	Hamster	Leydig cells	IHC	309, 310	
SP	Guinea pig	Leydig cells	IHC		310
SP	Mouse	Testis extract	PCR		312
SP	Bovine	Testis extract	PCR		312
TGFα	Rat	Sertoli cell	Northern, Western		494
TGFα	Rat	PMC	Northern, Western, ICC		494, 496
TGFα	Rat	Leydig cells	IHC		496
TGFβ	Rat	Sertoli cells	RRA, Northern, PA, Western, IHC	FSH ⇓	468, 470, 472
TGFβ	Rat	PMC	RRA, Northern, PA, Western		468, 470
TGFβ	Rat	Leydig cells	IHC		472
TGFβ	Rat	SpT, SpC	IHC		472
TGFβ	Pig	Sertoli cells	RRA, Bioassay		473
TGFβ	Mouse	Testis extract	Northern	FSH ⇓, E ⇑, Dex ⇑, T4 ⇑	469
TGFβ	Mouse	Germ cells	Northern		471
TNFα	Mouse	SpT	Bioassay, Northern, ISH		710
TNFα	Mouse	SpC	Northern, ISH		710
TNFα	Rat	Macrophages	Bioassay	LPS ⇑	711, 712
TRH	Rat	Testis extract	RIA, RIA/HPLC	Hypothyroidism ⇑	210, 212, 213
TRH	Rat	Leydig cells	IHC		214, 215
TRH	Dog	Testis extract	RIA/HPLC		211
ppTRH	Rat	Testis extract	Northern		214
ppTRH	Rat	Leydig cells	Northern		214
ppTRH	Human	Testis extract	Northern		217

⇑, Stimulates or increases; ⇓, inhibits or decreases; ⇔, no effect; IHC, immunohistochemistry; ICC, immunocitochemistry; S1-NPA, S1-nuclease protection assay; RNase PA, RNase protection assay; ISH, in situ hybridization; Northern, Northern blot analysis; Western, Western blot analysis; PA, protection assay; C, chromatography; GC, gel chromatography; ELISA, enzyme-linked immunosorbent assay; EIA, enzyme immunometric assay; ST, seminiferous tubule; TF, tubular fluid; IF, interstitial fluid; PMC, peritubular myoid cells; SpG, spermatogonia; SpC, spermatocytes; SpT, spermatids; SpZ, spermatozoa; eSpZ, elongated spermatozoa; bv, blood vessels; cm, conditioned medium; Dex, dexamethasone; T, testosterone; IFN-γ, interferon-γ; PRP, PACAP-related peptide; RB/CeSpT, residual bodies and cytoplasts from elongated spermatids; LPS, lipopolysaccharide.

TABLE 3. Receptors for regulatory peptides in the mammalian testis

Receptor	Species	Localization	Detected by	Modulation	Ref.
ActRII, IIB	Mouse	Testis extract	Northern		330, 331
ActRII, IIB2	Rat	Sertoli cells	Northern, ISH		359, 361
ActRII, IIB	Rat	Leydig cells	Northern, ISH		359, 360
ActRII	Rat	SpC/SpT	Northern, ISH		359, 360
ActRIIB2	Rat	SpG	ISH		361
ActRII	Rat	PMC	Northern		346
ANP	Rat	Leydig cells	LB, AR		812
ANP	Rat	Testis membranes	Cross-linking		813, 814
ANP	Mouse	Testis membranes	Cross-linking		813, 814
ANP	Mouse	Leydig cells	LB		811, 823
ANP	Mouse	Leydig tumor cells	LB, AL		808–810, 815, 816
AT-II	Rat	Leydig cells	LB, AR		790–792
AT-II	Monkey	Leydig cells	AR		790
AT-II	Human	Leydig cells	AR		790
AVP	Rat	Leydig cells	LB, AR	LH or GH ⇑	196, 199, 200
AVP[a]	Rat	PMC	LB, Northern		173
AVP	Mouse	Leydig cells	LB		180
CRH	Rat	Testis membranes	LB	Adrenalectomy ⇑	135, 137
CRH	Rat	Leydig cells	LB		136
CRH	Human	Testis extract	PCR		141
βEND	Rat	Testis membranes	LB		259
βEND	Rat	Sertoli cells	LB		260
EGF/TGFα	Mouse	Leydig tumor cells	LB, Western		500
EGF/TGFα	Mouse	Testis membranes	Western		506
EGF/TGFα	Mouse	Leydig cells, Sertoli cells	IHC		506
EGF/TGFα	Rat	Leydig cells	LB		501
EGF/TGFα	Rat	Sertoli cells	IHC, S1-NPA		495, 505
EGF/TGFα	Rat	PMC	LB, S1-NPA		495
EGF/TGFα	Rat	SpC/SpT	NPA		495
EGF/TGFα	Rat	Leydig cells precursors	LB		504
EGF/TGFα	Monkey	Testis extract	Western, Northern		507, 508
EGF/TGFα	Monkey	Sertoli cells, Leydig, PMC	IHC		505, 507
EGF/TGFα	Pig	Leydig cells	LB		502
EGF/TGFα	Human	Testis extract	LB		498, 509
EGF/TGFα	Human	Leydig cells	IHC, LB		509, 510
EGF/TGFα	Human	Sertoli cells	IHC		510
EGF/TGFα	Human	PMC	IHC		510
ET	Rat	Leydig cells	LB, AR (ETA)		733, 743
ET	Rat	Sertoli cells	LB		733
ET	Rat	PMC	LB, AR		738, 739, 742, 743
ET	Bovine	Testis extract	LB (ETA)		740
ET	Mouse	Leydig tumor cells	LB		737
ET	Human	Testis extract	LB		736
ET	Human	SpC/SpT, Leydig cells, PMC	IHC		736
ET	Marmoset	Testis extract	Northern (ETA, ETB)	High-fat diet ⇑	741
b-FGF	Rat	Testis neonatal cells	LB		560
b-FGF	Rat	Leydig cells	LB, IHC		555, 561
b-FGF	Rat	Sertoli cells	IHC, Western		555
b-FGF	Rat	SpG/SpC	IHC, Western		555
b-FGF	Pig	Leydig cells	LB		562
GnRH	Rat	Leydig cells	LB, AR		75–79
GnRH	Human	Leydig cells	IHC		72
GHRH	Rat	Leydig cells	LB		30
GHRH	Rat	Sertoli, germ, Leydig cells	RT-PCR	GHRH in Sertoli ⇑	35
GRP[b]	Rat	SpC	ISH		641
SLF (c-*kit*)	Mouse	SpG, SpC (13-18d)	Northern		627
SLF (c-*kit*)	Mouse	SpG, Leydig cells	ISH		629
SLF (c-*kit*)	Rat	SpG (9d)	Northern, ICC, Western		630
SLF (c-*kit*)	Human	Leydig cells, SpG	IHC		625
SLF (c-*kit*)	Human	SpT and SpZ acrosome	IHC		625
IGF-I	Rat	Sertoli cells	AL, LB, AR		423, 424, 399
IGF-I	Rat	Pachytene SpC	AR		399
IGF-I	Rat	Leydig cells	LB, RPA	LH, GH, FSH[c] ⇑	426–428, 430, 555
IGF-I	Mouse	Leydig cells, PMC, SpG	ISH		414
IGF-I	Human	Germinal epithelium	ISH, IHC		421
IGF-I	Human	SpC/SpT, Sertoli and Leydig	IHC		419

TABLE 3. Continued

Receptor	Species	Localization	Detected by	Modulation	Ref.
IGF-I	Pig	Sertoli cells	LB, AL		429
IGF-II	Rat	Sertoli cells	LB, AL		423
IGF-II	Rat	Germinal epithelium	ISH		432
IGF-II	Human	Germinal epithelium	ISH		421
IGF-II	Human	Whole testis	Western		433
IGF-II	Mouse	Sertoli cells	LB		431
IGF-II	Mouse	GC	LB		431
IL-1	Mouse	Testis membranes	LB		666
IL-1	Mouse	Leydig cells	AR, ISH		668, 669
NGF	Mouse	Testis extract	Northern		604
NGF	Rat	Testis extract	Northern	Testosterone, hCG ⇓	607, 609
NGF	Rat	Sertoli cells	ISH, Northern		605, 607
NGF	Human	Sertoli cells	IF		608
NGF	Human	Lamina propria, PMC	IHC		606
OT	Rat	Leydig cells	LB, AR		170–172
PACAP	Rat	GC	AR		54
PACAP (R1)	Rat	Testis extract	RT-PCR		56
PACAP (R2)	Human	Testis extract	Northern		57
PDGF	Rat	Leydig cells	LB, Northern, IHC, ICC		591, 595
PDGF	Rat	PMC	LB, Northern, IHC		591, 596
PDGF	Rat	Sertoli cells	Northern		592
SP	Human	Testis extract	PCR		312
SRIF	Human	Testis extract	Southern		228
TGFβ	Pig	Leydig cells	AL		474
TGFβ	Pig	Sertoli cells	LB, AL		475
TGFβ	Rat	Leydig, Sertoli, PMC, GC	Northern		476
TNFα	Mouse	Sertoli cells, Leydig cells	Northern		709
TNFα	Pig	Leydig cells	LB		713
TRH	Rat	Whole testis	In vivo binding		218
TRH	Rat	Testis extract	RT-PCR, Northern, LB		218–220
TRH	Rat	Leydig cells	RT-PCR		219

⇑, Increases; ⇓, decreases; Northern, Northern blot analysis; Southern, Southern blot analysis; S1-NPA, S1-nuclease protection assay; IHC, immunohistochemistry; ISH, *in situ* hybridization; LB, ligand binding; AR, autoradiography; RPA, ribonuclease protection assay; AL, affinity labeling; PMC, peritubular myoid cells; SpG, spermatogonia; SpC, spermatocytes; SpT, spermatids; GC, germ cells.

[a] AVP receptor type 1a.

[b] GRP receptor type BRS-3.

[c] Administration to hypophysectomized rats, binding to isolated Leydig cells.

primary site of GHRH-immunoreactive staining was the Leydig cells (28). In line with these results, authentic GHRH under positive gonadotropin control is actively released from cultured adult rat Leydig cells (30).

Interestingly, the GHRH mRNA found in the rat testis contains an exon 1 sequence different from that found in hypothalamus or placenta, and the initiation of GHRH transcription in the testis begins approximately 700 bp 5′ to that in placenta and 10.7 kbp 5′ to that in hypothalamus (31). Thus, the GHRH transcripts in each tissue have distinct exon 1 sequences and may use different promoters, suggesting that as yet unidentified spermatogenic-specific transcription factors may bind to the promoter region of testicular GHRH to regulate its expression (31).

Recently, a novel peptide, the putative 30-aa C-terminal peptide of the GHRH precursor, called GHRH-related peptide, has been found in abundance in adult rat germ cells by immunohistochemistry (32). Specific staining predominated in stage IV seminiferous tubules, in pachytene spermatocytes, and in the acrosomes of spermatids (32).

Moretti *et al.* (33) found immunoreactive GHRH-like material in human testis. They noted intense staining of the interstitial compartment with localization to the Leydig cells. The presence of GHRH-like peptides in human testicular tissue has been further analyzed by means of enzyme-linked immunosorbent assay (ELISA) and Northern blot of adult testis extracts (34). Both methodologies confirmed that the human testis is an extrahypothalamic site of expression for both immunoreactive GHRH-like peptides and GHRH gene.

2. Receptors. It has been shown that GHRH acts on the Leydig cells via a vasoactive intestinal peptide (VIP) receptor, directly stimulating cAMP production (30). Recently, the GHRH receptor mRNA has been detected in rat Sertoli cells and at lower levels in germ cells and Leydig cells; moreover, the treatment of Sertoli cells with GHRH has been found to increase the GHRH receptor expression in a dose-dependent manner (35).

3. Local functions. GHRH has direct actions on the Sertoli cell, including stimulation of cAMP production and c-*fos* and steel factor (SLF) mRNA expression (36). Accordingly, GHRH has been found to increase basal and FSH-stimulated cAMP formation in cultured adult and pubertal Sertoli cells with a

TABLE 4. Effects of the locally produced regulatory peptides on the testis *in vitro*

Substance	Species	System (age)	Effect	Ref.
ACT	Rat	Interstitial cells culture (7 d)	⇓ LH stimulated T production	272
ACT	Rat	Leydig cells culture (A)	⇓ hCG stimulated T production, not basal	370
ACT	Rat	Sertoli/germ cells coculture (23 d)	⇑ Germ cells proliferation	372
ACT	Rat	ST segments culture (A)	⇑ DNA synthesis	373
ACT	Rat	Leydig tumor cells culture	⇓ Proliferation and P accumulation	376
ACT	Rat	Sertoli cells culture (21 d)	⇓ FSH induced aromatase activity	345
ACT	Rat	Sertoli cells culture (21 d)	⇓ Basal and FSH stimulated AR mRNA	345
ACT	Pig	Leydig cells culture (21 d)	⇓ hCG stimulated DHEA accumulation	371
INH	Rat	Interstitial cells culture (A)	⇑ LH stimulated T production	272
INH	Rat	Interstitial cells culture (7 d)	⇑ LH stimulated T production	272
INH	Rat	Leydig cells culture (A)	⇔ On hCG stimulated and basal T	370
INH	Rat	ST segments culture	⇓ DNA synthesis	373
ANP	Rat	Leydig cells culture (A)	⇔ On T secretion, ⇑ cGMP levels	818
ANP	Rat	Leydig cells culture (A)	⇑ T secretion, Ca^{++} fluxes	821
ANP	Rat	Sertoli cells culture (20 d)	⇑ cGMP levels	392
ANP	Mouse	Interstitial cells culture (A)	⇑ T production	817
ANP	Mouse	Leydig cells culture (A)	⇑ Basal and hCG stimulated T production	811, 818–820
ANP	Mouse	Leydig tumor cells culture	⇓ hCG stimulated P secretion	822
AT-II	Rat	Leydig cells culture (A)	⇓ Basal and hCG-stimulated cAMP and T	791
AVP	Rat	Testicular cells culture (A)	⇓ hCG stimulated T production	176
AVP	Rat	Testicular cells culture (A)	⇓ hCG stimulated T production	203
AVP	Rat	Testicular cells culture (A)	⇑ hCG stimulated P production	203
AVP	Rat	Leydig cells culture (A)	⇓ LH stimulated T production	183
AVP	Mouse	Leydig cells culture (10-95 d)	⇑ T production	180
βEND	Rat	Sertoli cells culture (20 d)	⇓ FSH stimulated INH production	271
βEND	Rat	Sertoli cells culture (A)	⇓ Basal and FSH stimulated ABP production	260
βEND	Rat	Fetal organ culture (20 d)	⇓ Basal and FSH stimulated Sertoli cells proliferation	273
βEND	Rat	Sertoli cells culture (6 d)	⇓ FSH stimulated Sertoli cells proliferation	274
CRH	Rat	Leydig cells culture (A)	⇑ βEND secretion, ⇓ basal and hCG stimulated T and cAMP production	132, 142, 136, 138
CRH	Mouse	Leydig cells culture (A)	⇑ Basal T and cAMP production	143
DYN	Rat	Leydig cells (A)	⇔ Basal and hCG stimulated T production	270
EGF	Rat	Leydig cells culture (A)	⇓ hCG stimulated T production	512
EGF	Rat	Leydig cells culture (A)	⇓ 17α-hydroxylase and 17,20lyase activities	501
EGF	Rat	Leydig cells culture (A)	⇑ C-19/C-21 steroids	513
EGF	Rat	Leydig cells culture (15 d)	⇑ C-19/C-21 steroids	513
EGF	Rat	Sertoli cells culture (7-31 d)	⇑ ABP production (decrease with age)	521
EGF	Rat	Sertoli cells culture (20 d)	⇑ INH production	342
EGF	Rat	Sertoli cells culture (16 d)	⇑ Lactate production	519
EGF	Rat	Sertoli cells culture (16 d)	⇓ FSH stimulated E synthesis	519
EGF	Rat	Sertoli cells culture (20 d, A)	⇑ Transferrin and ABP secretion	522–525
EGF	Rat	ST cultures (A)	⇑ INH production	518
EGF	Rat	Sertoli-SpGenic cell coculture (20 d)	⇑ ODC activity	526
EGF	Rat	PMC culture (20 d)	⇑ ODC activity	526
EGF	Mouse	Leydig tumor cells culture	⇓ hCH receptors and hCG stimulated steroidogenesis	500
EGF	Mouse	Leydig cells culture (A)	⇑ C-19/C-21 steroids	513
EGF	Mouse	Leydig cells culture (15 d)	⇑ C-19/C-21 steroids	513
EGF	Mouse	Leydig tumor cells culture	⇓ hCH/LH receptor mRNA	511
EGF	Mouse	Leydig tumor cells culture	⇑ T (acute treatment)	515
EGF	Mouse	Testis fragments (A)	⇓ IFSH stimulated SpGA proliferation	529
EGF	Pig	Leydig cells culture (21 d)	⇑ hCG stimulated T production	504
			⇓ hCG stimulated DHEA production	504
EGF	Pig	Leydig cells culture (21 d)	⇑ T (acute treatment)	514
			⇓ hCH receptors (long term treatment)	514
EGF	Pig	Sertoli cells culture (21 d)	⇑ DNA synthesis	439
EGF	Pig	Sertoli cells culture (21 d)	⇑ Lactate production	528
EGF	Bovine	Testis membranes (immature)	⇓ FSH binding	520
EGF	Human	Leydig cells culture (A)	⇑ Basal T production, not hCG stimulated	450
ET	Mouse	Leydig tumor cells culture	⇑ Progesterone production	737
ET	Mouse	Leydig tumor cells culture	⇑ c-*jun* and c-*myc* mRNA expression	737
ET	Rat	Leydig cells culture (A)	⇑ Basal and hCG stimulated T production	743, 744
ET	Rat	PMC culture (20 d)	Induces morphological changes	739
ET	Rat	PMC culture (18 d)	⇑ DNA synthesis and contraction	742
ET	Rat	Sertoli cells culture (9 d)	⇓ FSH stimulated cAMP and E accumulation	745
b-FGF	Rat	Leydig cells culture (8 d)	⇓ LH stimulated T production	560, 565
b-FGF	Rat	Leydig cells culture (25 d)	⇓ 5α-Reductase activity	565
b-FGF	Rat	Leydig cells culture (25 d)	⇓ 3β-HSD activity, hCG binding (low conc.)	566, 567
b-FGF	Rat	Leydig cells culture (25 d)	⇑ 3β-HSD activity, hCG binding (high conc.)	566, 567

TABLE 4. Continued

Substance	Species	System (age)	Effect	Ref.
b-FGF	Rat	Sertoli cells culture (20 d)	⇑ c-*fos* mRNA	569
b-FGF	Rat	Sertoli cells culture (20 d)	⇑ Transferrin mRNA and secreted protein	555
b-FGF	Rat	PMC culture (20 d)	⇑ PAI-1 mRNA expression and secretion	516
b-FGF	Rat	Whole testicular cells culture (A)	⇔ On basal and hCG stimulated T	501
b-FGF	Mouse	Sertoli tumor cells culture	⇑ Sulfated glycoprotein-1 mRNA	570
b-FGF	Pig	Leydig cells culture (25 d)	⇑ LH stimulated T production	562
b-FGF	Pig	Leydig cells culture (21-28 d)	⇑ Aromatase activity	564
b-FGF	Pig	Leydig cells culture (21 d)	⇑ IGF-I secretion	514
b-FGF	Pig	Sertoli cells culture (21 d)	⇑ IGF-I secretion	514
b-FGF	Pig	Leydig cells culture (21 d)	⇑ c-*jun* mRNA, ⇑ stimulatory action of hCG on c-*fos* and *jun*-B	568
b-FGF	Pig	Sertoli cells (21 d)	⇑ DNA synthesis and proliferation	439
a-FGF	Rat	Leydig cells culture (25 d)	⇓ 3β-HSD, hCG-stimulated 5α-reductase activity, hCG binding	567
GnRH	Rat	Leydig cells culture (A)	⇓ hCG stimulated A-diol and T production in long incubation	92, 93, 102
GnRH	Rat	Leydig cells culture (A)	⇑ T production in short incubation	99, 102
GnRH	Rat	Leydig cells culture fetal (20.5 d)	⇓ LH stimulated T production	81
GnRH	Rat	Decapsulated testis (1-60 d)	⇑ T production in short incubation	82
GnRH	Monkey	Interstitial cell culture (A)	⇔ Basal and hCG stimulated T secretion	120
GnRH	Human	Organ culture (A)	⇔ On steroidogenesis	118
GHRH	Rat	Sertoli cells culture (4-60 d)	⇑ Basal and FSH stimulated cAMP more in prepubertal than adult cultures	26
GHRH	Rat	Sertoli cells culture (20 d)	⇑ c-*fos* and SLF mRNAs expression	36
GHRH	Rat	Leydig cells culture (A)	⇑ LH stimulated T and cAMP production	30
IGF-I	Rat	Sertoli cells culture (13 d)	⇑ DNA synthesis	423
IGF-I	Rat	Leydig cells culture (34 d-A)	⇑ Basal and hCG stimulated T production higher in pubertal than adult	427, 444, 448
IGF-I	Rat	Leydig cells culture (A)	⇑ hCG stimulated T production, not basal	445
IGF-I	Rat	ST segments culture (A)	⇑ SpG DNA synthesis	442
IGF-I	Pig	Sertoli cells culture (21 d)	⇑ DNA synthesis, proliferation	439
IGF-I	Pig	Leydig cells culture (21 d)	⇑ Basal and hCG stimulated T production LH/hCG binding	401, 429, 440, 402
IGF-I	Human	Leydig cells culture (A)	⇑ hCG stimulated T production	450
IGF-II	Rat	Sertoli cells culture (13 d)	⇑ DNA synthesis, lactate production	423
IGF-II	Rat	ST segments culture (A)	⇑ SpG DNA synthesis	442
IL-1	Rat	Leydig cells culture (A)	⇑ hCH binding and T formation	671, 673
IL-1	Rat	Leydig cells culture (A)	⇑ Basal and hCG stimulated T production	679
IL-1α	Rat	Leydig cells culture (A)	⇔ On basal and hCG stimulated T	680
IL-1α	Mouse	Leydig cells culture (A)	⇓ cAMP stimulated T production	674
IL-1α	Pig	Leydig cells culture (21-28 d)	⇓ hCG and cAMP stimulated T production	678
IL-1β	Rat	Leydig cells culture (9 d)	⇓ hCG stimulated T production ⇑ P	677
IL-1β	Rat	Leydig cells (19 d)	⇑T production (short term)	670
IL-1β	Rat	Leydig cells (19 d)	⇓ LH stimulated T production (long term)	670
IL-1β	Rat	Leydig cells culture (A)	⇓ hCG stimulated T production and P450sec	676
IL-1β	Rat	Leydig cells culture (A)	⇓ hCG stimulated T production	671
IL-1β	Rat	Leydig cells culture (A)	⇔ On basal and hCG stimulated T	680
IL-1β	Rat	Leydig cells culture (10 d-20 d-A)	⇑ proliferation of immature cells, not A	689
IL-1β	Rat	Leydig cells culture (A)	⇑ IL-1α mRNA expression	659
IL-1β	Rat	Leydig cells culture (A)	⇓ IGF-I mRNA expression	690
IL-1β	Rat	Sertoli cells culture (7-10 d)	⇓ FSH stimulated aromatase activity	681
IL-1β	Rat	Sertoli cells culture (19 d)	⇑ Transferrin secretion	682
IL-2	Rat	Leydig cells culture (A)	⇓ hCG stimulated T production	683
IL-2	Rat	Sertoli cells culture (A)	⇑ Transferrin secretion	692
IL-2	Mouse	Leydig cells culture (A)	⇓ LH stimulated T production	684
IL-6	Rat	Sertoli cells culture (A, 19 d)	⇑ Transferrin secretion	692, 682
NGF	Human	ST cultures (A)	Maintain normal morphology of the seminiferous epithelium	611
NGF	Rat	ST cultures (A)	⇑ DNA synthesis at the onset of meiosis	605
OT	Rat	ST Leydig cells depleted (A)	⇑ Contractility	158
OT	Rat	Testicular cells culture (A)	⇓ hCH stimulated T production	175
OT	Rat	Leydig cells culture (A)	⇔ On T production	181
OT	Rat	Leydig cells short term culture (A)	⇑ T production	182
OT	Rat	Perfused testes (A)	⇓ T production	178
OT	Rat	Testicular microsomes (A)	⇓ Androgens biosynthesis	179
OT	Mouse	Leydig cells culture (10-95 d)	⇑ T production	180
PACAP	Rat	Sertoli cells culture (15-60 d)	⇑ Sertoli cells cAMP, lactate, E, INH secretion	58
PACAP	Rat	SpC culture (A)	⇑ Protein synthesis	59
PACAP	Rat	SpT culture (A)	⇓ Protein synthesis	59

TABLE 4. Continued

Substance	Species	System (age)	Effect	Ref.
PDGF	Rat	Leydig cells culture (25 d)	⇓ hCG-stimulated 5α-reductase activity	565
PDGF	Rat	Leydig cells culture (25 d)	⇓ 3β-HSD activity, ⇓ hCG stimulated T formation	563
PDGF	Rat	Leydig cells culture (A)	⇑ LH stimulated T production	598, 599
PDGF	Rat	PMC culture (20 d)	⇑ ODC activity after 6h exposure	400
PDGF	Rat	PMC culture (17 d)	⇑ Proliferation, ⇑ release of collagen laminin, fibronectin	596
PDGF	Rat	PMC culture (5 d)	Induces chemotaxis	591
PDGF	Rat	PMC culture (20 d)	⇑ TGFβ induced contraction	483
PDGF	Rat	Sertoli/SpGen cells coculture (20 d)	⇑ ODC activity with EGF after 6h exposure	400
PDGF	Rat	Sertoli/SpGen cells coculture (20 d)	⇓ ODC activity after 24 h exposure	400
PModS	Rat	Sertoli cells culture (20 d)	⇑ Transferrin, ABP, and INH secretion	385, 388, 389
PModS	Rat	Leydig cells culture (A)	⇔ On T and INH production	390
SLF	Mouse	SpG (8 d)	⇑ DNA synthesis, thymidine incorporation	623
SP	Rat	Sertoli cells culture (21 d)	⇓ Transferrin and lactate release, ⇔ on aromatase activity	314
SP	Hamster	Leydig cells culture immatu	⇑ T production	312
SP	Hamster	Leydig cells culture (A)	⇑ Basal and hCG stimulated T production	312
SP	Hamster	Leydig cells culture (young and A)	⇑ LH binding in young, ⇓ LH binding in A	313
TGFα	Rat	Leydig cells culture (21 d)	⇑ DNA synthesis	441
TGFα	Rat	PMC culture (5-60 d)	⇑ Proliferation, migration	494, 495
TGFα	Rat	Sertoli cells culture (20 d)	⇔ On growth and transferrin production	494
TGFα	Rat	Sertoli/PMC cells coculture (20 d)	⇑ Transferrin production	494
TGFβ	Rat	Leydig cells culture (8 d)	⇓ LH stimulated T production	480
TGFβ	Rat	Leydig cells tumor culture	⇓ Growth and steroidogenesis	376
TGFβ	Rat	PMC culture (20 d)	⇑ Production proteins, migration, colony-formation	468
TGFβ	Rat	PMC culture(20 d)	⇑ Contractility	482, 483
TGFβ	Rat	PMC culture (10-35 d)	⇓ TGFα-induced DNA synthesis	470
TGFβ	Pig	Leydig cells culture (21 d)	⇓ LH-stimulated T production, LH-binding sites, ⇔ on proliferation	474, 478
TGFβ	Pig	Leydig cells culture (21 d)	⇑ LH-stimulated T production (low concentration)	474, 481
TGFβ	Pig	Sertoli cells culture (21 d)	⇑ Lactate production	475
TGFβ	Human	Spermatozoa	⇑ Expression of a 50 kDa protein	484
TNFα	Rat	Leydig cells culture (A)	⇑ Basal and LH stimulated T secretion	679
TNFα	Rat	Leydig cells culture (A)	⇔ On basal and LH stimulated T secretion ⇑ Inhibitory effect of IL-1β	715
TNFα	Rat	PMC culture(20 d)	⇑ PAI-1 mRNA expression and secretion	516
TNFα	Pig	Leydig cells culture (21 d)	⇓ hCG-stimulated but not basal T secretion	713
TNFα	Mouse	Leydig cells culture (A)	⇓ Basal and cAMP-stimulated T secretion	716
TRH	Rat	Leydig cells culture (A)	⇓ hCG-stimulated T secretion	208

⇑, Stimulate or increase; ⇓, decrease or inhibit; ⇔, absence of effect.

higher potency in pubertal than in adult cultures (26). Moreover, it has recently been shown that GHRH-related peptide specifically activates the expression of SLF by Sertoli cells to a higher extent than GHRH without increasing intracellular cAMP levels, or transferrin, androgen binding protein (ABP), or inhibin (INH) α-subunit transcripts (32).

Spontaneous and transgenic mutants may help to clarify the roles of the peptide described above. Mice homozygous for the spontaneous *little* (*lit*) gene mutation are normally proportioned dwarfs (37) carrying a missense mutation of the gene encoding for the GHRH receptor (38, 39). Both male and female *little* mice exhibit delayed sexual maturation (37). Although fertility in *little* males was initially reported to be reduced (37), this has subsequently been shown to be diet related (38), and the allometric reduction of all reproductive organs with body size was not associated with impairment of steroidogenesis or spermatogenesis (40) or with fertility (41). However, direct examination of GHRH and GHRH receptor expression in the gonads of *little* mice is lacking and, therefore, whether or not this animal model might provide further clues toward understanding the *in vivo* roles of GHRH in the testis is uncertain.

On the other hand, the expression of human GHRH in transgenic mice results in elevations of serum GH and stimulation of linear growth (42). Both males and females carrying the GHRH fusion gene are fertile and transmit the gene. These data appear to exclude the severe GHRH-mediated consequences for male reproductive physiology. However, whether this lack of testicular effect in both overexpressing and nonexpressing GHRH applies also to animals other than mice is not predictable.

B. Pituitary adenylate cyclase-activating peptide (PACAP)

PACAP is a novel member of the secretin/glucagon/VIP/GHRH family of peptides that was originally isolated from ovine hypothalamic tissues based on its ability to stimulate the accumulation of cAMP in rat pituitary cell culture (43). PACAP exists in two forms: a longer form of 38-aa residues (PACAP-38) and a shorter one (PACAP-27) corresponding to the amino-terminal 27 residues of PACAP-38 (44). Both PACAPs are derived from a 175-aa precursor, which in addition gives rise to a 29-aa peptide designated PACAP-related peptide (PRP) (45). To date there is no known function for PRP.

TABLE 5. Effects of regulatory peptides on the mammalian testis *in vivo*

Substance	Species (age)	Administration/model	Effect	Ref.
ANP	Human (A)	iv, single injection	⇑ Spermatic vein T levels	825
AVP	Rat (A)	IT injection	⇔ On T production	181
AVP	Rat hypox (A and 22 d)	sc 5 days + FSH sc	⇓ Testicular hCG receptors content and androgen biosynthesis	204
AVP	Rat (A)	Brattleboro strain AVP-deficient	⇔ On basal and hCG stimulated plasma T nor basal or stimulated T from isolated Leydig cells ⇑vs. control rats	206
AVP	Rat (A)	IT injection	⇓ Testicular blood flow	205
CRH	Rat (5 d)	IT injection of anti-CRH-antiserum	⇓ Serum T concentration, and T production of the treated testis *in vitro*	144
CRH	Rat (pubertal or A)	IT infusion for 2 h	⇑ βEND in IF of pubertal animals ⇔ in the adult	145
CRH	Monkey (A)	iv infusion, 4 h	⇓ Serum T concentration	146
EGF	Mouse (A)	ip, single injection	⇑ Testicular DNA synthesis	517
EGF	Mouse (8 d)	sc, single injection	⇑ Testicular ornithine decarboxylase	527
EGF	Mouse (A)	Sialoadenectomy-induced decrease of circulating EGF	⇓ Spermatogenesis without affecting plasma levels of T and FSH	530
EGF	Mouse (A)	Streptozotocin-induced diabetes with reduced circulating EGF	⇓ Spermatogenesis reversed by EGF administration	531
EGF	Mouse (A)	Sialoadenectomy induced decrease of circulating EGF	⇓ Sperm counts, motility and fertility with ⇑ of IT and plasma levels of T	532
EGF	Mouse (A)	Sialoadenectomy induced decrease of circulating EGF	No reduction of EGF, ⇔ on fertility	533
EGF	Mouse (A)	Sialoadenectomy induced decrease of circulating EGF	⇔ On fertility parameters	534
βEND	Rat (5 d)	IT injection of nal or nalm	⇑ Test hypertrophy, ⇑ ABP, ⇓ T	265
βEND	Rat hypox (A)	IT injection of β-EP	⇓ T response to LH	266
βEND	Rat (5 d)	IT injection of β-EP antiserum	⇑ Sertoli cell proliferation	273
ENK	Rat (5 d and 10 d)	IT injection	⇓ Basal T secretion of the removed testis *in vitro*	296
ET-1	Rat (A)	IT injection	⇓ Testicular blood flow	734
GnRH	Rat hypox (22 d and A)	sc, 5 days, once a day	30–60% ⇓ testicular LH receptors content	89
GnRH	Rat hypox (A)	sc, 3 days, every 8 h	⇑ Testicular GnRH binding capacity	77
GnRH	Rat hypox (A)	sc, 6 days, every 8 h	⇑ Testicular GnRH binding capacity, ⇓ basal and hCG stimulated T secretion by testis *in vitro*	112
GnRH	Rat hypox (A)	LH, sc, 2 days, every 8 h	⇑ Testicular GnRH binding capacity prevented by LH	113
GnRH agonist	Rat fetus decapitated (18.5–20.5 d)	sc, microcapsules, 2 days	⇓ Basal and LH stimulated T secretion by testis *in vitro*	97
GnRH antagonist	Rat (A and 30 d)	IT continuous infusion, 7–14 days, minipump	⇓ 16–32% testicular T, and LH, FSH and Prl receptors	110
GnRH agonist or antagonist	Monkey (A)	Testicular artery infusion over 1 min	⇔ T concentration in the testicular vein over 120 min sampling	120
GnRH agonist	Human acquired hypogonadotropinism	sc, 4–6 days, once a day	No modification in hCG-induced T secretion	115
GnRH agonist	Human prostate cancer patients	sc, 6 days, intranasally 6 months	No difference in LH receptors and hCG induced T secretion *vs.* control untreated patients in removed testis	116
GnRH agonist	Human prostate cancer patients	sc, 1 yr, once a day	hCG treatment for 3 days completely reverses the inhibition of intratesticular steroids	117
GnRH	Ram (A)	Testicular artery infusion over 1 min	⇔ On testicular vein concentration profile of T *vs.* control not infused testis	121
IGF-I	Mice Snell dwarfs (dw/dw) (immature)	im, 7 days, every 8 h	⇑ Testicular LH receptors and acute response to hCG	451
IGF-I	Monkey (prepubertal)	im, 18 days, once a day	⇔ On T response to acute hCG stimulation	454
IL-1β	Rat (A)	IT, single injection	⇑ Leukocyte accumulation and vascular permeability	685
IL-1β	Rat (A)	ip, 3 injections, 12-h intervals	⇓ IGF-I mRNA expression in Leydig cells	690
INH	Hamster (A)	IT injection, 4 days, once a day	⇓ Number of intermediate and B SpG in the injected testis not in the contralateral testis injected with control fluid	375
NGF	Rat (A)	Continuous testicular infusion, minipump, 14 days	⇑ Testicular ABP mRNA	612
OT	Rat (A)	*In situ* testicular infusion	⇓ Plasma and testicular levels of T	177
OT	Rat (A)	IT injection	⇔ On T production	181
OT	Rat (A)	Immunization against OT	⇑ Degeneration SpC at stages XIV-I	183
SLF	Mouse (10 d, A)	ip and iv injection anti-c-*kit* antibody	Block SpGA mitosis	635
TGFα	Mouse (8 d)	sc, single injection	⇑ Testicular ornithine decarboxylase	527
TNF	Rat (A)	iv, continuous infusion, 24 h	⇓ Plasma T, ⇑ LH and FSH, damage of the germ cells	714

IT, intratesticular; IF, interstitial fluid; nal, naloxone; nalm, nalmephene; ⇑, increase; ⇓, decrease; ⇔, absence of effect.

TABLE 6. Developmental expression of regulatory peptides and regulatory peptide receptors in the rat testis, relative changes

Substance/receptor	System	Detection	Age, days					Ref.
			Fetus	0–15	16–25	26–45	A	
INH-α	ST	IHC	+	+++	+	nd	++	343
INH-α	TE	S1-NPA	nd	+++	++	++	+	321
INH-α	TE	Northern	nd	++	+++	++	+	348
INH-α	TE	RIA	nd	++++	+++	++	+	347
INH-βA	ST	IHC	−	++	++	nd	+	343
INH-βA	TE	S1-NPA	nd	++	+	+	+	321
INH-βB	ST	IHC	+	++	++	nd	+	343
INH-βB	TE	S1-NPA	nd	++	++	++	+	321
ACTRII	TE	Northern	nd	+	++	+++	nd	363
ACTRII 6-kb	TE	Northern	nd	++	++	+	+	364
ACTRII 3-kb	TE	Northern	nd	+	+	++	++	364
ACTRIIB	TE	Northern	nd	+	+	+	+	364
AT-II R	TM	LB	nd	+++	++	+	nd	790
AT-II R	TM	LB	nd	+++	++	+	nd	792
AVP	TE	Northern	nd	−	−	+	++	164
AVP-R	PMC	LB	nd	nd	−	nd	+	173
AVP-R	LC	LB	nd	nd	++	+	++	180
CRH	TE	RIA	nd	++	+	nd	+++	130
βEND	TE	RIA	nd	+	++	+++	++++	251
EGFR	TE	S1-NPA	nd	+++	++	+	+	495
ppENK	TE	Northern	nd	−	+	++	+++	280
b-FGF	TE	S1-NPA	nd	+++	++	+	+	552
GnRH-R	TE	LB	−	+	++	+++	++++	81
GnRH-R	TE	LB	nd	+	++	+++	++++	82
GHRH	TE	mRNA	−	+	++	+++	+++	25
GHRH	IC	IHC	nd	+	++	+++	++	26
IGF-I	TE	mRNA	nd	+++	++	+	+	25
IGF-I	GC	IHC	nd	+	+	++	+++	407
IGF-I	SC	IHC	nd	++	+	−	−	407
IGF-I	LC	IHC	nd	+	−	−	−	407
IGF-IR	TM	LB	nd	nd	++	nd	+	424
IGF-II	TE	mRNA	nd	+++	++	+	+	25
IL-1	ST	Bioassay	nd	−	+	++	+++	654
IL-6	SC	Bioassay	nd	nd	+	++	nd	663
PDGF-A	TE	Northern	++	+++	nd	nd	−	591
PDGF-B	TE	Northern	++	+++	nd	nd	−	591
PDGFR-α	TE	Northern	++	+++	nd	nd	−	591
PDGFR-α	TE	Northern	nd	+++	++	+	+	592
PDGFR-β	TE	Northern	++	+++	nd	nd	−	591
POMC	TE	Northern	nd	+	++	+++	++++	251
TGFα	TE	S1-NPA	nd	+++	++	+	+	495
TGFα	LC	IHC	nd	++	+	nd	++	496
TGFα	ST	IHC	nd	−	−	nd	−	496
TGFβ1	TE	S1 NPA	nd	++	+	+	+	470
TGFβ2	TE	S1 NPA	nd	++	+	+	+	470
TGFβ3	TE	S1 NPA	nd	++	+	+	+	470
TGFβ1	SC	IHC	+	+	+	+	+	472
TGFβ1	LC	IHC	+	+	+	−	−	472
TGFβ1	GC	IHC	−	−	+	+	+	472
TGFβ2	SC	IHC	+	−	−	−	−	472
TGFβ2	LC	IHC	++	+	+	−	−	472
TGFβ2	GC	IHC	−	−	−	−	+	472
TGF-βR-I	TE	Northern	nd	++	+	−	−	476
TGF-βR-II	TE	Northern	nd	++	+	−	−	476
TGF-βR-III (6 kb)	TE	Northern	nd	++	+	−	+	476
TGF-βR-III (3.5 kb)	TE	Northern	nd	−	−	+	++	476
TRH	TE	RIA	nd	+	+	++	+++	364

The relative abundance referred to the data from each single study was subjectively graded (nd, not determined; −, negative; +, positive, + to ++++). ST, Seminiferous tubules; TE, testicular extracts; TM, testicular membranes; IC, interstitial cells; GC, germ cells; SC, Sertoli cells; LC, Leydig cells; PMC, peritubular myoid cells.

PACAP shares binding sites with VIP in a variety of tissue types. There are three cloned receptors for PACAP, designated PVR1, PVR2, and PVR3. The PVR1 binds PACAP 100- to 1000-fold more potently than VIP and couples, through G proteins, to the activation of both adenylate cyclase and phospholipase C. PVR2 and PVR3 bind PACAP and VIP with approximately equal affinities and are coupled, probably through the G protein Gs, to the activation of adenylate cyclase. Five splice variants of the PVR1 receptor have been described. PACAP does not act as a classic hypophysiotropic

TABLE 7. Regulatory peptides and regulatory peptide receptors expression during the stages of the rat seminiferous cycle

S/R	Localization	Detection	I	II	III	IV	V	VI	VII	VIII	IX	X	XI	XII	XIII	XIV	Ref.
ActR	SpT	AR	−	−	−	−	−	−	+	+	−	−	−	−	−	−	365
ActR-II	pSpc	ISH	−	−	−	−	−	−	−	−	−	−	−	−	+	+	350
ActR-II	rSpT	ISH	+	+	+	+	−	−	−	−	−	−	−	−	−	−	350
ActR-IIB2	SpG	ISH	−	−	−	−	−	−	−	−	+	+	+	−	−	−	361
ActR-IIB2	SC	ISH	−	−	−	−	−	−	−	−	+	+	+	−	−	−	361
INH	ST[c]	RIA	+++	++	++	++	++	+	+	+	+	++	++	++	++	++	351
INH-α	ST[d]	Northern	++++	++	++	++	++	++	+	+	+++	+++	+++	+++	++++	++++	349
INH-α	ST[d]	Northern	++	++	++	++	+++	++	++	+	+	++	++	++	+++	++	351
INH-βB	ST[d]	Northern	++++	+	+	+	+	+	++	++	+++	+++	+++	+++	++++	++++	349
INH-βB	ISH	++	++	++	−	−	−	+	+	−	−	−	−	++	++	350	
INH-βB	ST[d]	Northern	++	++	+	++	++	++	++	++	++	++	+	++	+++	++	351
INH-βA	SC	ISH	−	−	−	−	−	−	−	+	++	++	++	−	−	−	350
EGF	ST[c]	RIA	++	+	+	+	+	+	+	+	++	++	++	+	++	++	408
bFGF	pSpC	IHC	−	−	−	+	+	+	++	++	++	+	+	+	−	−	554, 555
bFGF	SpG	IHC	+	+	+	+	+	+	+	+	+	+	+	+	+	+	554
bFGF R	SC, SpT	IHC	+	+	+	+	+	+	+	+	−	−	−	−	−	−	555
GHRH	e/iSpT acr.	IHC	−	−	+	+	+	+	−	−	−	−	−	−	−	−	26
IGF-I	ST[c]	RIA	+	+	+	+	+	+	+	+	+	+	+	+	+	+	408
IL-1	ST[d]	Bioassay	+	+	+	+	+	+	−	+	+	+	+	+	+	+	655
IL-6	ST[d]	Bioassay	+++	++++	++++	++++	++++	++++	+	+	++	++	++	++	+++	+++	663
LNGFR[a]	SC	IHC	−	−	−	−	−	−	+	+	+	+	+	−	−	−	605
LNGFR	ST[d]	Northern	−	−	−	−	−	−	+	+	−	−	−	−	−	−	605
NGF	rSpT	IHC	+	+	+	+	+	+	+	+	−	−	−	−	−	−	605
NGF	pSpC[e]	IHC	−	−	−	−	−	+	+	+	+	+	+	+	+	+	605
NGF	pSpC[f]	IHC	−	−	−	−	−	−	−	−	+	+	+	+	+	+	605
NGF TrkA[b]	ST[d]	Northern	+	+	+	++	+	+	++	++	+	+	+	+	+	+	605
PACAP	SpT acr.	IHC	−	−	−	−	+	+	++	++	−	−	−	−	−	−	50
TGFβ1	SpC	IHC	+	+	+	+	+	+	+	+	+	+	+	+	+	+	484
TGFβ1	SpT	IHC	+	+	+	+	+	+	+	−	−	+	+	+	+	+	484
TGFβ2	SpT	IHC	−	−	−	−	+	+	+	+	−	−	−	−	−	−	484

The expression intensity with reference to the data from each single study was subjectively graded (−, negative; +, positive, + to ++++). ST, seminiferous tubule; SC, Sertoli cells; pSpC, primary spermatocytes; e/iSpT, early intermediate spermatids; rSpT, round spermatids; acr., acrosome.

[a] Low-affinity NGF receptor.

[b] NGF receptor TrkA.

[c] Stage syncronization of seminiferous epithelium after withdrawal and replenishment of vitamin A.

[d] Seminiferous tubule microdissected and stages recognized according to light absorption criteria.

[e] Mainly pachytene SpC.

[f] Mainly pachytene, diakinetic, and dividing SpC.

TABLE 8. Transgenic models with local effects on male reproduction

Substance	Models	Viability	Effects on reproduction	Other abnormalities	Ref.
ActRII	KO homozygote	Normal	Reduction of FSH levels, delayed fertility	A small percentage shows micrognathia, cleft palate, eyelid closure defects, and die within minutes of delivery	383
pENK	Overexpression in the testis	Normal	Grossly abnormal testicular morphology and infertility in one offspring, low sperm motility in another, reduced fertility in some offspring. Normal peripheral levels of LH and testosterone	No	290
IGF-I	KO homozygote	Normal	Infertility; lack of sex drive; reduction of testis size; vestigial vas deferens, seminal vesicles and prostate; 18% of normal spermatogenesis; 18% of normal serum testosterone and reduction of response to LH; immature Leydig cells	Dwarfism; delayed bone development	414, 457
IL-2	Overexpression	Reduced	Testicular atrophia, absence of spermatogenic cells	Infiltration of lymphocytes into the cerebellar tissue, motor ataxia	706
INH α-subunit	KO homozygote	Lethal at 4 wk	Development of gonadal stromal tumors, regression of spermatogenesis parallel to the enlargement of the tumor mass, increased FSH levels, infertility	No	377
OT	Overexpression in the testis	Normal	50% decrease of intratesticular T and DHT, no effect on testicular morphology, sperm production and fertility	No	166
TGFβ1	Overexpression	Reduced	Atrophy of testis, thickened tubular basement membranes	Hepatic fibrosis, renal failure, arteritis, myocarditis, atrophy of pancreas	489
TrkA	KO homozygote	Lethal at 8 wk	Some animals that reach adulthood are infertile	Loss of sympathetic neurons and sensory neurons responsive to temperature and pain	614

factor that stimulates or inhibits anterior pituitary hormone release, but instead modulates the responses to factors such as GnRH directly or indirectly or has more general effects on gene expression or cell growth and differentiation (46).

1. Expression, localization, and production. Immunoreactive PACAP-38 has been found in the rat testis (47, 48) that exceeds even the total amount of immunoreactivity in the entire brain (47). Extracts of adult rat testes contain all the PACAP precursor-derived peptides: PACAP-38, PACAP-27, and PRP (49). PACAP mRNA has been found near the perimeter of the seminiferous tubules in early germ cells by *in situ* hybridization (50), and immunohistochemical studies have shown PACAP-38 staining in spermatids near the lumen at stages VII and VIII of the seminiferous cycle, in spermatogonia and primary spermatocytes, but not in mature spermatids or spermatozoa (49). An unusual PACAP mRNA shorter than that reported in the rat cortex and hypothalamus has been isolated in rat testis (51, 52). This smaller form of PACAP mRNA is also present in human, murine, and bovine testis (52).

Cloning of the PACAP-38 cDNAs showed the expression of the corresponding mRNAs and peptide in human testis (53).

2. Receptors. The PACAP PVR1 receptor has been found in the most mature stages of the adult rat germinal cells by autoradiography (54). Although testicular cell membrane preparations showed some specific PACAP-27 binding, the rate was too low relative to the protein content to generate informative displacement profiles (54, 55). The five spliced variants of PVR1 receptor are coupled differently to adenylyl cyclase and/or phospholipase C stimulation, and the form denominated PACAP-R *hop* is the predominant one in the testis (56).

Recently, the cloning of the gene encoding the human type I VIP receptor, also called type II PACAP receptor or PVR2, has been achieved (57). The gene is selectively expressed in various human tissues including the testis (57).

3. Local functions. PACAP stimulates cAMP accumulation in Sertoli cells from 15-day-old rats, and this property declines with the increasing age of the donor animals (58). This effect is additive with submaximal, but not maximal, concentrations of FSH. Furthermore, PACAP increases the Sertoli cell secretion of lactate, estradiol, and INH in a concentration-dependent manner (58). The PACAP-mediated stimulation of the Sertoli cell cAMP accumulation is not altered by a VIP antagonist, suggesting that PACAP is acting via the PVR1 receptor on these cells (58). In addition to the effects on Sertoli cells, PACAP increases the synthesis of both secreted and intracellular proteins in spermatocytes, but decreases the synthesis of both spermatid-secreted and intracellular proteins (59).

Despite the distinct possibility of PACAP effects within the testis, direct proof of an *in vivo* function is still lacking.

C. GnRH

GnRH is a decapeptide synthesized in the cell bodies of hypothalamic neurons that selectively stimulates the gonadotrope cells of the anterior pituitary to release LH and FSH (60). The GnRH receptor is a seven-transmembrane region protein that on activation initiates a series of events that start

with G protein-mediated stimulation of phosphoinositide (PI) turnover followed by elevations in $[Ca^{2+}]i$ and activation of protein kinase C (PKC) (61, 62).

1. Expression, localization, and production. In 1980, Sharpe and Fraser (63) reported the presence of a factor with GnRH-like activity in the testicular interstitial fluid of human (h) CG-treated adult rats (63). Later, it was demonstrated that seminiferous tubules from the rat and the stumptailed macaque (*Macaca arctoides*) and medium conditioned by cultured rat Sertoli cells contained a factor with GnRH-like receptor-binding and biological activity (64, 65), immunologically distinct from native GnRH (64). Paull *et al.* (66) found a GnRH-like immunoreactivity in the cytoplasm and nuclei of germ cells using an antiserum directed to the center of the molecule, but not with antisera directed to the end of the molecule. The partial isolation and characterization of GnRH receptor-binding activity from adult rat testis acetic acid extracts revealed the presence of two factors chemically distinct from the native decapeptide, with approximate molecular weights of 68,000 and 6,000, respectively (67). This partially purified material led to a dose-dependent inhibition of LH-stimulated testosterone production in a mixed Sertoli-Leydig cell monolayer culture similar to that seen with synthetic GnRH (68). Low concentrations of GnRH-like factors have been found in adult rat testis extracts by RIA (69, 70). This low concentration has been partially attributed to the presence of a testicular GnRH-peptidase associated with the Sertoli cells, one of the putative sources of rat testicular GnRH. GnRH-peptidase content has been found to be much higher in adult testis than in immature testis (71).

Recently, GnRH mRNA has been found at a specific, although not specified, stage of spermatogenesis within the seminiferous tubules of both mature rat and adult human testes (72). In the rat, the expression of the GnRH mRNA was identified in Sertoli cells and spermatogenic cells of some seminiferous tubules. In humans, the GnRH mRNA was localized only in some spermatogenic cells in some seminiferous tubules, suggesting that the mRNA was expressed at specific stages of tubular development (72).

2. Receptors. GnRH-specific low-affinity binding sites were originally identified in testicular membrane preparations from adult rats by Marshall and collaborators (73, 74). Subsequent *in vitro* studies demonstrated high-affinity receptors located in the interstitial cells of the adult rat testis (75–78). In keeping with these results, quantitative autoradiography confirmed the presence of GnRH binding sites distributed on adult rat interstitial cells (79). Moreover, GnRH-binding sites have been found in the testis of the frog *Rana esculenta* (80).

The ontogeny of the GnRH receptors in the rat testis has been described. GnRH receptors were not detectable in homogenates of acutely excised 20.5-day fetal testes or in freshly prepared fetal Leydig cells, but were clearly present starting from 3 days of culture (81). These receptors were also readily detectable postnatally in the testes of 5-day-old rats and increased markedly during maturation (81, 82).

After Leydig cell binding, GnRH stimulates the inositol lipid metabolism, which triggers a cascading mechanism that ultimately results in the generation of increased cytosolic free calcium levels, enhanced PKC activity, and liberation of arachidonic acid (62, 83, 84).

In contrast to what was observed in the rat, GnRH receptors have not been found in adult human (85) or mouse testes (86, 87), leading to the assumption of a species-specific expression in the male gonad.

However, the picture has been further complicated by the recent immunohistochemical finding of GnRH receptors in adult human Leydig cells (72).

3. Local functions. The first hypotheses of a direct effect of GnRH and its agonists on testicular physiology came from the observation that the *in vivo* administration of pharmacological doses of GnRH exerted paradoxical inhibitory effects on male reproductive functions in immature and adult hypophysectomized rats (88–91) and inhibited the *in vitro* gonadotropin stimulation of androgen production by cultured testicular cells (75, 92–94). GnRH agonists inhibited LH-dependent steroid production and abolished the acute testosterone response to hCG in cultured testicular cells from fetal and neonatal rats (81, 95, 96). This inhibitory effect of GnRH on testicular basal and LH-stimulated testosterone secretion has also been confirmed by *in vivo* experiments in the rat fetus (97). However, different experimental models gave contrasting results.

Short-term incubations of adult rat Leydig cells with GnRH resulted in increased phospholipid turnover, prostaglandin E and testosterone production (86, 98–100), and activation of the cytochrome P450 enzyme (101), whereas chronic exposure decreased the response to hCG (102).

In short-term incubations the GnRH analog buserelin had a direct positive effect on testicular testosterone production by *R. esculenta* minced testes (103) and decapsulated rat testes between 1 and 60 days of life (82). A number of studies have been conducted on different frog species demonstrating that GnRH-like material acts directly on testes promoting androgen production (104–107) and primary spermatogonial mitosis (106, 108, 109).

In an elegant experiment, Huhtaniemi *et al.* (110) examined the functions of the GnRH receptors in the adult and immature rat testis, blocking the receptors by a 7-day *in situ* infusion of a potent GnRH antagonist. The infusion of the antagonist resulted in a dose-dependent decrease of the testicular GnRH receptors up to 90%; the circulating levels of gonadotropins, PRL, and testosterone were unaffected, but there was a subtle yet significant decrease (16–32%) in the testicular content of testosterone, and of LH, FSH, and lactogen receptors (110). GnRH agonists have been shown to stimulate testicular blood flow in hypophysectomized rats, an effect that is mediated via the Leydig cells and is presumed to reflect one of the actions of testicular GnRH (4, 111).

Initial studies investigating the factors regulating the GnRH receptor expression in the testis indicated that the *in vivo* administration of GnRH induced an increase in the number of its own receptors in both intact and hypophysectomized rats (77, 112). Furthermore, it has been demonstrated that the administration of LH to hypophysectomized rats prevents the posthypophysectomy increase of GnRH receptors, as well as reducing the high levels of receptors in previously hypophysectomized animals (113). Experimental

adult rat unilateral cryptorchidism induces a significant reduction in the number of receptors for LH, FSH, and PRL, but the number of GnRH receptors is unaffected (114).

Studies conducted in humans showed that the administration of a potent GnRH agonist for 6 days did not inhibit the hCG-induced increase in plasma testosterone levels or the testicular steroidogenic pathway in patients with gonadotropin deficiency (115). The chronic treatment of elderly men with disseminated prostatic cancer with a GnRH agonist resulted in inhibition of both the Δ^4 and Δ^5 pathways, with a subsequent decrease in the intratesticular testosterone concentration (116, 117). The ability of exogenous hCG to reverse both the reduction in Δ^4 and Δ^5 intratesticular steroid content and the intratesticular enzyme activities induced by the GnRH analog suggests that GnRH does not have a direct inhibitory effect on testicular testosterone biosynthesis in man (116, 117). In support of this evidence, no effect has been found with high concentrations of a GnRH agonist on steroid conversion in human testicular tissue *in vitro* (118). The lack of local effects of GnRH in human testis has been confirmed in other primates (119, 120) as well as in other species (87, 121).

Genetically hypogonadal mice (*hpg/hpg*), homozygous for a deletion mutation in the gene encoding GnRH and GnRH-associated peptide (122), cannot synthesize or release hypothalamic GnRH and GnRH-associated peptide. This accounts for diminished production of gonadotropins, which is responsible for the arrested development of their gonads and sterility. Thus, these endogenous mutants cannot help to define the importance of GnRH in testicular function, considering also the reported lack of expression of the GnRH receptors in the mouse testis (86, 87).

Based on present evidence, the physiological significance of testicular GnRH-like peptides and of the direct gonadal actions of GnRH and its agonists is as yet unresolved. The data reported above indicate that the local effects of testicular GnRH are subtle and species-specific.

D. CRH

CRH, a 41-aa peptide, is the key hormone controlling hypothalamic-pituitary-adrenal function. It now appears that the actions of CRH go beyond its role as a hypothalamic releasing factor. Through actions in the brain and in the periphery, CRH coordinates the endocrine, autonomic, behavioral, and immune responses to stress (123). The CRH receptor is a glycoprotein that belongs to the family of the seven-transmembrane G protein-coupled receptors, with a higher molecular weight in peripheral tissues and a lower molecular weight in the brain (124, 125).

1. Expression, localization, and production. CRH mRNA is present in the testis at levels comparable to those found in the midbrain (126) and is most abundantly distributed in the testis of MT-CRH transgenic mice (127). Immunoreactive CRH has been found to be present in the testis of rat, guinea pig, sheep, and man (128, 129) and localized in the Leydig cells, germ cells, and epididymal sperm (130, 131). In the rat, testicular CRH concentrations fluctuated with age, showing high levels at 10 days of age, a marked reduction at 20 days, a significant increase at 60 days, and maximal levels at 90 days (130). The increase of immunoreactive CRH concentrations at the time of full spermatogenic activity and sperm production led to the suggestion that, in adult life, most testicular CRH is localized in germinal cells (130). However, recent immunohistochemical studies indicate that in adult rat testis, CRH is mostly localized to interstitial cells, and that purified Leydig cells show consistent cytoplasmatic immunostaining for CRH (131). In adult human testis, CRH immunostaining has also been shown to be mainly localized in the interstitial compartment (132). CRH was isolated and characterized from testicular extracts and found to be, apart from a microheterogeneity at position 39, identical in amino acid sequence to the hypothalamic peptide (128).

Adult rat Leydig cells in culture release a consistent amount of CRH (132). LH/hCG, cAMP, and 5-hydroxytryptamine (5HT) are potent acute stimuli for its production (132, 133). Furthermore, the 5HT2 receptor subtype is found in the Leydig cells and mediates the serotonin stimulation of CRH secretion (133). Extrapolating from other tissues, it is conceivable that the mechanism of action of 5HT on binding to the 5HT2 receptor in Leydig cells involves the activation of PI hydrolysis and stimulation of PKC (133), an intracellular messenger system that appears to mediate the stimulation of CRH release in Leydig cells (132). Studies from the same group showed that 1) serotonin is a more effective stimulus than hCG in stimulating CRH secretion, and 2) gonadotropin-induced CRH release is inhibited by the 5HT2 receptor antagonist ketanserin, indicating that stimulation of CRH by hCG results from the action of endogenously released serotonin (133).

In vivo studies have shown that acute immobilization stress is a stimulus for testicular CRH mRNA expression and transcription into CRH protein (134). This model of stress was also characterized by a marked reduction (80%) in testosterone serum concentration but no change in LH serum levels. Therefore, the involvement of testicular CRH in stress-induced testosterone inhibition was suggested.

2. Receptors. CRH-binding sites have been demonstrated in whole testis (135) and isolated adult rat Leydig cells (136). Adrenalectomy of adult rats induced, after 14 days, an increase of CRH binding in testis membranes by approximately 215% above sham-operated controls, suggesting that glucocorticoids may be a regulator of peripheral CRH receptors (137). Scatchard analysis of CRH binding data on intact cells and purified Leydig cell membranes revealed a single class of high-affinity binding sites with a dissociation constant (K_d) of 0.1 nM, and showed that CRH receptors are present in low abundance [500/800 per cell *vs.* 20,000 for LH/hCG and 2,000 for angiotensin II (AT-II)] (136). Subsequent studies in rat Leydig cells have shown that, in contrast to corticotropes, CRH receptors interact with a G protein different from Gs, which is linked to phospholipase C, possibly Gq, G11, or an isoform not yet described (138).

Recently, two types of hCRH-receptor cDNAs were identified (type I and type II hCRH-R) (125). hCRH-RII is identical to type I except that it contains a 29-aa insert in its first cytoplasmic loop, suggesting that hCRH-RI and hCRH-RII result from alternative splicing of a single gene. Studies on

the signaling properties of the two receptors showed that hCRH-RI is coupled to stimulation of cAMP accumulation and PI hydrolysis. In contrast, hCRH-RII is deficient in signaling through both effectors, especially PI turnover in COS-7 cells (139). Since the CRH receptor in rat Leydig cells is mainly coupled to PKC activation, it can be excluded as an CRH-RII subtype. Interestingly, an additional CRH receptor has been identified, which was shown to be expressed at high levels in the heart and at low levels in the brain and lungs, and found to be significantly different (30%) from that of the pituitary gland (140). Functional studies have demonstrated that this "peripheral" CRH-receptor recognizes CRH and the CRH-related amphibian peptide sauvagine and is coupled with Gs and adenylate cyclase (140). Thus, it is conceivable that, in addition to the pituitary type, there might be distinct peripheral CRH receptor subtypes capable of coupling with different intracellular signaling pathways (*i.e.*, adenylate cyclase and/or phospholipase C).

PCR analysis of human tissues revealed CRH receptor transcripts in the brain, pituitary gland, and testis (141).

3. Local functions. CRH receptors in rat Leydig cells are coupled to stimulatory actions on β-endorphin (βEND) production (142) and inhibition of LH-induced steroidogenesis (132, 136, 138). *In vitro* studies showed that CRH acts rapidly (in minutes) in the fetal and adult rat Leydig cell to exert highly effective negative autoregulation of the Leydig cell steroidogenic response to the LH stimulus (132, 136). Similarly, intracellular and extracellular cAMP production stimulated by gonadotropin were significantly reduced by CRH treatment of rat Leydig cells (136, 138). Studies performed in both purified mouse Leydig cells and in a mouse cell line derived from a Leydig cell tumor (MA-10 cells) led to different results, showing that CRH had a stimulating effect on cAMP accumulation and steroid production (143). In the same studies, experiments performed on partially purified rat Leydig cells (60–80% Leydig cells) showed that CRH had no effect on basal and hCG-stimulated steroidogenesis. These results indicate that mouse Leydig cells respond differentially from rat Leydig cells to CRH, suggesting that CRH action in the male gonad is species-specific. Mice and rats might have different forms of CRH receptors on Leydig cells, which couple to different signal pathways and have opposite actions on steroidogenesis. This is consistent with the large number of differences between mouse and rat Leydig cells found by others (143). In highly purified rat Leydig cells (90–95% pure), it has been found that the inhibition of hCG-induced steroidogenesis by CRH was maximal (150–200% reduction) at the earliest incubation times (30–60 min) and much less evident at later time points (60–120 min, 20–40% reduction) (136). The marked reduction of CRH effects on hCG-induced steroidogenesis in rat Leydig cells after prolonged incubation was related to the temporal degradation of the peptide in culture, which was complete after 180 min (136). These observations might explain the above reported inconsistency of CRH inhibition on hCG-induced steroidogenesis observed in rat Leydig cells (143). These latter studies were performed in partially purified Leydig cell populations, which can be contaminated by high CRH-degrading activity, and CRH actions were assessed after incubation for only 2 h, a time point at which CRH inhibition has been reported to be minimal (136). Taken together, these results strengthen the concept that extreme caution must be used in examining the actions of a peptide in the testis based solely upon *in vitro* methodologies.

To further complicate the picture, *in vivo* studies performed in neonatal (5-day-old) rats showed that the intratesticular injection of an anti-CRH-antiserum led to a significant decrease in serum testosterone levels (144). These findings indicate that in the neonatal period in the rat, testicular CRH might be a local stimulator of steroidogenesis. A developmental regulation of CRH action in the testis is also indicated by the finding that intratesticular injection of CRH *in vivo* causes a significant increase of βEND secretion in interstitial testicular fluid in pubertal but not adult rats (145). In adult unrestrained intact male rhesus macaques, a 4-h infusion of CRH caused a prompt decrease in testosterone levels without significant changes in LH levels (146), leading to the suggestion that CRH may directly inhibit testosterone production by Leydig cells.

Both CRH deficient and transgenic mice have been generated (147, 127). CRH-deficient mice require glucocorticoid for lung maturation during fetal life (147). Despite marked glucocorticoid deficiency, these animals exhibit normal postnatal growth, fertility and longevity, suggesting that the major role of glucocorticoid is restricted to the prenatal period, and that the lack of CRH does not impair reproductive potential.

As pointed out earlier, analysis of CRH mRNA distribution in transgenic mice has revealed that transgene expression is primarily detected in all the classic expression sites of endogenous CRH and in the testis (127). Mapping of CRH within the testis by *in situ* hybridization revealed hybridization signal over seminiferous tubules and in an interstitial pattern consistent with Leydig cell expression. The CRH-expressing transgenic mice were developed using the mouse MT-1 promoter fused to the rat CRH gene. Therefore, the tissue distribution of rat CRH in these animals may more closely resemble MT-1 expression than CRH, and for this reason the CRH mRNA distribution does not necessarily represent the normal tissue-specific expression of CRH. The transgenic mice developed a Cushing-like syndrome; male animals bred successfully.

In conclusion, CRH actions in the testis may vary depending upon the species and the period of life examined; it becomes apparent that, at least in adult male rats and rhesus macaques, CRH may have direct inhibitory effects on steroidogenesis and locally mediate the detrimental effect of stress on testicular function. However, further studies are needed to verify whether the testicular effects of CRH observed in some animal species *in vitro* have significant pathophysiological consequences *in vivo*.

E. Oxytocin (OT)

OT is a nonapeptide involved in parturition and lactation that is synthesized in the hypothalamus and secreted by the posterior pituitary (148). The OT gene consists of three exons encoding a preprohormone that is processed in several mature peptides, including the nonapeptide hormone (149, 150).

Exon I encodes a signal peptide, the OT hormone, and the N terminus of a carrier molecule, neurophysin-I. Exon II encodes the bulk of neurophysin-I, and exon III encodes the C terminus of this molecule. A single class of OT receptors has been characterized that shows a structure with seven-transmembrane domains typical of the G protein-coupled receptors (151).

1. Expression, localization, and production. Immunoreactive OT was first identified in the human and rat testis in 1984 (152). Since then, OT immunoreactivity has been found in testes of other mammals (153, 154) and birds (155). Immunohistochemical studies have shown OT immunoreactivity in the interstitial tissue, probably the Leydig cells, of rat and dog testes (156, 157). The depletion of the Leydig cell population in the adult rat by the drug ethan-1,2-dimethanesulfonate (EDS) causes a reduction in the levels of OT immunoreactivity in testicular extracts to undetectable levels by RIA, confirming the Leydig cell origin of OT in the rat gonad (158). Accordingly, immunocytochemical studies have revealed OT localization in purified rat Leydig cells (159). Cultured Leydig cells from adult rats release significantly more OT into the medium over a 3-day period than was present in the cells at the beginning of the experiment (160). Furthermore, the production of OT by these cells is significantly reduced by treatment with the protein synthesis inhibitor cycloheximide, providing further evidence for a Leydig cell production of OT (160). An OT-like peptide is also secreted by purified guinea pig Leydig cells in culture (161). Interestingly, in the hypogonadal mouse (*hpg/hpg*), no OT can be found in the testis, but the treatment with LH or with testosterone causes the appearance of testicular OT (162). Using HPLC and specific RIA, authentic OT has been identified in the testis of the Australian marsupial bandicoot (*Isoodon macrourus*) but not in the possum (*Trichosurus vulpecula*) testis (163).

OT gene transcripts are not detectable by Northern hybridization of rat testicular extracts, but the authentic hypothalamic-type mRNA can be detected using highly sensitive PCR analysis (164). Normal cattle have relatively high levels of testicular OT mRNA (155). *In situ* hybridization in bovine testicular tissue sections localized OT transcripts to the seminiferous tubules. It is thought that its expression is localized in Sertoli cells. The same testicular distribution of bovine OT RNAs was also shown by *in situ* hybridization in a transgenic mouse bearing a bovine OT transgene but not in the normal wild type mice (165, 166). Bovine and sheep testis contain moderate levels of an OT gene transcript as revealed by Northern blot analysis (167). *In situ* hybridization localized this mRNA within the seminiferous tubules, possibly in the Sertoli cells. Conflicting with this result, in the same study, immunohistochemistry analysis showed that both OT and the syngenic neurophisin I epitopes were clearly restricted to the Leydig cells, being expressed here at low levels. It has been suggested that the absence in the OT protein within the tubules is probably due to a lesion of OT gene expression at the posttranscriptional level, whereas, the low level peptide expression in the Leydig cells can be attributed to the presence of functional transcripts in these cells, which are below the level of significant detection for the *in situ* hybridization assay (167).

In humans, evidence for OT gene transcription in the testis was found in three of five experiments only by using a highly sensitive assay, based on a modification of the PCR, sufficient to detect one mRNA molecule/cell (168).

The effect of increasing doses of LH (0.001–100 ng/ml) on OT production from highly purified adult rat Leydig cells in culture has been tested (160). Maximal secretion of OT occurred with 0.1 ng/ml LH. Since there is a prolonged delay in the peak rate of OT production relative to the testosterone peak, it has been postulated that OT production could be indirectly regulated by LH through an intermediate factor, probably testosterone. This hypothesis is in line with the reported observation that testosterone alone stimulates high levels of testicular OT in the absence of LH in *hpg/hpg* hypogonadal mice (162).

2. Receptors. A high density of OT receptors has been found in tunica albuginea and the epididymis of prepubertal pigs (169). Subsequent studies revealed the presence of OT receptors in the adult rat testis with ligand-binding characteristics similar to mammary and uterine OT receptors and an autoradiographic localization consistent with binding to Leydig cells (153, 170, 171).

The apparently straightforward action of OT on the contractility of the seminiferous tubules and the claim of a partial characterization of a tubular receptor (172) contrast with the inability to demonstrate functional OT receptors in rat testicular myoid cells at any stage of development (172, 173).

3. Local functions. The contractility of the seminiferous tubules is enhanced by OT (172, 174). This effect is confirmed by the observation that the tubules from testes in which immunoreactive OT could not be detected are always quiescent (158, 162).

There are conflicting reports on the influence of OT on steroidogenesis. Adashi and Hsueh (175, 176) showed that OT inhibits the gonadotropin-stimulated androgen biosynthesis in isolated rat Leydig cells through testicular recognition sites similar to those mediating the pressor actions of the neurohypophysial hormones. This was supported by Nicholson *et al.* (177, 178), who used continual-release OT implants *in vivo* and the perfused whole-testis model, and Kwan and Gower (179), who reported that OT completely inhibited androgen biosynthesis in incubated microsomal fractions from rat testis. However, Tahri-Joutei and Pointis (180) found that OT stimulated testosterone production by purified murine Leydig cells with an effectiveness more pronounced at puberty, while Sharpe and Cooper (181) found OT to have no effect on testosterone production either *in vitro* or *in vivo* in the rat. Using short-term cultures of isolated rat Leydig cells, OT significantly increased basal testosterone production in a dose-dependent manner without affecting LH-stimulated testosterone production (182).

It has been shown recently that transgenic mice, which overexpress OT in the testis, have a 50% decrease in the levels of intratesticular testosterone and dihydrotestosterone (DHT) without detectable effects on testicular morphology, sperm production, or fertility parameters (166). While the

results with the transgenic mice seem to be consistent with the *in vitro* and *in vivo* results in the rat (175–179), they contradict the *in vitro* experiments in the mouse (180). At this point the most reasonable explanation is that unlike the *in vitro* studies, transgenic models cannot distinguish between direct or indirect effects on Leydig cells and experience different exposure time compared with *in vitro* experiments (long-term *vs.* short-term). It is interesting to note that destruction of Leydig cells from adult rat testis using EDS, which results in the loss of OT (158), has very little impact on spermatogenesis, provided that high intratesticular levels of testosterone are maintained by exogenous administration (183). However, what the authors of the latter study observed was a 2- to 3-fold increase in the number of degenerating meiotic spermatocytes at stages XIV-I of the seminiferous cycle, and this was hypothesized to be related to the absence of OT, since active immunization of adult rats against OT caused a similar change.

Taking into account the possible differences between species, the important consistent finding must be that OT modulates androgen levels, possibly via direct action on the Leydig cells themselves, and regulates the extent of germ cell degeneration in the final stages of meiosis without severe physiological consequences to male fertility.

F. Arginine vasopressin (AVP)

AVP is principally an antidiuretic hormone. The substrate for production of the 9-aa AVP is a 164-aa precursor molecule. This molecule consists of a signal peptide, AVP itself, a specific neurophysin, and a glycosylated moiety. After synthesis in neurons of the hypothalamus, AVP migrates along neuronal axons into the posterior pituitary.

Two classes of cell surface AVP receptors, which are seven-membrane-spanning G-protein-coupled, mediate the actions of AVP. The V1 receptor occupancy induces an increase in PI hydrolysis and cytosol-free calcium. A subtype V1a receptor is found in the anterior pituitary. The V2 receptor, which is expressed only in the kidney, is coupled through the G-stimulatory protein to adenylate cyclase, cAMP, and a cAMP-dependent protein kinase (184).

1. Expression, localization, and production. Adult rat and pig testes contain an immunoreactive AVP-like peptide that behaves like authentic AVP by chromatographic criteria (185, 186). AVP-like peptides have also been identified in the testis of the adult homozygous Brattleboro rat, a genetic mutant deficient in hypothalamic, pituitary, and circulating AVP, suggesting that the production of hypothalamic and peripheral AVP may involve different biosynthetic pathways (187, 188). Immunoreactive AVP has been detected in the interstitial fluid of adult rat testis, and the disruption of spermatogenesis was associated with a decrease in AVP concentration (189).

The detection by Northern blotting of a mRNA in the rat testis, which was considerably shorter than that in the hypothalamus and which hybridized to a specific AVP probe, was initially reported by Ivell *et al.* (190). Structural analysis revealed that while exons II and III of the testicular RNAs are identical to those of the hypothalamic mRNA, the hypothalamic exon I, which encodes the AVP nonapeptide, is not represented, suggesting major structural differences between hypothalamic and testicular AVP gene-related mRNAs (164, 191). No function could be ascribed to these testicular AVP-like RNAs because of the lack of open reading frames and the apparent lack of association with translationally active polysomes (164). Although a developmental analysis of the transcripts showed that their detection correlated with spermatogenesis and the appearance of dividing germ cells, no other physiological manipulation was able to influence the levels of these aberrant transcripts (164). Subsequently, a testis-specific promoter for the rat AVP gene has been described, and an *in vitro* synthesized RNA corresponding to the longer testicular AVP gene-derived transcript was not able to act as a template for protein synthesis (192). Again, the aberrant testicular AVP gene-derived RNAs expression was closely associated with the integrity of germ cells and ongoing spermatogenesis (193). Accordingly, transgenic mice overexpressing the rat hypothalamic AVP gene were shown to have tissue-specific mRNA expression in the hypothalamus, temporal lobe, parietal cerebral cortex, cerebellum, posterior pituitary, pancreas, and lung, similar to the tissue distribution of endogenous and ectopic mouse and rat AVP expression but not in the testis (194).

Nevertheless, by applying the great sensitivity of the PCR, hypothalamic-like AVP mRNAs could be detected in rat testis beginning around late puberty (164) as well as in mouse Leydig cells and rat and mouse Leydig tumor cell lines where they are probably translated to give authentic AVP (195). Both normal and aberrant AVP gene transcripts could not be detected in human and baboon testis by PCR (168).

As a whole, these observations indicate that expression of functional AVP transcripts in the testis depends on the animal species (they are present in rat and mouse but not in primates) and are detectable only with methods more sensitive than Northern hybridization; these transcripts can be responsible for the AVP-like immunoreactive peptides found in the testis; the process of AVP gene expression differs significantly between neuronal and testicular tissues and involves differential splicing of the known AVP gene; the function of the aberrant testicular AVP gene-derived RNAs that are unable to encode the corresponding peptides is unknown.

2. Receptors. Specific, high-affinity, low-capacity binding sites for AVP of the V1 subtype have been identified in the Leydig cells of adult rat testis (196, 197), in testicular interstitial cell preparations from Brattleboro rats (188), and in the tunica albuginea of porcine testis (169). AVP-binding sites were found in Leydig cells from prepubertal, pubertal, and adult mice, with no marked differences in the affinity and a 50% decrease in receptor number in the pubertal period (180). AVP induces phospholipase C stimulation in enriched adult rat Leydig cell preparation (198). The autoradiographic localization of V1 receptors in the adult rat testis showed a binding to small arteries, the seminiferous tubule epithelial surface, and in a reticular interstitial pattern between seminiferous tubules consistent with binding to Leydig cells (199). In rats, the testicular AVP receptor concentration declines after hypophysectomy, and the treatment of hypoph-

ysectomized animals with LH or GH restores the receptor levels (200). Rat cultured PMC express a functional AVP receptor pharmacologically and structurally identical to the V1a subtype under developmental control, with no evidence of expression on cells prepared before puberty and postpubertal appearance (173).

3. Local functions. AVP produces a dose-dependent inhibition of gonadotropin-stimulated androgen biosynthesis in cultured testicular cells mediated by specific testicular recognition sites similar to those mediating the pressor actions of AVP but distinct from those involved in the antidiuretic effect (175, 176). Subsequent studies have confirmed that long-term treatment with AVP inhibits LH-induced testosterone production by Leydig cells *in vitro* (180, 182, 183, 201–203), and an *in vivo* experiment confirmed the antigonadal activity of AVP (204). These effects have been accounted for by a decreased testicular LH- binding capacity (204) and reduced 17α-hydroxylase activity induced by AVP (201, 203).

In contrast, after acute treatment, AVP induces a stimulatory effect on the steroidogenic activity of mouse Leydig cells, which is more pronounced at puberty compared with prepubertal and adult periods (180). The intratesticular injection of low doses of AVP in the adult rat *in vivo* caused a dose-dependent decrease in total testicular blood flow, without any major effect on vasomotion, interstitial fluid volume, and testosterone production (205). The lack of an important *in vivo* role for AVP in the control of Leydig cell function was suggested by the reported normal plasma LH and testosterone levels as well as testicular testosterone production capacity in the AVP-deficient Brattleboro rats (206). However, the subsequent demonstration of AVP-like peptides in the testis of Brattleboro rats (188, 189) has reopened the quest for definitive proof of the influence of AVP or AVP-like immunoreactive compounds in the testis.

To summarize, further studies are needed before one can draw any conclusion about the physiological relevance of AVP in the male gonad.

G. TRH

TRH is the key regulator of the synthesis and secretion of TSH in animals and humans and plays additional roles as a neurotransmitter/neuromodulator in the central nervous system (CNS) (207). Extrahypothalamic loci of function for TRH have also been demonstrated (208). TRH acts on the target cells through a membrane receptor which is a member of the seven-transmembrane region, G-protein-coupled receptor family (209).

1. Expression, localization, and production. Relatively high levels of TRH and its immediate precursor TRH-Gly have been found in the testis of sexually mature rats and dogs (210–213), and mRNA for pre-proTRH (ppTRH) has been identified in the rat testis (214).

Developmental studies on testicular ppTRH mRNA expression showed no expression at the earliest stages of postnatal development (day 8), while hybridization signals were found on day 20 and increased progressively up to day 70. TRH peptide concentrations measured by RIA at the same developmental periods paralleled the ppTRH mRNA expression. ppTRH mRNA and TRH peptide were colocalized to Leydig cells by Northern blot analysis and immunohistochemistry of enriched testicular cell elutriates, respectively (214). Subsequent studies have confirmed, through the use of EDS treatment of adult rats, that the Leydig cells are the only source of authentic TRH and TRH-like peptides in the rat testis (215).

Interestingly, the TRH-potentiating peptide (Ps4), which is a connecting peptide that links two copies of the TRH progenitor sequence and has the same distribution pattern of TRH in the CNS, is present in very low concentrations in peripheral tissues except in the testis where it is expressed in large amounts (216).

Methimazole-induced hypothyroidism has been found to increase the concentration of immunoreactive TRH and TRH precursor in the rat testis (212); however, others have reported that testicular mRNA concentrations are completely unaffected by experimental alterations in thyroid status (208).

In humans, the cloning and characterization of the ppTRH gene has led to the recognition of ppTRH gene expression in the testis (217).

2. Receptors. The *in vivo* binding of a hybrid protein consisting of TRH linked to a fragment of diphtheria toxin that specifically binds to TRH receptor showed a displaceable binding in the testis of adult rats (218). Specific binding sites for TRH and PS4, and the mRNA for TRH receptor, have been detected in adult rat testis (216, 219, 220) and purified rat Leydig cells (219).

3. Local functions. TRH can partially inhibit the LH/hCG-induced testosterone secretion from rat Leydig cells *in vitro* (208, 217).

Although the above data suggest a potential autocrine role for TRH in the regulation of Leydig cell function, an *in vivo* effect of this peptide in the testis remains to be demonstrated.

H. Somatostatin (SRIF)

SRIF is the hypothalamic 14-aa cyclic peptide that together with GHRH regulates the GH release from the pituitary. In addition to the pituitary, this peptide is present and plays an inhibitory role in the normal regulation of three organ systems: the CNS and the hypothalamus, the gastrointestinal tract, and the exocrine and endocrine pancreas (221). A family of SRIF receptors that may mediate the distinct biological effects of SRIF has recently been cloned. The different cloned SRIF receptor subtypes have been designated SSTR1, 2, 3, 4, and 5 based upon the order in which they were isolated. The SRIF receptors are membrane-bound receptors coupled to pertussis-toxin-sensitive G proteins (222, 223).

1. Expression, localization, and production. SRIF-like immunoreactivity has been detected in rat (224) and human (225) testis but not in boar testis (226). In rat testis, the levels of SRIF-like immunoreactivity decreased after hypophysectomy (224). However, no testicular evidence of pre-pro-SRIF gene expression could be detected in the adult rat (227).

2. Receptors. RT-PCR used to characterize the distribution of mRNA encoding the SRIF receptor SSTR5 in human tissues failed to detect mRNA expression in fetal or adult testis (228).

The paucity of data, together with the absence of studies on the local functions of SRIF, do not allow us to hazard any conjecture on the effects of this peptide within the testis.

I. Opioids

Three families of endogenous opioid peptides are recognized. βEND, enkephalins (ENK), and dynorphins (DYN) are the defined peptides with morphine-like activity. The endogenous opioid peptides are widely distributed in the brain and peripheral nervous system and play important roles in modulating endocrine, cardiovascular, gastrointestinal, and immune functions. Each family is derived from a distinct precursor polypeptide. These precursors are called pro-enkephalin (pENK), POMC, and prodynorphin (pDYN) (229). The POMC precursor polypeptide yields the opioid βEND, and the nonopioid peptides, ACTH and α-MSH. pENK contains six copies of Met-ENK and a single copy of Leu-ENK. The third opioid precursor, pDYN, yields DYN-A, DYN-B, and α- and β-neoendorphin. Three types of opioid receptors, termed δ, κ, and μ, which differ in their affinity for the opioid ligands and their distribution in the nervous system have been characterized (230). The δ- and μ-receptors bind ENK and END, and the κ-receptors potently bind DYN. These receptors are members of the superfamily of the seven-transmembrane-spanning receptors and share a high degree of amino acid sequence similarity with approximately 50% of the residues being identical. The opioid receptors are coupled to adenylyl cyclase and inhibit the formation of cAMP through pertussis toxin-sensitive GTP-binding regulatory proteins (230).

1. βEND

a. Expression, localization, and production. The expression of testicular POMC mRNA has initially been localized in the cytoplasm of most Leydig cells of adult rat testes by *in situ* hybridization (231). Subsequent studies showed that POMC mRNA is expressed in purified preparations of Leydig cells and in interstitial macrophages of the adult rat testis (232–234). Further *in situ* hybridization studies in the mouse demonstrated that POMC mRNA is most abundant in a subpopulation of somatic Leydig cells that are found in the interstitial regions associated with discrete tubule stages (IX-XII) of the cycling seminiferous epithelium (235), indicating that the expression of POMC mRNA by Leydig cells is influenced by spermatogenic cells (236). In the mouse and hamster, RNA gel-blot experiments showed that the gene for POMC is expressed also by germ cells and, in particular, by pachytene spermatocytes (237). The size of POMC mRNA transcripts detected by Northern blot analysis in the somatic and germ cells of the testis is 400 nucleotides shorter than that of the pituitary gland, *i.e.*, 800 *vs.* 1200 nucleotides (237–239). The short testicular POMC mRNA lacks exons 1 and 2 and cannot serve as a template for the POMC signal peptide (entirely coded by exon 2) (238, 240), which is necessary in pituitary cells for the processing of POMC during precursor migration from the rough endoplasmic reticulum to the secretory granules via the lamellar Golgi complex (241). Thus it is unlikely that the POMC-derived peptide βEND found in the testis derives from the small POMC transcript. Subsequent studies performed with the S1 nuclease mapping technique have shown that a very small (<1%) but definite amount of the normal (1200 nucleotides) POMC transcript was present in rat and human testis (238, 240, 242), and in purified Leydig cells and Leydig cell lines in rodents (243). The low level of expression of the translatable POMC transcript is consistent with the low production of βEND in the testis. This is also supported by the observation that an increased expression of the pituitary-size POMC mRNA in human Leydig cell tumors was associated with a dramatic increase (1,000-fold) in βEND concentrations compared with normal testis (244).

Immunohistochemical studies showed that βEND is confined to the interstitial cells of rodent (245) and human testis (246) and is present in the interstitial cells, canaliculi of the efferent system, spermatogonia, and spermatocytes in the frog, *Rana esculenta* (247). Acetylated END forms (N-acetyl-βEND, N-acetyl-αEND, and N-acetyl-γEND 1–27) were also found to be present in the rat testis and exclusively localized to spermatogonia and primary spermatocytes (248). γEND-generating endopeptidase activity has been shown to be abundant in the rat testis and to be mainly associated with the germinal cell fraction of the tubules (249); thus, this enzyme can be operative in processing βEND into α- and γEND inside the rat spermatogenic cells.

Studies in mice and rats showed that βEND in the testis is developmentally regulated, with peaks at birth and after puberty (250, 251). In the mouse, at day 16 of fetal life, 50% of interstitial testicular cells stained positively for βEND; the number dropped to 12% by day 5 of age, increased again at midpuberty, and reached a maximum in adult age (100%) (250). An analogous developmental pattern has been shown in the rat (251). Total testicular βEND levels were very low and barely detectable from 5–20 days of age, rose sharply in parallel with testis weight from 20–60 days, and then remained unchanged through 150 days of age. In these latter studies it was shown that most of βEND from prepubertal testes chromatographed like authentic βEND, while with the onset of puberty and in adult life much of the total βEND chromatographed like its precursor β-lipotropin (251).

In fetal and adult testis, Leydig cell-derived βEND is hormonally regulated (252). In fetal rat Leydig cells in culture, LH is a potent stimulus for βEND production (253), while androgens, GnRH, and, to a lesser extent, dexamethasone (Dex) are inhibitory signals (252, 253). Immunohistochemical studies showed that *in vivo* hCG treatment increased 4-fold the number of interstitial cells positive for βEND in prepubertal mice (250) and elevated by 100% the βEND concentrations in interstitial fluid (254). These findings, coupled with the observation that testicular POMC gene is positively regulated by gonadotropins (255), demonstrate that LH is a stimulus for the synthesis and release of POMC-derived peptides in Leydig cells throughout rodent life. In addition to LH, CRH is another important stimulatory signal for βEND production from adult rat Leydig cells (142, 145). In contrast to LH, which is a systemic regulator, the CRH signal generated inside Leydig cells may act as a relevant autocrine

regulator of βEND production (256). Finally, it must be noted that alcohol is a direct exogenous stimulant of βEND secretion in adult rat testis (257, 258), and it has been suggested that alcohol may act through testicular βEND to suppress the synthesis and release of testosterone (258).

b. Receptors. βEND-binding sites were initially found on membranes obtained from adult rat testis (259). Subsequent studies showed that Sertoli cells have specific binding sites for opiates (260); no other testicular cell has so far been found to bind βEND. Opioid receptors (μ, δ, and κ) have been cloned (261). RNA blotting studies have shown no detectable expression of κ-receptor mRNA in mouse testis (262). Since βEND is a μ-δ opioid ligand, it is possible that μ- and/or δ-opioid receptors are expressed in Sertoli cells; this possibility must be verified.

c. Local functions. The intratesticular role of βEND has been analyzed in various studies (239, 256, 263). *In vivo* studies have proposed that βEND in the testis inhibits steroidogenesis (264–267) and is involved in the stress-induced attenuation of the steroidogenic testicular response to gonadotropins (268). The lack of opioid-binding sites on Leydig cells (260) and the absence of direct βEND effects on Leydig cell steroidogenesis (182, 269, 270), would exclude the direct participation of intratesticular βEND in the regulation of testosterone secretion. Thus, it is possible that βEND may be an indirect modifier (via Sertoli cells) of Leydig cell steroidogenic activity. In accordance with this hypothesis is the observation that βEND significantly inhibits FSH-induced INH production by Sertoli cell cultures obtained from pubertal rats (271). INH is one of the Sertoli cell factors known to amplify testosterone production by Leydig cells in response to LH (272); the paracrine inhibition of its production by βEND would result in a reduction of Leydig cell steroidogenic capability.

βEND exerts other important inhibitory actions on Sertoli cell function, which include the production of ABP (260) and Sertoli cell growth (273, 274). Regarding the latter aspect, it was initially shown that naloxone treatment of fetal rat testes in culture increased basal and amplified FSH-induced Sertoli cell division (273). Subsequent studies demonstrated that βEND inhibited FSH-induced proliferation of neonatal rat Sertoli cells by 50% and that this action involved Gi and was exerted at a point before intracellular production of cAMP (274). These studies indicate that testicularly produced βEND may act as a local antagonizer of FSH stimulation of the Sertoli cell. This inhibitory action can be of great importance in fetal and neonatal age, which is a period of life in which there is high Leydig cell βEND content (250) and production (252, 253) and corresponds to the proliferative period of Sertoli cells (16). Since the ultimate number of the Sertoli cells dictate the output of sperm cells at maturity both in rodents and human (16), it cannot be excluded that in the fetus, during late pregnancy, and in perinatal life an increase in testicular END might be involved in the pathogenesis of some oligozoospermias of the adult. A candidate trigger of this event could be stress, which, in adult rats, has been shown to stimulate testicular CRH gene expression and protein synthesis (134) and testicular βEND release into the interstitial fluid (275).

Mice lacking βEND by site-directed mutagenesis have recently been generated (276). The resulting transgenic animals display no overt developmental or behavioral alterations and have a normal functioning hypothalamic-pituitary-adrenal axis. However, they lack the opioid analgesia induced by mild swim stress and show significantly greater nonopioid analgesia. Homozygous mutant male and female mice have normal fertility based on the onset of puberty, average litter size, and the number of consecutive litters in established mating pairs. No obvious changes in endocrine function have been documented in the βEND-deficient mice despite the extensive experimental data base concerning opiate effects in the hypothalamus (229). While it is possible that βEND plays no role in the regulation of the hypothalamic-pituitary-adrenal axis and hypothalamic-pituitary-gonadal axis, a more likely explanation, in light of the analgesia studies, is that subtle compensating mechanisms establish themselves to maintain normal functioning of these critical neuroendocrine systems. Considering the inhibitory effects of βEND, which include reduction of testosterone secretion and neonatal Sertoli cell growth, transgenic animals overexpressing βEND in the gonads could help to clarify the suggested roles of this opioid peptide in testicular development and function.

2. Enkephalin (ENK)

a. Expression, localization, and production. High levels of pENK expression are found in somatic and germ cells, including Sertoli and peritubular cells in immature testis and Leydig and spermatogenic cells in adult testis (237, 277–281).

The analysis of postnatal development of ppENK mRNA in the rat testis showed little signal before postnatal day 20 and an increasing positivity after day 30 (282). The protein precursor for Met-ENK is encoded on transcripts of 1.4 kb in brain tissues and in those peripheral organs, such as adrenal, that are known sites of synthesis of the peptide. In the rat and mouse testis the major pENK transcript is 1.7 kb and is localized to the germ-line (283, 284). However, low levels of the conventional 1.4-kb transcript could also be detected in Sertoli cells and at lower frequency in Leydig cells (281). In humans and hamster, the germ-line appears to express only the conventional 1.4-kb mRNA (281, 283, 284). Different transcriptional initiation sites may be responsible for these differences (283, 284). These transcripts appear to be efficiently translated and associated with polysomes (285).

Extensive studies of a "cassette" sequence in the 5′-flanking region of the pENK gene have revealed that responses to several transcription factor proteins can be conferred in tissue culture cells in constructs containing <200 bases of the pENK gene 5′-flanking sequence (286, 287). Because of this distinctive feature, high testis expression and low expression in other organs have been obtained in transgenic mice containing segments of the 5′-flanking region of the rat pENK connected to a chloramphenicol acetyltransferase reporter (288) or the human 5′-flanking region connected to β-galactosidase (289). Subsequently it has been shown that high levels of expression of the pENK gene in the testis in transgenic mice can be achieved with an even shorter "cassette" region of the pENK gene promoter, further supporting a role for pENK expression on testicular and spermatogenic development and function (290).

FSH and various compounds that activate the cAMP sys-

tem transiently increase the pENK mRNA content in Sertoli cells isolated from 20-day-old rats (281, 282). Similarly, hCG or a cAMP analog treatment of cultured adult rat Leydig cells leads to a rapid increase in pENK mRNA levels (291), while the phorbol ester O-tetradecanoylphorbol 13-acetate increases the ppENK mRNA abundance in cultured peritubular cells prepared from testes of 20-day-old rats through a cAMP-independent pathway (292). A low content of pENK-derived Met-ENK sequences was found in adult testis from rat, hamster, and cattle (277). The immunohistochemical localization of ENK in adult rat testicular tissue cultures revealed positive staining in some interstitial cells, and 1 h after hCG addition specific ENK immunoreactivity was visualized in Sertoli cell cytoplasm in some tubules (293). A positive immunoreactivity for Met-ENK, which increased when the cAMP stimulator forskolin was added to the cells, was also observed in Sertoli cell cultures (294).

Interestingly, a DNA-binding factor with high affinity and specificity for the Leu-ENK-encoding sequences in the pDYN and pENK genes has been characterized (295). This factor, named Leu-ENK-encoding sequence DNA-binding factor, was present at highest levels in rat and mouse testis, cerebellum, and spleen and was generally higher in late embryonal compared with newborn or adult animals. The specific functional role of this factor is unknown. However, it could contribute to the regulation of the expression of pDYN and pENK gene transcription during development.

pENK transcripts have been detected also in human testis, and human spermatozoa show a positive immunoreactivity for Met-ENK molecules localized in the acrosome where they are stored, presumably until release at fertilization (285).

b. Receptors. As discussed above, in the βEND section, opioid-binding sites are exclusively localized in Sertoli cells in adult rats. It is still unknown which class of opioid receptor is represented on these cells.

c. Local functions. In immature rats the *in vivo* intratesticular injection of a synthetic ENK analog suppresses in a dose-dependent manner the basal testosterone secretion from the injected testes excised and placed in culture (296). The effect seems to be a consequence of the local action of the ENK analog since its systemic injection produced no change in steroidogenesis (296).

As reported above, transgenic mice containing bases −193 to +210 of the human pENK gene and an additional 1 kb of 3′-pENK-flanking sequence driving expression of bacterial chloramphenicol acetyltransferase, and the same promoter and flanking sequences driving expression of a rat pENK cDNA, have been generated (290). The animals showed dramatic expression of the transgene in the testis, while much lower expression was observed in the brain and other ENK-producing tissues. High levels of expression in the testis can thus be achieved with a very short promoter region and do not require intron A sequences previously considered necessary. Moreover, the effect of altered ENK expression on testicular function has been analyzed. No male founder or transgenic offspring of the animals expressing the human pENK gene revealed reproductive difficulties. In contrast, one founder of the mice expressing the rat pENK gene showed no reproductive success despite normal mounting, intromission, and other motor behaviors associated with reproduction. In this animal, no sperm were seen upon dissecting the epididymis, the testes were significantly smaller compared with the testes of age-matched controls, and the seminiferous tubules were grossly abnormal, with degenerating pachytene spermatocytes and early germ cells including spermatogonia. Mature spermatids were not present in any of the tubules. Sertoli cells in the seminiferous tubules were often abnormal and incorrectly oriented. A second male founder also displayed reduced fertility, and motility measurements of epididymal sperm of this animal revealed that only 43% of the sperm cells were motile. Lack of reproductive success was also observed in two offspring from two transgenic lines. The four infertile animals displayed blood LH and testosterone levels within normal limits. In summary, overexpression of the pENK cDNA appears to have induced grossly abnormal testicular morphology in one offspring, low sperm motility in another, and reduced fertility in others (290).

These findings suggest that the role of ENK in testicular function merits closer scrutiny.

3. Dynorphin (DYN).

a. Expression, localization, and production. Immunoreactive DYN (297) and pDYN mRNA have been found in testicular extracts of adult rat testis (298, 299). In the rat, pDYN-derived peptides are found in the Leydig cells by immunohistochemistry, and immunoreactive DYN has also been demonstrated in guinea pig and rabbit testicular homogenates by RIA (299). Accordingly, pDYN mRNA and pDYN-derived peptides are synthesized in the R2C rat Leydig tumor cell line and are positively regulated by cAMP analogs (300). The chromatographic characterization of DYN immunoreactivity in guinea pig and rat testis showed that both authentic DYN-A and DYN-B are present in this tissue and that the immunoreactive forms of the peptides are similar to those found in brain and pituitary (301). Through the use of rat testicular cell fractionation procedures and Northern blot analysis, Sertoli cells were also found to be the site of pDYN mRNA synthesis, and *in situ* hybridization to fixed adult tissue confirmed this result (302). Moreover, the treatment of primary cultures of rat Sertoli cells with cAMP resulted in a transient increase in steady state pDYN mRNA and immunoreactive DYN levels (302). Immunoreactive DYN has been also detected in the testicular perifusion effluent of perifused adult rat testicular fragments (270).

b. Receptors. Since DYN is the major ligand for the κ-opioid-binding sites and the κ-receptor mRNA has not been found in the mouse testis (262), a diffuse involvement of DYN in testicular function, at least in this animal species, is unlikely.

c. Local functions. The treatment of rat perifused testicular fragments and purified Leydig cells with DYN did not alter either basal or hCG-stimulated testosterone secretion (270).

In summary, at this point in our knowledge, despite the demonstration of the presence of DYN mRNAs and peptides in the testis, a functional involvement of DYN in the physiology of the male gonad is rather unlikely and should be considered only speculative.

J. Substance-P (SP)

The undecapeptide SP belongs to a family of related peptides, the tachykinins, which are widely distributed in both the central and peripheral nervous system where they function as neurotransmitters/neuromodulators and share a common terminal amino acid sequence (303). The receptors for SP belong to the family of tachykinin receptors classified as NK1, NK2, and NK3. SP, neurokinin A, and neurokinin B are the preferred endogenous ligands for the three receptor types, respectively (304). The cloned SP receptor and the other tachykinin receptors are membrane proteins with seven-transmembrane helix patterns typical of G protein-coupled receptors (305).

1. Expression, localization, and production. A selective immunostaining for SP has been reported in the Leydig cells of adult human testicular tissue (306, 307), isolated human Leydig cells (308), and in fetal and adult Leydig cells from golden hamster and guinea pig (309–311). Using a modified PCR assay, SP mRNA could be detected in extracts of human, mouse, and bovine testes, but not in rat or boar testes (312). Sequencing analysis of the PCR products from human testis showed that the transcripts expressed encode both SP and neurokinin-A neuropeptides (312).

2. Receptors. PCR analysis was able to demonstrate the presence of mRNA for both SP and neurokinin-A receptors in human testis (312).

3. Local functions. Angelova *et al.* (311) reported that, *in vitro*, synthetic SP can stimulate the production of testosterone by hamster Leydig cells from neonatal and prepubertal animals, whereas a clear inhibition of both basal and hCG-stimulated steroid production was observed in adult Leydig cell cultures (311). The influence of SP on the binding characteristics of the LH receptor in purified Leydig cells from golden hamster was also studied (313). A significant increase in the binding capacity was estimated in the Leydig cells from young animals, while SP reduced the number of LH binding sites in adult hamsters (313). Sertoli cells from immature rats respond to 24 h SP treatment with an increase of transferrin and lactate release (314).

Further studies are required before one can speculate that SP might have a regulatory role on the testicular function *in vivo*.

K. Neuropeptide Y (NPY)

NPY is a 36-aa peptide member of the pancreatic-polypeptide family, isolated and extensively distributed in the brain, in sympathetic neurons innervating cardiovascular and respiratory system, gastrointestinal, and genitourinary tract (315). Considerable interest has focused on the importance of NPY in the control of basic vegetative functions. NPY stimulates food intake, modulates circadian rhythms, and regulates the release of several hypothalamic hormones (316). NPY appears to act upon at least four types of receptors called Y1, Y2, Y3, and an atypical Y1 receptor that mediates the feeding response stimulated by NPY (317).

1. Expression, localization, and production. NPY mRNA is expressed in immature rat Leydig cells and Sertoli cells (318). Leydig cells treated for 12 h with LH, interleukin-1α (IL-1α), or IL-1β exhibited an increase in NPY mRNA expression, which is also observed after forskolin treatment. FSH treatment increased NPY mRNA levels of Sertoli cells in a dose-dependent manner. Moreover, a germ cell factor and a Sertoli cell factor stimulated by FSH increased NPY gene expression in Leydig cells. A positive NPY-like immunoreactivity in cultured immature Leydig and Sertoli cells was also shown by immunocytochemistry (318).

To date, no evidence has been published for the presence of NPY receptors in the testis or for local *in vivo* or *in vitro* actions of the peptide .

Erickson *et al.* (319) have recently reported the results of knocking out NPY (319). The knockout mice appear to be normal in all respects, except for a propensity for seizures. Both male and female mutants are fertile, demonstrating that the neuroendocrine systems regulating growth and reproduction as well as the sympathetic innervation of the vas deferens are functional in the absence of NPY.

In light of these data, any hypothesis on a possible involvement of NPY in the local regulation of testicular function seems premature.

III. Peptides Originally Identified in the Male Gonad

A. Inhibin (INH) and activin (ACT)

INH and ACT are structurally related gonadal dimeric glycoproteins named for their regulatory effects on FSH synthesis and release. These substances belong to a larger superfamily that includes transforming growth factor β (TGFβ), Müllerian inhibiting substance, the decapentaplegic gene complex of *Drosophila*, the bone morphogenic proteins (BMPs), and the vegetal growth factor gene of *Xenopus* (320). INHs are heterodimers of 32 kDa composed of an α-subunit and one of two related β-subunits (βA and βB). Both INH-A (α-βA) and INH-B (α-βB) suppress the FSH release from the anterior pituitary. ACTs are β/β-dimers of which the βA/βA and βB/βB homodimers and the βA/βB heterodimer have been purified and characterized and shown to stimulate FSH secretion from the pituitary. The mRNAs encoding INH/ACT subunits are most abundant in the ovary and the testis but can be found in a variety of other tissues (321). In addition to their endocrine role, these proteins have been shown to affect the growth and differentiation of a number of cell types, including cultured rat anterior pituitary cells, gonadal and neuronal cell lines, and hematopoietic progenitor and erythroleukemia cells (321).

Two distinct binding proteins for INH and ACT have been identified, follistatin and α-2 macroglobulin. Follistatin, initially discovered for its ability to suppress pituitary FSH release (322), was subsequently found to bind ACT directly (323) and, with lesser affinity, INH (324, 325). It is a single-chain protein highly cysteine-rich, product of a single gene, with different molecular weight forms attributed to alternative mRNA splicing. It is expressed in a variety of tissues, particularly gonads, kidney, and pituitary. The suggested biological role of follistatin is the involvement in the neu-

tralization of ACT activity (326). α-2 Macroglobulin is an high molecular weight endopeptidase inhibitor present in high levels in human serum (327, 328), known to bind proteases and a number of growth factors, including TGFβ and nerve growth factor (NGF) (329). It is unknown whether binding of ACT or INH to α-2 macroglobulin alters their bioactivity or immunoreactivity.

Two genes coding for the ACT receptors have been cloned. One (ActRII) (330) encodes for a protein of 494 aa consisting of an ACT-binding domain, a single membrane-spanning domain, and an intracellular serine/threonine-specific protein kinase domain. The second (ActRIIB) encodes potentially four different ACT receptor isoforms belonging to the protein serine/threonine kinase receptor family, which are produced by alternative mRNA splicing (331).

To date, a high-affinity receptor for INH has not been found by molecular or biochemical techniques. However, although the identified ACT receptors exhibit a low affinity for INH (330, 331), a separate high-affinity receptor for INH is widely believed to exist, since in some systems in which ACT and INH are both active, INH exerts its activity with a much higher potency (332).

Excellent reviews have been written on the subject, to which the reader can refer for a complete description (332–334).

1. Expression, localization, and production. All three INH subunits have been found in the fetal, postnatal, and adult testis. In the rat, α-subunit, βB-subunit, βA-subunit, and follistatin mRNAs have been found starting from day 14 post coitum (pc) by *in situ* hybridization (335, 336). The βA-subunit mRNA signal was localized in the interstitial tissue, the βB-subunit was localized in the tubules, and the α-subunit message was present in both the interstitial tissue and the seminiferous tubules (335, 336). The expression and localization of α- and βA-subunit mRNA and protein have been evaluated by *in situ* hybridization and immunocytochemistry in fetal sheep testes collected at various times of gestation (337). As in the rat, the expression of the α-subunit mRNA was localized within the seminiferous cords and in a small number of Leydig cells of the developing fetal testis; the expression progressively increased with gestational age (337) as confirmed also by immunoassay and bioassay (338). No expression of INH βA-subunit mRNA or protein was detected at any stage of gestation (338). Bioactive and immunoactive INH were found to be produced and to increase throughout gestation in bovine fetal testis (339). Roberts *et al.* (340) have studied the testicular postnatal distribution of immunostaining and mRNA signal of the INH subunits in the rat (340). In 12-day-old rats, immunostaining and mRNA signal for the α-subunit were found in Leydig cell clusters. The βA- and βB-subunit staining and βA-subunit message were detected in isolated interstitial cells. Positive immunostaining for each subunit was localized in a Sertoli cell-like pattern in the seminiferous tubules. In addition, there was a positive mRNA signal for the α- and βB-subunit over regions containing these cell types (340). The treatment with hCG induced a dramatic increase in the α-subunit immunohistochemical staining and mRNA signal in Leydig cell clusters (340). In adult rats, α- and βB-subunit staining and α-subunit mRNA signal were observed in the interstitial cells. As in the immature animals, all three subunits were localized in a Sertoli cell-like pattern in the tubule, and a positive mRNA signal for the α- and βB-subunits was found over these cells; contrary to what was observed in the immature animals, the hCG treatment did not modify the intensity of the signals (340). Purified Leydig cells from adult male rats synthesized and secreted INH *in vitro* as measured by Northern blot analysis, RIA, and *in vitro* bioassay; LH stimulated immunoactive INH production in a dose-dependent manner (341). Moreover, it has been established that FSH and to a lesser extent epidermal growth factor (EGF), but not testosterone, are able to stimulate the production of INH by immature Sertoli cells in culture (342).The comparative distribution of the three INH subunits in the rat testis during prenatal and postnatal development has been studied using an immunohistochemical approach (343). Both α- and βB-subunit staining were present in the seminiferous epithelium of fetal, neonatal, pubertal, and adult rat with a distribution consistent with a localization in Sertoli cells. The predominant localization of the βA-subunit was in the nuclei of pachytene and zygotene spermatocytes (343).

Interstitial cell cultures from immature rat and pig testis and immature rat Sertoli cell cultures secrete ACT (344, 345). Cultured PMC from pubertal animals express high levels of βA-subunit mRNA and some α- and βB-subunit mRNA and secrete immunoreactive and bioactive ACT-A (346). In extracts from mature rat testis, INH α-subunit RNA can easily be detected, whereas the βA- and βB-subunits are expressed at 50 and 15 times lower levels, respectively (321). Moreover, INH α-, βA-, and βB-subunit mRNA levels are 4- to 10-fold higher in 8- to 15-day-old animals when compared with mature animals (321). These age-dependent changes have also been observed by the measurement of immunoreactive testicular INH levels during development (347), and a positive effect of FSH on the INH α-subunit expression either *in vivo* or in Sertoli cell cultures *in vitro* has been reported (348).

The expression of INH α- and βB-subunit in the rat seminiferous epithelium is stage dependent; Northern blot assays of seminiferous tubule fragments extracts revealed that the expression of both subunits is highest in stages XIII-I and lowest in stages VII-VIII (349). The seminiferous epithelium stage-dependent expression of INH βA- and βB-subunit mRNAs was also analyzed by *in situ* hybridization. The βA-subunit was expressed in Sertoli cells starting at stage VIII of the cycle with a maximal expression during stages IX-XI, while the βB-subunit was maximally expressed in Sertoli cells at stages XIII-III (350). This finding has been confirmed by the evaluation of the INH α- and βB-subunit mRNA expression and the levels of bioactive and immunoreactive INH in rats with stage-synchronized spermatogenesis (351).

In human fetal testis α-, βA-, and βB-subunits were detected at the beginning of the second trimester of gestation by both mRNA analysis (352) and immunocytochemistry (353, 354). The α-subunit was found in both tubular and interstitial areas in early gestation, becoming predominant in the tubules by late gestation. The β-subunits were observed initially in the interstitium and subsequently also in the tubule by the end of gestation. Moreover, it has been shown that midgestation human fetal testicular cultures secrete de-

tectable immunoreactive INH α-subunit in response to FSH and hCG stimulation (353). In adult human testis, the INH α-subunit has been localized in the Sertoli cells and some Leydig cells (355, 356). In subsequent studies, in adult nonhuman primate species and in men, INH/ACT subunits were found in the cytoplasm of Sertoli cells and Leydig cells but not in germ cells (357).

2. Receptors. ActRIIB message is found in rat testis from 15–20 days pc (358). Two species of ActRII mRNA (6 and 4 kb) have been identified in mouse and rat testis (330, 359). Both messengers are expressed in Sertoli cells and at low levels in Leydig cell preparations. Spermatocytes and round spermatids express only the smaller mRNA, whereas elongating spermatids do not express ACT receptor mRNA (359). Attisano *et al.* (331) observed an equal abundance of ActRII and ActRIIB in mouse testis (331). In the rat, Cameron *et al.* found a clearly present ACT receptor message in the germ cells. ActRII is the predominant form with a strong signal in round spermatids and a moderate signal in pachytene spermatocytes. In contrast, ActRIIB was absent within the tubules but weakly expressed in Leydig cells (360). The ActRII and ActRIIB2 mRNA messages were localized in the rat seminiferous epithelium (350, 361). Maximal ActRII expression was seen in late primary spermatocytes at stages XIII-XIV and in early round spermatids at stages I-IV (350). ActRIIB2 mRNA was expressed maximally in stages IX-XI in type A1 and A2 spermatogonia and in Sertoli cells (361). In pubertal rat testis, ActRIIB2 expression was localized in Sertoli cells around primary spermatocytes and meiotically dividing cells (361). Woodruff *et al.* (362) have reported that ACT-A binds primarily to spermatogonia, while INH-A binds all germ cell stages (362). The changes in ACT receptor expression during sexual maturation in rat testis were examined by Northern blot analysis. Two ActRII mRNAs (6 kb and 3 kb) were detected. The large mRNA was low on day 7, and gradually increased by day 35, while the small mRNA was low on day 7, began to increase on day 21, and increased dramatically by day 35 (363, 364). The levels of expression of ActRIIB gene did not change during testicular development (364). *In situ* binding of recombinant human ^{125}I-labeled INH and ACT has been used to identify binding sites and potential target cell populations in rat testis throughout postnatal development (365). INH bound to islands of interstitial cells in animals of all ages. In contrast, ACT binding was seen on cells located basally within or around all seminiferous tubules, regardless of the age of the animal, as well as on round spermatids in stages VII-VIII of the cycle of the seminiferous epithelium in animals older than 30 days (365). ActRII mRNA has also been detected in pubertal PMC in culture (346). Cloning of the human ACT receptor from a human testis cDNA library reveals high evolutionary conservation (366).

Dynamic changes in the relative amount of the INH- and ACT-binding proteins could act to modulate ACT and INH bioavailability, and thus it is important to study the local testicular production of these substances. Both follistatin mRNA and immunoreactivity have been identified in adult testicular tissues (326, 350, 361, 367). In these studies, follistatin mRNA was localized in a similar stage-specific manner to that of the INH βA-subunit mRNA, with maximal levels in stages IX-XI Sertoli cells (350). Sertoli cell expression of follistatin mRNA was stimulated by EGF but not by FSH or testosterone (367). In contrast to the site of follistatin mRNA expression, follistatin protein was immunohistochemically identified in spermatogenic cells (particularly spermatocytes and spermatids) and Leydig cells, but not in Sertoli cells (368). One explanation for this discrepancy is that follistatin produced by one cell type may diffuse and associate with another cell type or with components of the extracellular matrix; alternatively, the lack of protein synthesis cannot be ruled out.

Sertoli cells synthesize and secrete α-2 macroglobulin *in vitro*, and immunoreactive α-2 macroglobulin can be measured in tubular and rete testis fluids (369). In the testis, this protein is believed to protect the seminiferous tubules and the rete testis from proteases released from spermatids and other cells. The impact of α-2 macroglobulin on ACT/INH activity in the testis is presently unknown.

3. Local functions. The first evidence that INH and ACT could serve as local modulators of testicular functions derived from the observation of their ability to modulate the LH-stimulated biosynthesis of androgen in rat cultured Leydig cells (272). ACT exerted an inhibitory action on LH-stimulated androgen biosynthesis at all stages of Leydig cell differentiation, whereas INH facilitated LH action in a mixed testis cell preparation from neonatal and adult rats (272, 370). In immature porcine Leydig cell cultures, ACT-A reduced the hCG-stimulated dehydroepiandrosterone accumulation, but increased the conversion of pregnenolone and dehydroepiandrosterone to testosterone, suggesting that ACT may alter the activity of either the cholesterol side chain cleavage enzyme or the 3β-hydroxysteroid dehydrogenase (3β-HSD)/isomerase (371). In cells obtained from pubertal and adult rats, ACT stimulated the proliferation of spermatogonial cells (372, 373) and caused a rapid reaggregation of Sertoli cells and germ cells in the absence of basement membrane or peritubular cells (372). This effect is accompanied by an ACT-stimulated increase in thymidine incorporation into germ-Sertoli cell cocultures and, interestingly, these effects can be differentially regulated by follistatin (374). However, ACT has a different function during rat gonadal development in that it inhibits thymidine incorporation in rat testes on day 14 pc and has no effect in testes on day 15 or 18 pc (358). INH decreases spermatogonial proliferation when injected locally into adult hamster testis without affecting the controlateral testis treated with control fluid (375). ACT-A has a slight inhibitory effect on proliferation of a rat Leydig cell testicular tumor line (376). ACT inhibits FSH-stimulated aromatase activity, androgen receptor mRNA expression, and androgen binding without significantly affecting FSH receptor mRNA expression in cultured Sertoli cell isolated from 21-day-old rats (345).

Targeted disruption studies have provided some interesting clues about local functions of INH/ACT in the testis *in vivo*. Male mice homozygous for a deletion of INH α-subunit develop mixed or incompletely differentiated gonadal stromal tumors as early as 4 weeks of age (377), and show a 200-fold increase in testicular expression of ACT βA-subunit mRNA accompanied by a tissue- specific

reduction in the expression of its type II receptor (378). The predisposition of INH-deficient mice to gonadal tumors identified INH as the first tumor suppressor protein with a gonadal specificity. The gonads and external genitalia of male and female INH-deficient mice were normal before tumor development by gross examination and detailed histology, suggesting that INH is not essential for normal sexual differentiation and development. Moreover, as mature spermatozoa were initially found in the homozygote αINH-deficient animals, INH did not seem necessary for the formation of mature germ cells. Only when the tumor becomes much more destructive was an arrest of spermatogenesis observed. Even in the contralateral tumor-free gonads of some male mice, germ cell maturation was arrested. This suggests that the tumors secrete a substance(s) that may be toxic for spermatogenesis. The exact mechanism of tumor formation in INH-deficient mice is still unclear. The deletion of INH α-subunit results in 2- to 3-fold elevation of serum FSH levels. The prolonged elevation of FSH could contribute to, but is not directly responsible for, the development of gonadal tumors, considering that humans who have FSH-secreting adenomas with FSH levels more than 20-fold higher than normal do not develop gonadal stromal tumors (379). The finding that ACT, but not INH, can stimulate the proliferation of gonadal tumor cell lines derived from the INH-deficient mice (380) suggests that the unopposed action of ACT, together with prolonged FSH action, might take part in tumorigenesis.

Also ACT-, follistatin-, and ActRII-deficient mice have been produced (381–383). Jaenisch and colleagues (381) created mouse strains deficient in the βB- subunit expected to be deficient in ACT-B, ACT-AB, and INH-B. Homozygous mutant embryos had defects in eyelid development. Homozygous mutant females were unable to rear offspring normally while homozygous males bred normally, and histological examination of their testes failed to detect overt abnormalities. Mice lacking the βA-subunit, thus unable to express ACT-A and INH-A, die within 24 h of birth. They have no whiskers or lower incisors and have a cleft palate (382). Surprisingly, defects in ActRII-deficient mice show little resemblance to those of other ACT-deficient animals (383). Most individuals developed into adults whose only significant problem was the suppression of FSH and the consequent defective reproductive performance that progressed to infertility in the females but only to a delay in fertility in males (383). The testes of the homozygous animals were small, but spermatogenesis could occur despite reduced FSH and without signal transduction through ActRII. Finally, follistatin-deficient mice are small and have a number of serious defects leading to death within a few hours of birth (384).

In conclusion, ACT and INH have local regulatory roles in the testis both in the control of androgen secretion and germ cell proliferation. Although the gene deletion studies indicate that many aspects of gonadal development proceed in the absence of INH and ACT, the identification of INH as a tumor suppressor gene with gonadal specificity is remarkable.

B. PModS

PModS is a nonmitogenic paracrine factor isolated from the conditioned medium of peritubular cells that modulates Sertoli cell function and whose secretion is increased by testosterone (385, 386). Further characterization of PModS showed that there are two forms of the substance, named PModS A and B, with respective mol wts of 54,000 and 56,000 (387). PModS stimulates transferrin, ABP, and INH secretion from Sertoli cells in culture (385, 388, 389), whereas it exerts no effect on Leydig cell basal and hCG-stimulated testosterone and immunoreactive INH production (390). PModS was found to dramatically increase mRNA levels for c-*fos*, but had no effect on c-*jun* mRNA. The treatment of Sertoli cells with an antisense c-*fos* oligonucleotide was found to inhibit the actions of PModS on transferrin expression, suggesting that PModS acts indirectly through transcription factors to induce Sertoli cell-differentiated functions (391). PModS stimulates cGMP levels in Sertoli cells, without influencing cAMP levels or calcium mobilization or the metabolism of PI, and increases phosphorylation of specific proteins (392). Since it has been observed that cGMP does not directly mediate the actions of PModS, it is postulated that tyrosine phosphorylation and alteration in the expression of transcription factors may mediate the Sertoli cell differentiation induced by PModS.

In spite of the reported *in vitro* effects and hormonal regulation of PModS, the lack of biochemical characterization of the substance, identification of receptors, and *in vivo* function do not allow the expression of a critical judgment on its role in the male gonad.

IV. Growth Factors

A. Insulin-like growth factors (IGFs) and IGF-binding proteins (IGFBPs)

The IGFs, their receptors, and IGFBPs are cellular modulators that play essential roles in the regulation of growth and development. The IGFs are single-chain polypeptides that are expressed ubiquitously. The two major forms are IGF-I and IGF-II, which share a 62% sequence homology. There are two known receptors that specifically recognize the IGFs: the IGF-I receptor, and the IGF-II receptor, which is identical to the cation-independent mannose-6-phosphate receptor. While the IGF-I receptor is the primary mediator of IGF action, the IGF-II receptor, which functions in the trafficking of lysosomal enzymes, has no known IGF-signaling functions. The insulin receptor can also bind the IGFs with low affinity, and hybrid IGF/insulin receptors that bind the IGFs and potentially transmit a cytoplasmic signal have been isolated. IGF-I and IGF/insulin receptors have a cytoplasmic portion that contains a highly conserved tyrosine kinase catalytic domain; signaling via the IGF-II receptor may involve GTP-binding protein activation.

The biological actions of the IGFs are modulated by a family of at least six IGF-binding proteins (IGFBPs) that are found in the circulation and in extracellular compartments. The IGFBPs can inhibit or enhance IGF effects and may also have ligand-independent effects.

The reader can refer to recent reviews on IGFs, IGF receptors, and IGFBPs for detailed information (393–395).

1. Expression, localization, and production. Testicular immunoreactive IGF-I was first discovered in media conditioned by seminiferous tubule cultures from adult rats, Sertoli cells cultured from immature rats (396), and in acid extracts of rat testes (397, 398). Sertoli cells and PMC prepared from sexually immature rats accumulate IGF-like activity in the medium, and the Sertoli cell accumulation is inhibited by cycloheximide and stimulated by cyclic nucleotide analogs (399). The partial characterization of this IGF immunoreactive material demonstrated that immature rat Sertoli cells in culture secrete a peptide that is the equivalent of human IGF-I (400). A high homology between the IGF-I-reactive material in culture media conditioned by porcine Sertoli cells and human IGF-I, whose secretion could be increased by FGF and EGF, was also observed by other groups (401–403). Evidence has been presented that apart from Sertoli cells and PMC, cultured Leydig cells from immature rats also produce IGF-I (404, 405). Peritubular cells display the highest production rate followed by Sertoli cells and Leydig cells.

A marked age-related change of IGF-I-like immunoreactive material has been reported in the rat testis by means of immunohistochemistry (406, 407). Up to 2 weeks after birth, all cells in the growing testis show IGF-I-like immunoreactivity; thereafter, the frequency declines and the Sertoli cells become negative. During puberty, there is a rapid increase in the number of spermatogenic cells showing IGF-I-like immunoreactivity. In the adult rat, IGF-I immunoreactivity is exclusively localized in the cytoplasm of spermatocytes. No significant stage-dependent changes have been observed in testicular IGF-I concentrations in stage-synchronized rat testes (408).

The expression of the mRNA for IGF-I was originally demonstrated in adult and immature rat testis (409–411). Subsequently, Leydig cells have been shown to express this gene; the expression was enhanced by GH treatment (412). The analysis of the ontogeny of IGF-I and IGF-II mRNA in the rat has revealed that both mRNAs are maximally expressed in the testes of day 2 animals and decrease with age (25). The quantification of mRNA for IGF-I during pig testicular development showed a steady increase from fetal 100–102 days up to 25 weeks postnatally (413). The pattern of expression of IGF-I gene expression in the mouse testis has been studied by *in situ* hybridization analysis (414). In the testis of prepubertal mice (postnatal day 14) IGF-I transcripts were localized in the interstitial compartment. In older animals (postnatal day 35) IGF-I transcripts were detected in the seminiferous epithelium, but not in the interstitial cells, with a strong signal evident only in spermatids. In immature hypophysectomized rats, the administration of LH, FSH, and GH all resulted in a significant increase in testicular IGF-I mRNA expression (411). It has been shown that IGF-I production by cultured immature pig Leydig cells and Sertoli cells is stimulated in a dose-dependent fashion by LH and FGF or FSH and FGF, respectively (415). Both FSH and GH trigger a modest increase of IGF-I levels in Sertoli cell cultures prepared from pubertal rats (399). FSH treatment partially prevents the marked decrease in intratesticular IGF-I in hypophysectomized or GnRH antagonist-treated rats but not in GnRH antagonist plus EDS-treated animals, providing *in vivo* evidence for the importance of Leydig cell-Sertoli cell interactions in regulating testicular IGF-I content (416, 417). This effect of FSH is potentiated by GH and testosterone (418).

In the human testis the immunohistochemical staining of IGF-I is preferentially localized in Sertoli cells; less evident staining is found in primary spermatocytes and in some Leydig cells (419, 420). The slight positive reactions at the levels of spermatocytes and Leydig cells have been interpreted as being due to IGF-I-receptor complexes. *In situ* hybridization studies in human testis failed to reveal mRNA for IGF-I (421). This disagreement with the immunohistochemical findings has been explained by an accumulation of circulating IGF-I bound to IGFBP-2 or to IGF-I receptors localized in the germinal cells or alternatively to a cross-reactivity in the immunological detection of IGF-I with IGF-II derived from local testicular synthesis within the seminiferous tubule.

No mRNA for IGF-II was detected in the adult rat testis (410); low mRNA expression, unmodified by hormonal treatments, was found in the immature hypophysectomized rat (411). In the pig, IGF-II mRNA was expressed in the fetal and immature testis, and its expression decreased with age (413). Human fetal testis contains abundant IGF-II mRNA, which significantly decreases from 13 to 25.8 weeks of gestation (422). IGF-II mRNA was abundantly localized in vascular cells and peritubular cells of adult human testis by *in situ* hybridization (421).

2. Receptors. Sertoli cells and pachytene spermatocytes from immature rats (399, 423, 424) and Sertoli cells from immature pigs (425) were found to possess IGF-I receptors. IGF-I receptors have been identified in both whole testicular homogenates and isolated Leydig cells from adult rats (398, 424, 426–428). IGF-I binds to total testis membrane fractions from immature and adult rat testes, the maximal specific binding being reduced between 21 days of age and adult life (395). It has been suggested that Leydig cell type I IGF receptors can be up-regulated by LH, FSH, and GH, with hCG/LH being the most important factor (428). In both pig (429) and rat (428, 430), Leydig cell LH/hCG up-regulates the IGF receptors.

Strong IGF-I receptor gene expression, which does not appear to change with developmental age, is evident in Leydig cells, PMC, and occasionally in spermatogonia of 14- and 35 day-old mice testis by *in situ* hybridization (414).

In human testis the major positivity for the IGF-I receptor was found in secondary spermatocytes, early spermatids, and in some Leydig cells, whereas Sertoli cells were less positive (419). *In situ* hybridization to localize mRNA for IGF-I receptors in human testis showed positive expression in the germinal epithelium (421).

Both Sertoli and germinal cells from adult rat and mice contain IGF-II receptors (423, 431). IGF-II receptor mRNA has been localized by *in situ* hybridization in the germinal epithelium of both human (421) and rat (432) adult testis. IGF-II receptor immunoreactivity was present in human testis from 23 weeks of gestation to 2 yr of age (433).

The actions of IGFs are modified by IGFBPs that may

enhance or inhibit the effects of IGFs. As reported above, at least six different IGFBPs have been identified in the rat. Northern analysis of the IGFBP3 and IGFBP2 mRNAs in rat tissues showed an abundant expression in the testis (434, 435). Cultured immature rat Sertoli, Leydig, and peritubular cells produce IGF-I BPs (404). Both ligand blot analysis and RNA blot hybridization indicated that, in the rat, cultured peritubular cells synthesize primarily IGFBP-2, while IGFBP-3 is predominantly synthesized by prepubertal Sertoli cells (436, 437). The expression and regulation of IGFBP-1, -2, -3, and -4 in purified adult rat Leydig cells have been evaluated (438). None of the testicular crude interstitial cells, purified Leydig cells, or seminiferous tubules expressed IGFBP-1 mRNA. Large amounts of IGFBP-2 were expressed in purified Leydig cells and small amounts were expressed in seminiferous tubules. IGFBP-3 mRNA was predominantly expressed in purified Leydig cells but not in seminiferous tubules, while small amounts of IGFBP-4 were expressed by the Leydig cells.

In the adult human testis IGFBP-1 mRNA was not detected; IGFBP-2 was expressed in both Leydig cells and Sertoli cells; IGFBP-3 was detected only in the endothelium of testicular blood vessels; IGFBP-4 was localized in the endothelium, Leydig cells, and interstitial connective tissue; IGFBP-5 was abundant in connective tissue and was detected at low levels in the Leydig cells; IGFBP-6 was present in some peritubular cells and in some cells of the interstitial compartment (421).

3. Local functions. IGF-I and IGF-II stimulate the proliferation of rat and pig prepubertal Sertoli cells (423, 439), and IGF-I has a small mitogenic effect on immature Leydig cells (401, 440, 441). Both IGFs stimulate spermatogonial DNA synthesis and have a maintaining effect on premeiotic DNA synthesis in the rat under *in vitro* conditions (442), and IGF-I induces the differentiation of mouse type A spermatogonia (443).

IGF-I stimulates testosterone production and potentiates hCG-induced testosterone formation by cultured Leydig cells of rodent (427, 444–448), and porcine origin (401, 429, 440); this effect is more pronounced in immature than in adult cells (448, 449).

The IGF-I potentiating effect on hCG-induced testosterone production by Leydig cells *in vitro* has been confirmed in human (450).

An intraperitoneal injection of recombinant IGF-I to immature Snell dwarf mice (dw/dw) for 7 days caused a significant increase of testicular LH receptors and an acute steroidogenic response to hCG similar to that induced by recombinant hGH treatment, suggesting that the effects of GH on the testis are probably mediated by IGF-I (451). These effects could not be observed in phenotypically normal control (dw/−) animals. The GH-deficient rat has been used as animal model in which to analyze the effects of GH on reproduction. These dwarf rats, although fertile, have a decreased testicular size (452). The administration of GH in the GH-deficient rats from days 21 to 49 of life does not induce significant differences in testicular IGF-I concentrations or in the number of germ cells per cross-section of the seminiferous tubules (418), although it has been suggested that testicular development in the first 30 days of life is pituitary hormone- and probably GH-dependent (453).

Neither GH nor IGF-I pretreatment enhanced acute gonadal responses to gonadotropin stimulation of prepubertal non-human primate testis *in vivo* (454).

Transgenic mouse models have provided invaluable information to our understanding of IGFs physiology. Wolf *et al.* (455) characterized the effects of elevated IGF-II on body and organ growth at 4 and 12 weeks of age in transgenic mice expressing human IGF-II under the transcriptional control of the rat phosphoenolpyruvate carboxykinase promoter (455). Body growth was not significantly influenced by elevated IGF-II, but transgenic mice displayed increased kidney and testis weight at the age of 4 weeks, suggesting that elevated IGF-II in postnatal life may have subtle time-specific effects on testis growth. Disruption of the IGF-II gene showed that IGF-II is essential for normal placental and fetal growth, but not for postnatal growth, since the viable and fertile IGF-II null mutants exhibit dwarfism at birth but grow postnatally at a normal rate (456). In contrast, the postnatal growth of IGF-I null mutants, which survive variably depending on genetic background, is reduced (457). The IGF-I null mutation also has a dramatic impact on the development and physiology of the reproductive systems of both sexes (414). Homozygous mice are infertile dwarfs. The testes are reduced in size and sustain spermatogenesis only at 18% of the normal level. The most likely explanation for the infertility of IGF-I null males is predominantly the absence of sex drive, which is apparently due to inadequate serum testosterone levels probably insufficient for perinatal androgenization. Interestingly, androgen deficiency in the mutants can be correlated with an apparent retarded differentiation of Leydig cells. This assumption is supported by the following considerations. The Leydig cells lack the membranous whorls and produce low levels of testosterone both in basal conditions and after LH stimulation, and basal and LH-stimulated androstenedione production is much higher in the IGF-I null mutants than in wild type controls. All these are features of immature Leydig cells. Does testicular IGF-I have some degree of autonomy from other hormonal stimuli? The answer seems to be yes. GH receptor genes do not appear to be expressed in the testis (458–460). In contrast to the IGF-I null mutants, male *little* mice carrying a missense mutation of the gene encoding the receptor for GHRH (38, 39, 41), *dr/dr* rats, which have a base substitution in the GH gene (461, 462), and *dw/dw* rats, which have a deficit in GHRH signal transduction pathway (463), are fertile. All these natural mutants have low serum levels of IGF-I. Thus, in addition to being apparently GH-independent, the testicular functions of IGF-I seem to be served by its local production without an endocrine contribution by its circulating form. As a whole, these results strongly suggest that testicular IGF-I has a determinant role in Leydig cell development and differentiation and that the lack of this substance in the testis induces infertility. More direct proof of this action will derive from selective testicular targeted deletion studies.

B. Transforming growth factor-β (TGFβ)

TGFβ is a polypeptide composed of two disulfide-linked monomers, synthesized as large precursors. It belongs to a large family of polypeptides including ACT/INH, Müllerian inhibiting substance, the decapeptaplegic (dpp)/Vg-related factors, and the BMPs (464). At least five different TGFβ molecular species are recognized (213). The TGFβ receptors comprise multiple components. The main TGFβ-binding components are membrane proteins called receptor types I, II, and III, respectively. TGFβ receptor types I and II are involved in signaling, whereas the type III receptor, otherwise known as betaglycan, regulates access of the TGFβ to the signaling receptors. A model in which the type I receptor requires the type II receptor for ligand binding and both receptors upon heterodimeric receptor complex formation are required to activate serine/threonine kinase domains has been suggested (465, 466). TGFβ has a wide range of effects on cell proliferation, differentiation, and organization. Depending on the conditions, TGFβ can either inhibit or stimulate proliferation; the ability of TGFβ to elicit multiple cellular responses of opposite sign is the subject of great debate (467).

1. Expression, localization, and production. Both Sertoli cells and peritubular cells isolated from 20-day-old rats synthesize and secrete TGFβ in culture (468). Reverse phase chromatography of secreted proteins indicates that Sertoli cells primarily produce TGFβ1, and peritubular cells produce both TGFβ1 and TGFβ2, while a mixed population of germinal cells at various stages of development was not found to contain the TGFβ message. TGFβ3 mRNA has been detected in substantial amounts in murine adult testis (469).

The developmental expression of multiple forms of TGFβ (TGFβ1, TGFβ2, and TGFβ3) in whole testis and isolated peritubular cells and Sertoli cells from prepubertal, midpubertal, and late pubertal rat testes has been determined using nuclease protection analysis (470). TGFβ1 and TGFβ2 mRNA expression was predominant in the immature testis and decreased at the onset of puberty. TGFβ3 mRNA expression peaked at an early pubertal stage. Peritubular cell mRNA expression of TGFβ1, TGFβ2, and TGFβ3 decreased during pubertal development upon differentiation of this cell type. Sertoli cell expression of TGFβ1 increased slightly and plateaued during pubertal development, TGFβ2 mRNA was evident only in immature prepubertal cells, and TGFβ3 mRNA increased transiently at the onset of puberty. Immunoblot analysis indicated that both cultured peritubular cells and Sertoli cells can produce the proteins for TGFβ1, TGFβ2, and TGFβ3 (470). It has been shown that, in the mouse, the germinal cells express a unique transcript for TGFβ1 of 1.8 kb (471). The precise *in vivo* localization of TGFβ1 and TGFβ2 during rat testicular development has been determined by means of immunohistochemistry (472). TGFβ1 immunoreactivity was detected in Sertoli cells throughout testicular development. TGFβ2 was found in fetal Sertoli cells but became undetectable rapidly after birth. In fetal animals the Leydig cells contained TGFβ1 and TGFβ2; after birth TGFβ1 persisted whereas TGFβ2 became undetectable. Before puberty, TGFβ1 and TGFβ2 were absent in a portion of the Leydig cells; when the adult stage was reached, TGFβ1 was no longer detectable and TGFβ2 staining was faint to absent. During spermatogenesis, TGFβ1 predominated in spermatocytes and early round spermatids, but as the spermatids elongated around stages VIII-IX of the cycle, the TGFβ1 levels declined. TGFβ2 was undetectable in spermatocytes and early round spermatids, but as spermatogenesis progressed, around stages V-VI, the spermatids rapidly acquired a TGFβ2-positive reaction (472).

TGFβ-like activity has been identified in conditioned medium obtained from cultures enriched in immature porcine Sertoli cells by both bioassay and RRA (473), and the release of TGFβ-like material is modulated by hormonal treatment. FSH decreases the release of TGFβ-like material to undetectable levels in a dose-dependent way, while the TGFβ secretion is enhanced by estradiol, Dex, or T_4 treatment; the inhibitory action of FSH seems to be predominant since the stimulatory action of steroid and thyroid hormones is not observed in the presence of FSH (473). An analogous FSH-induced reduction of TGFβ secretion by cultured rat Sertoli cells has been reported by others (470).

2. Receptors. TGFβ specifically binds to immature porcine Leydig cells and Sertoli cells (474, 475). The testicular cell expression and developmental appearance of mRNAs for the three high-affinity TGFβ receptors has been examined in the rat (476). Transcripts for receptors I and II were essentially detected in the immature 10-day-old testis, whereas receptor III mRNAs were present throughout testicular development. Somatic cells contained mRNAs for the three receptor types extending also to the PMC the previous observation of the presence of TGFβ receptors on Leydig cells and Sertoli cells. In germ cells, transcripts for types I and II were in low abundance compared with type III mRNA, which was expressed in three different transcript forms (476).

3. Local functions. TGFβ1 appears to play a role in reproductive function both during embryogenesis and in the adult. TGFβ1 inhibits proliferation of cultured primordial germ cells (PGC) isolated from 8.5 pc mouse embryos (477). Cultured genital ridges exert a chemotropic effect on PGC, which is blocked by antibodies to TGFβ1, and TGFβ1 mimics the chemotropic effect of genital ridges. These observations suggest that TGFβ1 may be one of the factors that modulate the migration of PGC to the genital ridges *in vivo* (477). TGFβ has a potent inhibitory effect on Leydig cell steroidogenesis. In immature pig and rat Leydig cells, TGFβ reduces the number of hCG receptors, and the cAMP and testosterone response to this hormone, without affecting Leydig cell proliferation (478–480). A potent TGFβ inhibition of both growth and steroidogenesis was also observed in a rat Leydig cell testicular tumor line (376). Morera *et al.* (474) reported that TGFβ exerts a biphasic effect on hCG-stimulated testosterone production: stimulatory at low concentrations and inhibitory at high concentrations. TGFβ1 at a concentration of 1 ng/ml was found to enhance the hCG-stimulated testosterone formation in primary cultures of purified immature porcine Leydig cells, and this effect was additive with EGF, as a result of a complex interaction occurring at the levels of cholesterol substrate availability in the mitochondria and of 3β-HSD/isomerase activity (481).

TGFβ has been found to influence peritubular cell function and migration, increasing the production of extracellular matrix and promoting colony formation (468). TGFβ elicits an increased contractility and shape changes of purified pubertal rat PMC embedded in collagen (482), and a synergistic effect of TGFβ and platelet-derived growth factor (PDGF) on collagen gel contraction by PMC has been demonstrated (483). TGFβ1 stimulates lactate production and glucose uptake in Sertoli cells isolated from immature porcine testes (475), but has no effect on transferrin production by differentiated Sertoli cells (468). TGFβ1 inhibits the TGFα-induced PMC DNA synthesis at each stage of pubertal development but neither stimulates nor inhibits Sertoli cell DNA synthesis or growth (470) and enhances the expression of a 50 kDa protein in human spermatozoa (484).

A role for TGFβ in testicular immunosuppression has been suggested on the basis of three observations: antibodies neutralizing TGFβ reverse the suppression of rat peripheral blood lymphocyte proliferation induced by rat abdominal testis extract, recombinant TGFβ1 dose-dependently inhibits testicular IL1-like factor-induced proliferation of murine thymocytes, and extracts of seminiferous tubules contain a TGFβ-like growth inhibitor of a mink lung epithelial cell line (485).

Despite the above data, preliminary results obtained with the generation of mice homozygotes for the disrupted TGFβ1 allele showed no gross developmental abnormalities until about 20 days after birth when the animals succumbed to a multifocal inflammatory disease (486, 487). The only reported testicular abnormality in these animals was a mild to moderate inflammation of the serosa of the testis; however, since all the animals die before puberty, the potential effect of the gene knockout on adult testicular function is unknown. Nevertheless, it has been later reported that one homozygous mutant male survived to reproductive age and was able to impregnate two superovulated females (488). The pregnancy progressed to term, but the outcome of the pregnancy, in terms of number and viability of offspring, was uncertain because the offspring were cannibalized.

A transgenic mouse model, in which mature TGFβ1 is overexpressed in the liver and causes hepatic fibrosis, has been generated (489). The transgenic line expressing the highest level of the transgene also had high (>10-fold over control) plasma levels of TGF-β1. Several extrahepatic lesions developed in these animals, including glomerulonephritis and renal failure, arteritis, and myocarditis, as well as atrophic changes in the pancreas and testis. The atrophic changes in the testis consisted of thickened tubular basement membranes, small and more widely spaced seminiferous tubules, and more prominent Leydig cells. This thickening of the basal membranes is consistent with the reported *in vitro* stimulatory effect exerted by TGFβ on the extracellular matrix production by PMC (468).

TGFβ3 null mutant mice have also been generated by gene targeting (490). Within 20 h of birth, homozygous TGF-β3 −/− mice die with unique and consistent phenotypic features including delayed pulmonary development and defective palatogenesis.

In conclusion, despite the established *in vitro* suppression on Leydig cell differentiated functions, the stimulatory effects on PMC, and the developmentally regulated expression of TGFβs and TGF receptors within the testis, the potential local role of TGFβ *in vivo* is unknown. The reported normal fertility in one homozygous mutant TGFβ1-deficient mouse seems to suggest that TGFβ1 action is not an absolute requirement for testis development and function. However, breeding studies involving intercrosses of heterozygous mice carrying one disrupted TGFβ1 allele have shown a reduction in both heterozygous and homozygous mutant offspring compared with the wild type (488). The decrease in the number of homozygous mutants is consistent with the role exerted by TGFβ1 in embryological development and implantation. Nevertheless, the reduced number of the heterozygotes also seen has raised the possibility that TGFβ1 may be important in haploid germ cell function. It could be that in the absence of a functional TGFβ1 gene, germ cell function or survival is impaired leading to a reduction in the frequency of conceptions involving the mutant germ cells.

C. TGFα/EGF

EGF and TGFα together with amphiregulin belong to the EGF family of growth factors. A variety of basement membrane and extracellular matrix proteins, as well as plasminogen activator and mammalian clotting factors, also contain EGF-like domains. EGF is a 53-aa polypeptide first isolated from the submandibular gland of the male rat; TGFα contains 50 aa and has approximately 40% sequence homology to EGF. Both EGF and TGFα prohormone molecules have hydrophobic transmembrane domains near the COOH termini, and either prohormone can be produced as an integral membrane protein. The mature EGF is a proteolytic processed polypeptide of the EGF precursor that exhibits eight EGF-like repeats in addition to the mature EGF (491). EGF and TGFα appear to be the major EGF family peptides involved in mammalian development, and both have been shown to bind to a single EGF receptor (EGFR). EGFR has intrinsic tyrosine kinase activity (492, 493).

1. Expression, localization, and production. Several lines of evidence indicate that TGFα and EGF are produced within the testis. Peritubular cells and Sertoli cells isolated from 20-day-old rats contain a mRNA species that hybridizes in a Northern blot analysis with a human TGFα cDNA probe, and an immunoblot with a TGFα antiserum confirmed the production of TGFα by both Sertoli cells and peritubular cells (494). The analysis of the developmental expression of TGFα in whole testis and isolated cell types showed that the TGFα gene was predominant early in testis development and decreased during puberty and that TGFα mRNA content was greatest in prepubertal peritubular cells but remained relatively constant in Sertoli cells, with a slight decline at the later pubertal stages (495).

The testicular immunohistochemical localization of TGFα at various ages has also been described (496). In neonatal rats intense staining was seen in Leydig cells. In the 21-day-old rat, TGFα was visualized exclusively in most but not all the Leydig cells, as well as in interstitial cell cultures, but not in Sertoli cells, germ cells, or in Sertoli cell cultures. In contrast to the observations in tissue sections, PMC, which contam-

inated the Sertoli cell preparations, showed intense staining for TGFα. In adult testis, all the Leydig cells were positive and no staining was found in the seminiferous tubules. After EDS treatment, no significant staining for TGFα could be detected in the interstitium, confirming the Leydig cell origin of the immunoreactivity.

Immunoreactive EGF has been also observed in mouse and human testis (497, 498). In mouse, mature EGF-positive immunostaining was localized in Sertoli cells, pachytene spermatocytes, and round spermatids, while EGF precursor immunostaining was limited to pachytene spermatocytes and round spermatids (499). In vitamin A-synchronized rat testis, testicular EGF levels significantly higher at stages IX-XIV of the cycle of the seminiferous epithelium have been demonstrated (408). In contrast, Northern blot analysis did not detect EGF expression in isolated rat peritubular, Sertoli, and germ cells (494).

2. Receptors. The presence of EGFR in testicular cells of several species has been demonstrated by binding, Northern blot, and immunocytochemistry. Specific binding sites have been shown in a clonal strain of Leydig tumor cells (500), in enriched interstitial cell preparations from adult rats (501), in immature porcine Leydig cells (502), as well as in murine Leydig cells (503). Specific EGF binding was also observed in isolated interstitial cell preparations from both intact and Leydig cell-depleted adult rat testis, suggesting that EGF binds to Leydig cells and to an interstitial cell fraction, possibly the precursor of the mature Leydig cell (504). Immunocytochemical data established that EGFR is present in Sertoli cells of adult and immature rat testes (505). In immature rats, the receptor was also found in presumed peritubular cells and in the cell surface of Sertoli cells where it exhibited autophosphorylation upon stimulation with EGF (494, 506). The EGFR gene expression during the rat testis pubertal development showed that EGFR mRNA levels were higher in the early pubertal stages, with a predominant localization in peritubular cells and a low level of expression in Sertoli cells, pachytene spermatocytes, and round spermatids (495). Scatchard analysis confirmed the presence of high-affinity receptors on peritubular cells; however, no functional receptors were detected on Sertoli cells from any stage of development examined (495). In adult mice, Western blotting of testis membrane fractions revealed a specific band corresponding to EGFR (506).

In adult nonhuman primates, a positive immunostaining for EGFR was shown in Leydig cells, Sertoli cells, and PMC, and immunoblotting of testicular membrane preparations revealed a specific band corresponding to EGFR (505, 507). Moreover, changes in the concentration of EGFR mRNA in the testes of monkeys approaching puberty have been reported (508). In humans, specific EGF binding activity was detected in testicular membrane preparations (498). Subsequent studies have confirmed the presence of EGFR in a particulate fraction of human testicular tissue (509). Cross-linking experiments revealed major binding species in a region that is thought to represent a proteolysed form of the receptor, and immunohistochemical localization of EGFRs demonstrated their presence in the interstitial tissue (509). EGFR has been observed in PMC and Sertoli cells of adult human testis by means of immunofluorescence (510).

3. Local functions. TGFα and EGF have been shown to influence Leydig cells, Sertoli cells, and PMC functions. The effects vary depending on time of exposure and animal species. In the MA-10 tumor cell line, EGF induces a substantial decline in the number of LH receptors with a corresponding reduced ability of hCG to stimulate steroidogenesis (500, 511). Similarly, EGF inhibits the gonadotropin stimulation of testosterone production by primary cultures of adult rat Leydig cells (512). EGF capability to decrease the hCG-stimulated testosterone production can also be exerted via inhibition of 17α-hydroxylase and 17,20-lyase activities (501).

Contrasting results have been obtained by other authors. Verhoeven and Cailleau (513) reported a stimulatory effect of EGF on basal and LH-stimulated androgen production, while an inhibitory effect was only observed in cells that, after a prolonged culture in the absence of LH, were acutely challenged with LH and EGF (513).

In porcine immature Leydig cells, EGF enhances the gonadotropin action on testosterone formation through an increase in the availability of cholesterol substrate and in the activity of 3β-HSD (504). Acute treatment of pig Leydig cells with EGF stimulated testosterone secretion (2-fold) without affecting cAMP production; long-term treatment produced a dose-dependent decrease of hCG receptor number but negligible effects on hCG-induced testosterone production (514). Similar acute EGF effects have been observed with a clonal strain of cultured murine Leydig tumor cells (515). In humans, EGF can stimulate steroidogenesis of isolated Leydig cells, without further enhancing the steroidogenesis induced by maximal concentrations of hCG (450). TGFα interacts synergistically with LH to promote DNA synthesis of immature rat Leydig cells in culture (441) and stimulates peritubular cell proliferation, migration, and colony formation (494) but has no effect on Sertoli cell growth and transferrin production (495). Very recently, it was reported that TGFα increases the mRNA levels and the secretion of plasminogen activator inhibitor I in peritubular cells from 20-day-old rat testes (516). These data suggest that TGFα may be involved in the control of net protease activity of the seminiferous tubule influencing the restructuring events occurring at specific stages of spermatogenesis and the extracellular matrix turnover.

Intraperitoneal injection of EGF stimulates DNA synthesis in adult mice testes, the maximal stimulation occurring 8 h after injection (517).

The addition of EGF to the culture medium of seminiferous tubule segments from adult rats (518) and rat primary Sertoli cell-enriched cultures (320) results in the stimulation of INH production.

Moreover, nanomolar concentrations of EGF induce a 2-fold increase of lactate production and inhibit by more than 50% FSH-stimulated estradiol synthesis of prepubertal rat Sertoli cells (519). It has been reported that EGF contains three sequence homologies with human FSH-β, the hormone-specific subunit of FSH, and this homology seems to be of some significance since EGF can inhibit the *in vitro* binding of [^{125}I]human FSH to calf testis membranes (520).

Prepubertal rat Sertoli cells respond to EGF with a small but significant rise in the secretion of both transferrin and ABP (521–523) whereas cells from older animals are less responsive (524, 525). EGF stimulates DNA synthesis and proliferation of immature pig Sertoli cells (439) and, alone or in combination with PDGF and TGFβ, stimulates ornithine decarboxylase (ODC) activity in Sertoli cell-spermatogenic cell cocultures and cultured peritubular cells (526). Accordingly, TGFα or EGF single subcutaneous injections to 8-day-old mice result in a 22-fold increase of testicular ODC activity (527). The treatment of porcine cultured Sertoli cells with EGF increases lactate production, glucose transport, and lactate dehydrogenase activity (528). EGF inhibits the FSH-stimulated germ cell differentiation of adult mice testicular fragments by blocking the proliferation of type A spermatogonia (529).

The original *in vivo* observation by Tsutsumi *et al.* (530) suggesting that submandibular gland EGF may control testicular function in an endocrine fashion gave rise to an array of studies regarding the possible role of circulating and intratesticular EGF in testicular physiology (530). Tsutsumi *et al.* reported that removal of the salivary glands of the mouse results in a decrease of circulating EGF to an undetectable level with a concomitant impairment of spermatogenesis, not accompanied by a modification of the circulating levels of testosterone or FSH. These alterations in spermatogenesis can be reversed by the administration of EGF. In line with this evidence, it has been reported that streptozotocin-induced diabetes in mice results in a decrease in EGF content in the submandibular glands, reduction in plasma EGF levels, and oligozoospermia, which can all be normalized by EGF administration (531). More recently, it has been shown that sialoadenectomy of sexually mature male mice results in a decline in sperm count, motility, and fertility that can be reversed by EGF administration (532). However, the submandibular gland and EGF involvement in the control of spermatogenesis has been questioned. Tokida *et al.* (533) reported that sialoadenectomy did not decrease plasma EGF or cause infertility in male mice, and Russell *et al.* (534) suggested that although sialoadenectomy decreased certain functional testicular parameters, the effects were negligible.

Mice homozygous for either disrupted TGFα or EGFR gene have been generated (535–540). The mice defective for the TGFα gene show dramatic derangement of hair follicles and curly whiskers but are healthy and fertile (535). The recruitment of other factors might account for the absence of predicted pathology in TGFα $-/-$ mice. In the case of TGFα, there is a distinct possibility of functional substitution, since TGFα is known to compete with EGF binding to EGFR, and therefore EGF (or a member of the EGF family) might be recruited to compensate for the TGFα deficiency. EGFR $-/-$ mice show periimplantation, midgestational, and postnatal mortality dependent on genetic background (537–539). The homozygous mutants that lived up to 3 weeks showed abnormalities in skin, kidney, brain, liver, and gastrointestinal tract. Organ systems with no gross histological differences between homozygous and wild type littermates included the pancreas, heart, skeletal muscle, skeleton, teeth, ovary at postnatal day 12, and testis at postnatal day 8 (538).

On the other hand, mice transgenic for TGFα under the control of the MT-1 promoter (495, 541, 542) overexpress the gene in several reproductive organs, including the seminal vesicles and the testis. The transgenic mice have no abnormal testicular morphology or alterations of the spermatogenesis. These observations suggest that TGFα overexpression may not perturb adult and prepubertal testis function. On the contrary, whether or not the absence of EGFR may impair testicular development or function is still unclear. Although the above results demonstrate that the testis is a site of EGF and TGFα production and that testicular germ cells and somatic cells are targets for EGF/TGFα, their potential roles in the testis is uncertain because conclusive evidence *in vivo* is lacking.

D. Fibroblast growth factor (FGF)

FGF belongs to a family of at least seven polypeptides that include acidic FGF (aFGF or FGF-1), basic FGF (bFGF or FGF-2), int-2 (FGF-3), kaposi sarcoma FGF (K-FGF or FGF-4), FGF-5, FGF-6, and keratinocyte growth factor (KGF or FGF-7) (543). FGFs are widely distributed in many tissues and exhibit a variety of actions, including stimulation of cell division and differentiation of cells derived from embryonic mesoderm and neuroectoderm, neuronal growth, and angiogenesis (544, 545).

The FGF receptors are single-chain transmembrane glycosylated proteins characterized as a subgroup of the tyrosine kinase superfamily with two or three extracellular immunoglobulin-like domains. Four distinct FGF receptor genes have been cloned. They are classified into FGFR-1/flg, FGFR-2/bek, FGFR-3, and FGFR-4 (546–548). The FGF receptor genes have a distinct pattern of expression during early embryonic development and during organogenesis.

The most widely studied FGFs are aFGF and bFGF, which exhibit 55% sequence homology (543, 545). Since both aFGF and bFGF lack a signal sequence to facilitate their secretion, they are not secreted in a soluble form, but in association with some compounds of the extracellular matrix from which they are released to exert their paracrine/autocrine role. Interestingly, FGF, apart from the high-affinity signaling receptors, also binds with lower affinity to cell surface proteoglycans that cannot transmit signals alone, but somehow modulate the ability of the growth factor or the signaling receptor to generate a biological response (549). Furthermore, it has been suggested that bFGF can act also in an intracrine fashion (550).

1. Expression, localization, and production. bFGF gene expression and protein production have been studied during rat prenatal and postnatal development. The immunohistochemical distribution of bFGF in the 18-day-old rat fetus revealed that the sex cords are completely negative but are underlined by a strongly positive basement membrane (551). The fibroblast-like cells of the interstitium show moderate immunoreactivity whereas the basement membrane and the peritubular cells are more strongly stained and in the Leydig cells the staining is both intracellular and cell surface associated (551). In whole testis, bFGF gene expression is predominant early in prepubertal testicular development and decreases with sexual maturity (552). Both freshly isolated

peritubular cells and Sertoli cells express bFGF mRNA and protein at relatively constant levels during pubertal development (552, 553), with a slight suppression at the late pubertal stages (552). Freshly isolated mature Leydig cells also express low levels of bFGF, and FSH increases the Sertoli cell production of bFGF-like proteins (552). Western blots of rat and mice testicular homogenates identified a 30-kDa bFGF-like protein (554). Immunohistochemical staining at various postnatal ages was also studied. In 5-day-old rat testis, prespermatogonia were immunoreactive (554). In the adult rat testis, the cytoplasm of pachytene spermatocytes and Leydig cells was positive while Sertoli cells were negative (554, 555). In mice a similar distribution of bFGF mRNA and protein is observed (555, 556). bFGF has also been isolated from bovine and human testis (557, 558).

FGF-6 mRNA can be detected in the adult but not in the pubertal mice testes (559).

2. Receptors. The presence of high-affinity, low-capacity receptors for FGF has been demonstrated in cultured testicular cells from neonatal rats (560) and in immature rat (561) and porcine (562) Leydig cells. The bFGF receptor was localized by immunohistochemistry in Leydig cells, round and elongated spermatids, and Sertoli cells. The presence of the receptors appeared more pronounced in stages I-VIII of the seminiferous epithelium (555).

3. Local functions. FGF inhibits LH-stimulated testosterone production by cultured neonatal rat testicular cells and cultured immature rat Leydig cells through a reduction of 17α-hydroxylase and Δ5–3β-HSD activity (560, 563) but enhances hCG-stimulated testosterone formation (481), and aromatase activity (564) in cultured immature porcine Leydig cells. Other studies have shown that bFGF inhibits hCG-stimulated 5α-reductase activity in cultured immature rat Leydig cells (565). Subsequently, it has been shown that treatment of immature rat Leydig cells with increasing bFGF concentrations had a biphasic effect on 3β-HSD activity and LH/hCG receptor number, with lower concentrations being progressively inhibitory and higher concentrations progressively stimulatory (566, 567). Interestingly, it has been demonstrated that the secondary increase in 3β-HSD activity after addition of higher bFGF concentrations can be blocked by heparin, while the inhibition of 3β-HSD by low bFGF concentration can be partially reversed by either insulin or IGF-I (566). The treatment with FGF alone or together with hCG of mixed testicular cells from adult rat did not affect testosterone production (501).

bFGF stimulates the secretion of IGF-I by pig Leydig cells, Sertoli cells, and Leydig cell-Sertoli cell coculture (514).

Unlike bFGF, aFGF inhibits 3β-HSD, hCG-stimulated 5α-reductase activity, and hCG binding in cultured immature rat Leydig cells both at low and high concentrations (567).

bFGF increases c-*jun* mRNA levels in immature cultured pig Leydig cells, but its effect on c-*fos* and *jun*-B mRNA is lower than that produced by hCG; furthermore, bFGF potentiates the stimulatory action of hCG on c-*fos* and *jun*-B, whereas, hCG potentiates the effect of bFGF on c-*jun* (568). bFGF has been shown to influence Sertoli cells because it stimulates the proliferation of immature pig Sertoli cells (410), increases c-*fos* and transferrin mRNAs in cultured rat Sertoli cells (553, 555, 569), and augments the production of extracellular sulfated glycoprotein-1 by a Sertoli cell line (TM4) (570). Finally, it has been reported that bFGF treatment of rat PMC induces a dose-dependent increase in the production of plasminogen activator inhibitor I, suggesting an involvement of this growth factor in the regulation of protease activity of the seminiferous tubule (516).

Although the above data demonstrate that testicular bFGF expression is developmentally regulated, and that bFGF can modulate Leydig cell, Sertoli cell, and PMC functions *in vitro*, the physiological role of this peptide remains unknown.

E. Platelet-derived growth factor (PDGF)

PDGF, a major mitogen for cells of mesenchymal origin, is widely expressed in normal and transformed cells (571–573). PDGF is composed of two polypeptide chains, named A chain and B chain, respectively, which can combine in three disulfide-linked dimers, AA, AB, and BB, with a mol wt of approximately 30,000 (574). The PDGF isoforms exert their biological actions via binding to cell surface receptors that belong to the protein tyrosine kinase family of receptors (575). Two receptor subunits have been identified, the α-subunit, which can bind both the PDGF A chain and PDGF B chain, and the β-subunit, which can bind only the PDGF B chain. These subunits, upon ligand binding, dimerize to form three high-affinity binding sites for the dimeric PDGF ligand: an $\alpha\alpha$-receptor, an $\alpha\beta$-receptor, and a $\beta\beta$-receptor (576). Due to the binding specificities of the α- and β-subunit, PDGF-AA can bind only to $\alpha\alpha$-receptors, PDGF-AB can bind to $\alpha\alpha$- and $\alpha\beta$-receptors, and PDGF-BB can bind to all three (577). Apart from being a mitogen, PDGF is a potent chemoattractant for a number of cell types both *in vitro* and *in vivo* and activates the early transcription of otherwise quiescent genes, several of which encode potent cytokines and others of which are protooncogenes (578). The widespread expression of the PDGF system in the developing embryo has been proposed to indicate multiple functions for PDGF in the prenatal development of mesenchymal structures (579–587). Mice deficient for PDGF-B (588), PDGFR-β (589), and PDGF-A (590) develop cardiovascular, hematological, renal, and pulmonary abnormalities in late gestation.

1. Expression, localization, and production. The expression of PDGF A chain and B chain genes was observed in rat testicular RNA 2 days before birth; it increased through postnatal day 5 and fell to low levels in adults (591). The predominant cell population expressing transcripts of the PDGF genes during prenatal and early postnatal periods were Sertoli cells, while in adult animals PDGFs were confined to Leydig cells. Accordingly, early postnatal Sertoli cells, but not cells from older animals, produced PDGF-like substances, and this production was inhibited dose-dependently by FSH, whereas cultured adult Leydig cells produced PDGF-like molecules under positive hCG control (591). This kind of developmental expression for mRNAs and proteins of the two PDGF subunits was also confirmed by immunohistochemistry and immunocytochemistry (591). Loveland *et al.* (592) examined the expression of mRNAs encoding the

PDGF subunits in Leydig cells, primary spermatocytes, round spermatids isolated from adult animals, and enriched preparations of Sertoli cells obtained from 20-day-old animals. Although Leydig cells and Sertoli cells contained mRNAs encoding both PDGF subunits, neither PDGF subunit mRNA was detected in the germ cells (592).

2. Receptors. *In situ* hybridization to localize the expression of the PDGFR β-subunit mRNA during organogenesis in the mouse embryo revealed the expression of the receptor throughout the mesenchyme of the testis starting from 12.5 day pc (593). Immunohistochemical studies conducted in the rat fetus have shown that little or no PDGFR-β immunoreactivity could be detected in day 13 rat fetus; by day 16, a strong immunoreactivity was seen among other tissues in the gonads, and the intensity of staining peaked at day 18 and decreased by day 21 (594). Strong PDGFR-β immunostaining has been reported in the sex cords of the developing gonad in a 12-week-old human fetus (594). Purified adult rat Leydig cells and PMC from neonatal rats possess specific high-affinity, low- capacity receptors for PDGF as revealed by ligand binding, immunohistochemistry, and Northern analysis (591, 595). Immunohistochemical analysis in the rat showed PDGFR-expressing cells scattered in the intertubular compartment in the fetal testis that become organized in a cellular layer corresponding to PMC during postnatal development (591). Purified PMC from prepubertal rats show PDGF receptors after 24 h in culture, and PDGF treatment increases the cytosolic Ca^{++} concentration in these cells (596).

3. Local functions. The combination of PDGF and EGF induces a significant activation of ODC in rat Sertoli cell-spermatogenic cell cocultures after 6 h of incubation (526). After 24 h of incubation, PDGF does not appear to affect activity if either EGF or TGF-β is present, while PDGF alone inhibits ODC activity in Sertoli cell-spermatogenic cell cocultures. Moreover, PDGF significantly stimulates ODC activity of PMC after 6 h of incubation both alone or in combination with EGF and TGF-β, while after exposure for 24 h no effect can be observed. These findings raise the possibility that PDGF has the ability to down-regulate ODC activity in Sertoli cell-spermatogenic cell cocultures but not in nonepithelial peritubular cells. This contrasting effect led to the suggestion that PDGF might play an important role in spermatogenic cell differentiation and a more mitogenic function in the peritubular cell population (526). The predicted activity on PMC was later confirmed since the PDGF treatment of cultured rat PMC stimulated cellular proliferation and increased the release of type IV collagen, fibronectin, type V collagen, and laminin (596) and synergistically with TGFβ stimulated the contraction of PMC in culture (483). In contrast, PDGF did not show any stimulation of spermatogonial differentiation of type A spermatogonia in organ culture of adult mouse cryptorchid testis (443). Moreover, PDGF shows a strong chemotactic activity for purified immature rat PMC (591). In cultured immature Leydig cells, PDGF inhibits hCG-stimulated 5α-reductase activity, basal Δ^5-3β-HSD activities, and hCG-stimulated testosterone formation (597). Also in cultured purified adult rat Leydig cells, exposed to a minimally stimulating dose and maximally stimulating dose of LH, PDGF-BB stimulates testosterone production in a dose-dependent manner (598, 599). PDGF markedly inhibits aromatase activity stimulated by cAMP of human adipose stromal cells (600).

Based on the above data, a tentative model of the developmental regulation that could be exerted by the PDGF system in the rat testis has been presented (591). In the prenatal and early postnatal period the PDGF molecules produced by the Sertoli cells could chemotactically attract the intertubular precursors of the PMC to the peritubulum and induce them to divide. In adults, PDGF could modulate Leydig cell production of testosterone in an autocrine way. However, *in vivo* evidence is needed to verify this assumption. The generation of PDGF-B-, PDGF-A-, and PDGF-β-receptor-deficient mice has not helped to address this question for the following reasons. Both PDGF-B and PDGF β-receptor knock-out mice die perinatally and show kidney glomerular defects due to the absence of mesangial cells, hemorrhages, anemia, and thrombocytopenia (588, 589). PDGF-A null allele is also lethal with two restriction points, one prenatally before embryonic day 10 (50% loss of embryos) and one postnatally (30% perinatal death, 20% postnatal day 20 death) (590). Postnatally surviving PDGF-A-deficient mice develop lung emphysema secondary to the failure of alveolar septation. This is apparently caused by the loss of alveolar myofibroblasts. The fact that these animals do not survive to reproductive age and the absence of description of the testis phenotype do not provide information on the developmental and regulatory effects of the PDGF system in the testis. However, it is interesting to note that in all cases of PDGF and PDGF receptor gene disruption, a myofibroblast-type cell is affected (mesangial cells and alveolar myofibroblasts), and during testicular development the PMC, which are also a myofibroblast-type cell, are the principal target of the PDGF system action.

In conclusion, although circumstantial evidence indicates an involvement of PDGF in the control of testicular development, further studies are needed to confirm this hypothesis.

F. Nerve growth factor (NGF)

NGF is a protein essential for the development and maintenance of sensory and sympathetic neurons of the peripheral nervous system (601). It interacts with two distinct receptor entities that are distributed among both responsive and nonresponsive cells. One, which has been identified as the protooncogene product of *trk* (generally designated TrkA), is a prototypical growth factor receptor containing a tyrosine kinase in its internal domain. The second receptor, called LNGFR (identifying it as the low-affinity NGF receptor), lacks this type of entity. It does possess a G protein consensus interaction site at the C terminus, but an activity involving such a molecule linked to this receptor has yet to be demonstrated (602).

1. Expression, localization, and production. A positive immunoreactivity for NGF has been reported in the adult mouse testis (603). This immunoreactivity appeared to be localized to the cells of the germ line. Most stages, from primary spermatocytes to mature sperm, were positive. The presence

of NGF mRNA and protein in adult rodent testis has also been demonstrated by *in situ* hybridization, Northern analysis, immunohistochemistry, enzyme-linked immunoassay, and a test of biological activity (604). NGF mRNA and NGF-like immunoreactivity was detected in spermatocytes and early spermatids of adult mouse and rat testis. The presence of NGF-like substance in a testicular extract was also confirmed by a bioassay. An analysis of the stage-specific expression of NGF during the cycle of the seminiferous epithelium in the rat revealed NGF mRNA and protein at all stages of the cycle (605).

A quantitative determination by immunoassay of NGF in human testis revealed the presence of 5.44 ng of β-NGF per g wet weight (606).

2. Receptors. A high level of LNGF receptor mRNA was detected in the mouse testis (604). Subsequently, the LNGFR mRNA was found in Sertoli cells of adult rat testis in tubules at stages VI to VIII of the seminiferous epithelial cycle, and its expression was down-regulated by testosterone (607). This characteristic distribution of NGF and NGF receptor has been confirmed in adult human testis (608). Further studies conducted in human testicular tissue have shown that NGF receptors are localized on cells of the lamina propria (606).

TrKA mRNA, encoding an essential component of the high-affinity NGF receptor, is present at all stages of the seminiferous epithelium (605, 609); in contrast, the expression of LNGFR mRNA was found only in stages VII and VIII of the seminiferous cycle (605). The LNGFR protein was present in the plasma membrane of stages VII to XI on the Sertoli cells apical part facing the lumen of the tubules and in the basal compartment (605). Further studies on LNGFR mRNA expression during testicular development have shown that LNGFR is expressed in the peritubular cells of the embryonic mouse testis (610). Immunohistochemical analysis in the rat showed LNGFR-expressing cells scattered in the intertubular compartment in the embryonic testis that become organized in a cellular layer that surrounds PMC during postnatal development (610). Furthermore, in peripubertal and adult mouse and rat testis, the expression of a shorter mRNA of 3.2 kb that cross-hybridizes to the LNGFR transcript (3.7 kb) has been identified. This shorter mRNA has been localized by *in situ* hybridization particularly in spermatids at stages VII-IX of the mouse seminiferous epithelium cycle (610).

3. Local functions. Addition of NGF to the culture medium of mechanically isolated seminiferous tubules from human testes stabilizes specific functions of the seminiferous tubules such as the maintenance of the myoid phenotype of the lamina propria, the prevention of thickening of the tubular wall, and the stabilization of Sertoli cell morphology and function (611). In the adult rat, NGF dose-dependently increases DNA synthesis of seminiferous tubule segments with preleptotene spermatocytes at the onset of meiosis while other segments remain nonresponsive (605). The infusion of NGF into adult rat testis *in vivo* causes an increase in the level of testicular ABP mRNA probably caused by prolongation of stages VII-VIII of the seminiferous epithelium that display the maximal ABP mRNA expression (612).

NGF- and TrkA-targeted mice have been generated (613, 614). Both mutants show reduced growth and survival rate and suffer complete loss of sympathetic neurons and sensory neurons responsive to temperature and pain. NGF (−/−) mice have a maximal life span of 4 weeks, which is somewhat shorter than the life span observed for TrkA (−/−) mice (8 weeks). The fertility potential of NGF mutant could not be analyzed, and a detailed histological analysis of nonneuronal tissue has not been conducted. Mice that are heterozygotes for NGF gene disruption display a mild phenotype but grow and breed normally. As reported above, the majority of TrkA (−/−) mice die after weaning stage; however, it has been reported that those that reach adulthood remain infertile (615). Also in this case the lack of histological analysis and the profound systemic effect of knocking out do not allow conclusions on the suggested role of NGF in the male reproductive system.

Mice carrying a mutation of the gene encoding the LNGFR have also been produced (616). The homozygous animals displayed a complex phenotype with deficit in cutaneous innervation and heat sensitivity but were viable and fertile. The observed phenotype is much less severe than might have been expected considering the expression patterns of the LNGFR gene. This indicates either that LNGFR has no essential role in the development of the many tissues that express it or that its function is redundant and its absence can be compensated for by another protein.

In conclusion, the data reported above suggest that NGF may be involved in the regulation of testicular morphogenesis and function. However, definitive *in vivo* experimental proofs are lacking.

G. Steel factor (SLF)

SLF, also called mast cell growth factor, stem-cell factor, or hematopoietic cell growth factor, is a protein whose gene maps to chromosome 10 in the vicinity of the *steel* (Sl) locus. The SLF protein can be expressed in a biologically active form as either a membrane-bound protein or as a soluble factor. SLF is the ligand for the c-*kit* protooncogene receptor encoded by the murine dominant *white spotting* (W) locus. The product of the c-*kit* protooncogene is a member of the PDGF family of tyrosine kinase receptors. The SLF/c-*kit* system is essential in melanogenesis, gametogenesis, and hematopoiesis during embryonic development and postnatal life (617, 618).

1. Expression, localization, and production. SLF gene transcripts have been detected in the mouse embryo along the migratory pathways of the PGC and in the gonads (619), and its postnatal testicular expression is relatively high at all ages (620). The forms of SLF mRNA present in the testis suggest that from postnatal day 5 onward the membrane-bound form of SLF predominates (620). As observed by *in situ* hybridization and immunohistochemistry, the SLF expression is distinct in Sertoli cells but not in germ cells from postnatal day 1 to postnatal day 9; thereafter the intensity of expression declines. SLF in Sertoli cells appears to be concentrated basally at the stage of the cycle of the seminiferous epithelium where it is known to interact with differentiating type A spermato-

gonia (620). Treatment of Sertoli cell cultures with cAMP analogs led to a significant increase in the SLF mRNA levels (621). The production of the membrane-bound form of SLF by mouse Sertoli cells has been demonstrated by means of a bioassay, based on the ability of Sertoli cell cultures from W^v/W^v but not Sl^d/Sl^d mice, to support the growth of cocultured mast cells (622). Further studies on the regulation of SLF production by mouse Sertoli cells have produced contradictory results. Rossi *et al.* (623) reported that FSH and cAMP were able to increase the mRNA levels for both the soluble form and the transmembrane form of SLF in cultured primary mouse Sertoli cells. The inductive effect of cAMP was more pronounced in cultures from 13-day-old animals than in cultures from 18-day-old animals (623). Tajima *et al.* (624) showed that the treatment of mast cell-Sertoli cell coculture with cAMP increased SLF production whereas FSH had no significant effect.

In human testicular tissue, a positive staining for SLF was demonstrated within the Leydig cells and less intensely in the area of Sertoli cells (625).

2. Receptors. *In situ* hybridization studies showed that c-*kit* is expressed during mice embryogenesis in the PGC before and after migration into gonadal ridges (626). The c-*kit* mRNA was found to be expressed at high levels in spermatogonia, and at lower levels in meiotic pachytene spermatocytes purified from 18-day-old mice (627). Rossi *et al.* (628) cloned a spermatid-specific 3.2-kb cDNA in mouse that encodes a truncated c-*kit* protein lacking the extracellular and transmembrane domains and part of the tyrosine kinase domain. *In situ* hybridization revealed c-*kit* transcripts in mouse spermatogonia beginning at 6 days after birth and labeling of Leydig cells at all ages examined (629). The synthesis of the c-*kit* receptor by isolated type A spermatogonia from 9-day-old rat testis was established by Northern blot hybridization, immunocytochemistry, and Western blot analysis and was found to be constitutively autophosphorylated on tyrosine with a significant increase in phosphorylated protein upon stimulation with the *kit* ligand SLF (630).

Normal human testis stained for c-*kit* in Leydig cells, in the cytoplasm of early spermatogenic cells, as well as in the acrosomal granules of the round spermatids and in the acrosome of testicular spermatozoa (625).

3. Local functions. After the demonstration that c-*kit* is allelic with *W* locus in mice and SLF is mapped to *Sl* locus, the relationship between *W* and *Sl* mutations has been clarified (631). The phenotype analysis of *W* or *Sl* mice proved that c-*kit* and its ligand are indispensable for gametogenesis, melanogenesis, and hematopoiesis (632). During gametogenesis, receptor activation by SLF acts primarily as a homing mechanism for migration or as a viability factor critical for clonal survival of PGC. Expression of the mRNA for c-*kit* and SLF at the destination site continues after migration is complete, suggesting that receptor signaling may also have a late role in local proliferation and differentiation. The transmembrane form of SLF supports the survival but not the proliferation of mouse PGC (633, 634). Soluble recombinant SLF stimulates, in a dose-dependent fashion, thymidine incorporation in cultures of isolated germ cell populations enriched in spermatogonia. Autoradiographic analysis shows that SLF selectively stimulates DNA synthesis in type A spermatogonia (623). Despite the evidence for postnatal expression of c-*kit* and SLF, whether c-*kit* and its ligand are required for the maintenance of the germ cells in postnatal life is difficult to address by phenotype analysis because virtually no germ cells are present in the gonads of *W* and *Sl* mice.

In a very interesting paper, Yoshinaga and colleagues reported that intraperitoneal injection of an anti-c-*kit* monoclonal antibody to prepubertal mice completely blocks the mitosis of type A spermatogonia but not the mitosis of gonocytes, spermatogonial cell precursors, or Sertoli cells, and intravenous injection of the same antibody into adult mice causes a depletion in the differentiating type A spermatogonia (635). These data demonstrate that the maintenance and/or mitosis of the differentiating type A spermatogonia requires c-*kit* and its ligand (635). In summary, the c-*kit* receptor system is essential in several stages in gametogenesis. During development it provides a proliferative, migratory and/or cell survival signal for PGC. Postnatally, during spermatogenesis c-*kit*/SLF is thought to facilitate proliferation and/or survival of spermatogonia.

H. Gastrin-releasing peptide (GRP)

GRP is a 27-aa peptide that belongs to the family of the bombesin (BN)-like peptides. The BN-like peptides are a diverse family of peptides originally characterized in frog skin and later found to have a wide distribution and range of actions in mammals including secretion of gastrointestinal peptide hormones, regulation of smooth muscle contraction, modulation of neuronal firing rate, and growth (636). The BN-like peptides have classically been divided into three subfamilies, the BN subfamily, of which GRP was considered the mammalian form; the ranatensin subfamily, of which neuromedin-B was considered the mammalian form; and the phyllolitorin subfamily, which to date has only been characterized in amphibians. After the identification and molecular cloning of a true amphibian gut GRP distinct from amphibian BN (637) and phylogenetic analysis of BN-like peptide prohormone sequences (638), the BN-like peptides were reclassified into GRP subfamily, neuromedin-B subfamily, and skin peptide subfamily.

Three receptors for BN-like peptides have been cloned to date, a GRP-preferring subtype (639), a neuromedin B-preferring subtype (640), and a third subtype, designated BN receptor subtype 3 (BRS-3), with an as yet unknown ligand (641). All the receptors belong to the seven membrane-spanning domain G protein-coupled receptor superfamily. A fourth BN receptor subtype (BB4), with high affinity for the BN-related peptides distinct from GRP, has been cloned very recently from frog brain and suggested to be present also in mammals (642).

1. Expression, localization, and production. Purified adult rat Leydig cells were found to produce immunoreactive GRP by RIA; the GRP production was not stimulated by hCG treatment of the cell cultures (643, 644). HPLC analysis of the Leydig cell-conditioned medium extract revealed the presence of two biologically active C-terminal fragments of the

GRP, GRP 18–27 and GRP 14–27. Immunohistochemical studies in the adult testis showed specific staining for GRP localized in the Leydig cells in both rat and human tissue (643, 644).

2. Receptors. Recently, human genomic and cDNA clones encoding a new BN-like peptide receptor subtype, called BN receptor subtype 3 (BRS-3), have been isolated and characterized (641). Chromosome mapping studies indicate that the BRS-3 gene is located in human chromosome X. Interestingly, mRNA expression in rat tissues is limited to the testis on secondary spermatocytes and has also been found to be widely expressed in a panel of human cell lines from all histological types of lung carcinoma (641). Expression of BRS-3 cDNA in *Xenopus* oocytes encodes a functional receptor that is activated by BN-like peptides (BN, GRP, Phe^8-phyllolitorin, and ranatensin) applied at 10^{-5} M concentration. Whether BRS-3 may be a low-affinity receptor for BN agonists examined thus far or for a high-affinity BN-like ligand yet to be defined is unknown. It is noteworthy that since BRS-3 maps on chromosome X and its mRNA expression is limited to secondary spermatocytes, which are haploid and possess either an X or Y chromosome, only half of these cells could potentially express BRS-3. Moreover, specific high-affinity, low-capacity binding sites have been found on cultured PMC isolated from prepubertal rat testis by using [^{125}I]GRP as ligand (L. Gnessi, unpublished results), and it has been reported that GRP can transmodulate the binding of PDGF to PMC by reducing the number, but not the affinity, of its receptors (645).

3. Local functions. To date no evidence for local testicular functions of GRP have been reported.

Although the above data demonstrate the presence of GRP-like substances and BN receptor subtypes in the testis, the potential physiological significance of testicular GRP remains to be established since *in vitro* and *in vivo* evidence supporting a local action of the peptide is lacking.

V. Immune Derived Cytokines

A. Interleukins (ILs)

The ILs are a family of cytokines produced by activated lymphocytes and macrophages. Here we provide a summary of the general characteristics of the ILs and/or IL receptors so far identified or shown to have an effect in the mammalian testis, IL-1, IL-2, IL-4, and IL-6.

IL-1 is the term for two polypeptides (IL-1α and IL-1β). Although both forms of IL-1 are distinct gene products, they recognize the same cell surface receptors and share the various biological activities. IL-1 is the prototype of the proinflammatory cytokines in that it induces the expression of a variety of genes and synthesis of several proteins that, in turn, induce acute and chronic inflammatory changes. An endogenous antagonist for the IL-1 receptor (IL-1ra) has recently been isolated and cloned and may play a role in modulating the effects of IL-1. IL-1α, IL-1β, and IL-1ra all bind to the two IL-1 receptor subtypes isolated and cloned to date. The IL-1 receptor type I (IL-1RtI) and IL-1RtII are both members of the Ig superfamily of receptors. The major difference between the type I and type II receptor is the truncated cytoplasmatic portion of the type II receptor. No clear picture has emerged from the studies on the signal transduction events after the binding of IL-1 to either one of its cell surface receptors (646).

Il-2 is a glycoprotein with an apparent molecular mass of 15.5 kDa, which plays a pivotal role in T cell activation (647). There are three forms of IL-2 receptors that can be distinguished on the basis of their affinity for the ligand. The receptor is an heterodimeric complex composed of three receptor subunits, IL-2Rα, IL-2Rβ, and IL-2Rγ. Independent IL-2Rα or IL-2Rβ subunits generate low and intermediate affinity receptors, whereas high-affinity receptors are formed when all receptor subunits are noncovalently associated also with IL-2Rγ, which plays a pivotal role in facilitating IL-2 binding by IL-2Rβ and in receptor signaling. The β- and γ-subunits have been shown to bind to cytoplasm protein-tyrosine kinases of the *jak* family (648).

IL-4 is a glycoprotein with an approximate molecular mass of 15–19 kDa. It is made in response to immunological recognition, principally, although not exclusively, by CD4$^+$ T lymphocytes. IL-4 has a wide range of functions on B cells, T cells, macrophages, hematopoietic precursor cells, and stromal cells (649). IL-4 mediates its functions by binding to a high-affinity receptor complex, composed of the IL-4-binding receptor chain and a common γ-chain. The proposed mechanism for signaling through the IL-4 receptor complex involves the activation of protein tyrosine kinases associated with the cytoplasmic domains of the γ-chain and the IL-4R chain (650).

IL-6 is a multifunctional cytokine that acts as a differentiating and proliferative factor on B cells, T cells, hepatocytes, and hematopoietic progenitor cells (651). Binding of IL-6 to the IL-6 receptor results in the homodimerization of a nonligand-binding 130-kDa signal-transducing molecule, gp130, activation of the tyrosine kinase *jak*2, and the consequent initiation of the *Ras*-dependent mitogen-activated protein kinase cascade (651, 652).

1. Expression, localization and production. In 1987 Khan *et al.* (653) reported the isolation of an IL-1-like factor from intact testis of adult rat. IL-1-like activity was found in the extracts and medium conditioned by cultures of normal and cryptorchid seminiferous tubules but not in interstitial cells or in culture media conditioned by 10-day-old rat Sertoli cells. This indicates that the testicular IL-1-like factor was produced by cells in the seminiferous tubules, most probably by Sertoli cells, in adult but not in prepubertal rats (653). The appearance of the IL-1-like factor during postnatal development and its cellular origin have been further investigated by a murine thymocyte proliferation assay (654). Very low IL-1 activity was seen in culture medium conditioned by seminiferous tubules from rats aged 10 or 20 days. From 30 days of age, increasing amounts were detected, with maximum levels being attained in adult animals. No IL-1 activity was found in medium conditioned by peritubular cells. Sertoli cell-enriched seminiferous tubules obtained from experimentally cryptorchid or from prenatally irradiated rats produced much higher levels of IL-1 activity than did those obtained from intact testes, suggesting that the testicular

IL-1-like factor is produced by Sertoli cells and that its appearance during development coincides with the initiation of active spermatogenesis (654). An IL-1-like factor is produced by adult rat seminiferous tubules segments containing all stages of the spermatogenic cycle except for stage VII (655).

Human testicular cytosol preparations exhibited significant IL-1-like activity that could be neutralized by specific IL-1 antibodies in a bioassay (656). An IL-1-like factor has been isolated from adult rat testis interstitial fluid (657). *In vitro* experiments have confirmed that both human and rat seminiferous tubules secrete IL-1, and cultured medium from rat Sertoli cells contains IL-1 bioactivity that could be neutralized by an anti-IL-1α antibody (658). However, further reports revealed that other cell types contribute to the production of IL-1 in the testis. Human recombinant IL-1β and bacterial lipopolysaccharide (LPS) cause a dose-dependent increase in IL-1α mRNA expression in purified mature rat Leydig cells, indicating Leydig cells as a potential source of IL-1 (659). Lin *et al.* (660) have demonstrated that both IL-1α and IL-1β mRNAs are expressed in IL-1β-stimulated adult rat Leydig cells, with IL-1β being the predominant species, and that in Leydig cells IL-1 mRNA can be induced by a single injection of hCG *in vivo.* Interestingly, the phagocytosis of residual bodies or cytoplasts from elongated spermatids, or even the phagocytosis of latex beads, induces the secretion of IL-1α by rat Sertoli cells *in vitro* (661). Freshly isolated macrophages from adult rat testis secrete a lymphocyte-activating activity that is responsive to bacterial LPS stimulation and is blocked by IL-1ra, a naturally occurring antagonist that specifically inhibits both IL-1α and IL-1β (662). However, the levels of IL-1 secreted by the testicular macrophages are considerably reduced compared with a population of resident macrophages from peritoneum cultured under identical conditions.

Sertoli cells prepared from rats of increasing ages were found to secrete IL-6 *in vitro*; LPS, latex beads, residual bodies, and cytoplasts from elongated spermatids markedly stimulated IL-6 secretion at all ages investigated (663). The levels of IL-6 varied throughout different stages of the seminiferous epithelium cycle; the highest levels were observed in stages II-VI and the lowest were seen in stages VII-VIII. Furthermore, FSH differentially stimulated IL-6 secretion during the seminiferous epithelial cycle (663). IL-6 is also secreted from enriched preparations of adult rat Leydig cells, and its release is increased in a dose-dependent manner by hCG and IL-1β (664). Cultured mouse Sertoli cells produce IL-6 and its production is enhanced by IL-1α, IL-1β, TNFα, and LPS and inhibited by interferon-γ (INFγ) (665).

2. Receptors. Specific IL-1 receptors have been identified in crude membrane preparations of mouse testis (666). IL-1α showed significantly higher specific binding than IL-1β. However, binding of IL-1α was species-dependent, with a barely detectable signal in rat and guinea pig testis (667). In autoradiographic studies, IL-1 receptors were heterogeneously distributed, with highest densities present in the interstitial areas of the mouse testis (668). Type 1 IL-1 receptor mRNA, localized in the mouse testis with *in situ* hybridization, confirmed the autoradiographic data with an intense signal observed over the interstitial cells (669). The paucity of IL-1 binding in the rat (667) is somewhat puzzling, since recombinant human IL-1 produces a variety of actions in the rat testis (see below), suggesting the possibility of the presence of a novel IL-1 receptor in the rat that may be important in mediating the effects of IL-1 in this species.

3. Local functions. Verhoeven *et al.* (670) showed that IL-1β alone stimulates steroid production in short-term cultures of enriched rat Leydig cells from 19-day-old animals, whereas LH-induced androgen formation is inhibited under longer incubation conditions (670). IL-1 is a potent inhibitor of adult Leydig cell function in that it diminishes hCG binding and blocks hCG-stimulated testosterone and cAMP formation in primary cultures of mature rat Leydig cells (671–673). The observed inhibitory effects of IL-1 on Leydig cell testosterone production are predominantly due to a block of the expression of P450c17 and P450 scc (674–676). A dose-dependent inhibitory action of IL-1β on hCG-stimulated testosterone production was found in a primary culture of neonatal rat testicular cells (677). In immature porcine Leydig cells, IL-1α and, to a lesser extent, IL-1β are potent inhibitors of hCG-stimulated testosterone production (678).

Only two studies have reported a stimulatory effect or a lack of effect of IL-1 on Leydig cell steroidogenesis. In one, an IL-1β mediated stimulation was observed in both basal and LH-induced testosterone production by adult rat Leydig cells in culture (679), while in another the lack of effect of both IL-1α and IL-1β on LH-stimulated testosterone production was reported (680).

These data suggest that the actions of IL-1 on Leydig cell steroidogenesis *in vitro* may be stimulatory, inhibitory, or ineffective under different culture regimens.

In the tubule, IL-1β inhibits FSH-induced aromatase activity in immature rat Sertoli cells (681) and stimulates Sertoli cell transferrin secretion (682).

IL-2 is also a potent inhibitor of adult rat Leydig cell steroidogenesis in primary culture. IL-2 inhibits hCG-stimulated testosterone and cAMP formation but has no effects on the binding of hCG to Leydig cells. It also blocks cAMP and forskolin-induced testosterone formation (683). Meikle *et al.* (684) reported that although IL-2 inhibits hCG-stimulated testosterone synthesis in isolated murine Leydig cells, it stimulates testosterone production in minced testes, suggesting an IL-2-mediated release of regulatory factors from other cells that are able to overcome the direct inhibitory effect of IL-2. Locally injected IL-1β, but not IL-1α, induces acute inflammatory-like changes in the testicular microcirculation of adult rats (685). IL-1α is able to enter the testis in intact form and to cross the blood-testis barrier, which is consistent with the finding of a direct action of IL-1α on gonadal function (686).

Intraperitoneal injection of the bacterial endotoxin LPS induces a significant increase of IL-1β concentration and a decrease in the density of IL-1 receptors in the testis (687).

IL-1 may act as a growth factor for spermatogonia and thus participate in the regulation of spermatogenesis (655, 688). A similar mitogenic effect on Leydig cells isolated from immature rats has been reported for IL-1β (689), and Lin *et al.* (690) demonstrated that IL-1β can inhibit IGF-I mRNA expression in adult rat Leydig cells, both *in vivo* and *in vitro.* Interest-

ingly, the ontogeny of testicular IL-1 activity during normal sexual maturation and photoperiod-induced regression in the seasonal breeding of bank voles is in accordance with a proposed role for IL-1 as a mitogen for germ cells (691). Testicular IL-1 activity can first be detected at puberty as spermatogenesis is activated and increases until full sexual maturity is reached, whereas it fails to appear when pubertal development and the initiation of spermatogenesis is arrested by light deprivation (691).

IL-2 and IL-6 increase the release of transferrin from rat Sertoli cells obtained by microdissection of IX-XI- and XIII-staged seminiferous tubule segments; moreover, IL-6 increases basal and FSH-induced transferrin secretion (682, 692).

It has recently been demonstrated that IL-4 is a positive regulatory factor for mouse PGC *in vitro* in that the IL-4 protein is present in the vicinity of proliferating PGCs *in vivo*. Both subunits of the high affinity IL-4 receptor are expressed in PGC-containing tissues, suggesting a developmental role for IL-4 as a survival factor for mouse PGCs *in vivo* (693).

Because of their pronounced effects on the immune system and the hematopoietic system, IL- and IL receptor-deficient mice have been generated (see Refs. 694 and 695 for review). The results from these studies indicate that ILs do not play unique roles in the development of the immune system and steady state hematopoiesis. However, impairment or modulation of the immune and/or inflammatory responses has been shown to occur in each of the IL/IL receptor-deficient mice, demonstrating that no IL is dispensable or completely redundant *in vivo*. Regarding the reproductive function, IL-1 (696), IL-2 (697), IL-2 receptor (698, 699), IL-4 (700, 701), and IL-6 (702, 703) deficient mice are healthy and fertile. Thus it appears that in normal conditions the testicular effects of these cytokines are either redundant or probably subtle.

Mice overexpressing IL-2, IL-4, and IL-6 have been generated. Mice with an IL-6 transgene driven by the Ig enhancer (Eμ-IL-6), analyzed at ages varying from 7 to 18 weeks, develop oligoclonal plasmacytoses; there is no information on testicular appearance or fertility in these animals (704).

IL-4 transgenic mice, despite their normal gross appearance at birth, develop severe runting and die within the first 2 weeks of life (705). They show severe hypoplasia of the thymus and marked lymphocyte depletion in the spleen. When transgene expression was attenuated, a marked increase in serum IgE levels and the appearance of an inflammatory ocular lesion resembling the inflammatory reaction observed in human allergic disease was observed. The few attenuated transgenic founders generated were capable of breeding.

Interestingly, male transgenic mice with the murine MT-1 promoter-human IL-2 fusion gene have atrophic testes and motor ataxia due to infiltration of lymphocytes into the cerebellar tissue (706). In these mice, the testes were depleted of spermatogenic cells without any apparent signs of an immunological response. Whether this dramatic effect could be ascribed to the reported potent inhibitory action of IL-2 on testosterone production in Leydig cells (619, 620) is unclear.

In summary, the effects of ILs in the testis are complex. The ILs secreted by various cellular testicular components may be involved in discrete local paracrine/autocrine modulatory actions. Nevertheless, it is important to notice that IL production can be induced within the testis under appropriate conditions, such as after LPS administration (659, 661–663, 665), and this could be a potential mechanism for the reduction of fertility and androgen secretion that accompanies testicular inflammation (707).

B. Tumor necrosis factor-α (TNFα)

TNFα is a potent cytokine, produced mainly by activated macrophages, that has numerous biological functions, including hemorrhagic necrosis of transplanted tumors, cytotoxicity, and an important role in endotoxic shock and in inflammatory, immunoregulatory, proliferative, and antiviral responses (708). Receptors for TNFα are expressed in the majority of mouse and human cell lines. Two distinct TNFα receptor subtypes (type I and type II) have been identified (709).

1. Expression, localization, and production. Recent studies have demonstrated that mouse spermatids secrete TNFα and that both pachytene spermatocytes and round spermatids contain mRNA for TNFα as detected by both Northern blot analysis and *in situ* hybridization (710). There was much less overall hybridization with TNFα antisense probes on sections of testis from a 16-day-old mouse compared with that in adult testis (710). Presumptive interstitial macrophages were also labeled (710). Testicular macrophages from adult rats release TNFα *in vitro*, while medium from cultured Sertoli cells, Leydig cells, and peritubular cells did not contain TNFα activity (711). Further studies revealed that testicular macrophages do not constitutively release TNFα both *in vivo* and *in vitro* and that the reported release of TNFα *in vitro* was due to the activating effect on macrophages of the collagenase used to isolate the cells (712). However, testicular macrophages release TNFα when exposed to bacterial endotoxins (712).

2. Receptors. Purified cultured porcine Leydig cells from immature animals appear to posses specific TNF receptors (713), and adult mice Sertoli and Leydig cells contain mRNA for the TNFα receptor (710).

3. Local functions. TNFα augments testosterone secretion in both whole testis cell cultures, as well as purified Leydig cells from adult rat, and produces maximal increases of hCG-induced testosterone secretion (679). The effect of TNF administered by continuous intravenous infusion on testicular function in rats has been also studied (714). Testicular weight decreases within 24 h, and germ cells damage is observed together with a fall in plasma testosterone and an increase in LH and FSH levels. The decrease in testosterone concentration and increase in gonadotropin levels suggest that TNF interferes with Leydig cell function; germ cell damage may be either direct or secondary through Leydig and/or Sertoli cell dysfunction. Moreover, TNFα enhances the inhibitory effect of IL-1β on Leydig cell steroidogenesis (715).

TNFα reduces hCG-stimulated, but not basal, testosterone secretion by cultured purified Leydig cells isolated from immature porcine testes in a dose- and time-dependent manner, antagonizing the LH/hCG action on testosterone for-

mation predominantly through a decrease in the availability of cholesterol substrate in the mitochondria (713). Subsequently, Xiong and Hales (716) reported that TNFα inhibits both basal and cAMP-stimulated testosterone secretion from isolated adult mouse Leydig cells, and this inhibition was parallel to the TNFα-mediated repression of the cholesterol side-chain cleavage enzyme P450scc and P450c17 mRNA and protein levels.

Interestingly, the experimental autoimmune orchitis induced in adult male mice can be attenuated when the affected animals are injected with neutralizing antibody to TNFα, but not neutralizing antibody to INF-γ (717).

A recent study has shown that TNFα stimulates proliferation of PGCs in culture, suggesting possible involvement of TNFα in the proliferative regulation of the PGCs during embryonic development (718).

However, homozygous TNF receptor-1 (719, 720) and TNF receptor-2 (721) gene-targeted mice show higher susceptibility to infections (719) and fail to develop germinal centers in peripheral lymphoid organs (720) but are viable, breed normally, and show no apparent phenotypic anomalies. The absence of defects in the germ cell compartment of the TNFα receptor mutants suggests that other signals *in vivo* (SLF, IL-4) may be a sufficient supply for PGCs during normal embryonic development. In light of the above results, TNFα may be considered one of the factors involved in the detrimental effects of inflammation on testicular function.

VI. Vasoactive Peptides

A. Endothelin (ET)

The ETs (ET-1, ET-2, and ET-3) are peptides of 21 aa produced in a variety of tissues where they act as modulators of vasomotor tone, cell proliferation, and hormone production (722, 723). The three ET isopeptides are each produced from corresponding prepropolypeptides that are encoded by separate genes (724). Longer intermediates termed big ET-1, -2, and -3 (38–41 aa) are first excised from the prepropeptides by proteases that cleave at sites that contain paired basic amino acids. Big ETs, which are biologically inactive, are then further cleaved at Trp-21-Val-/Ile-22 to produce the 21-residue mature peptides. The putative endopeptidase that catalyze the specific cleavage at Trp-21 has been termed ET-converting enzyme (ECE), which is a membrane-bound neutral metalloprotease (725).

ET-1 is produced mainly in endothelial cells and vascular smooth muscle cells, but it is also produced in neurons and astrocytes, endometrial cells, hepatocytes, kidney mesangial cells, and breast epithelial cells. The majority of ET-1 secretion from cultured endothelial cells is toward the vascular smooth muscle side of the cells, where it can bind to specific receptors and cause vasoconstriction.

ET-2 is produced predominantly within the kidney and intestine, with smaller amounts produced in the myocardium, placenta, and uterus.

ET-3 has been found in high concentrations in the brain (726) and may regulate important functions in neurons and astrocytes, such as proliferation and development. It is also found throughout the gastrointestinal tract, in the lung and kidney.

Two distinct ET receptor subtypes, A and B, have been cloned (727, 728). The two ET receptors differ in their ligand selectivities: ET receptor B binds to the three ET isopeptides with similar affinity, whereas ET receptor A does not bind ET-3. The receptors are members of the superfamily of receptors linked with guanine nucleotide-binding proteins.

1. Expression, localization, and production. Adult rat testis contains ET-1 mRNA (729, 730) and peptide (731) and ET-3 mRNA (732) in relative abundance. Further studies have demonstrated that cultured rat Sertoli cells, but not Leydig cells, from 20-day-old rats secrete a peptide with the immunological and chromatographic properties of ET-1 and that this secretion is greatly inhibited by FSH (733). Immunocytochemical studies also confirmed that ET-1 is exclusively localized in Sertoli cells in immature rats, but in adult testis some staining is also observed in the interstitium (733). Subsequent immunohistochemical studies in adult rat testis showed immunoreactive ET-1 in Sertoli cells, endothelial cells, and most, but not all, interstitial cells. The intratesticular levels of immunoreactive ET-1 are significantly increased after hCG treatment (734). The recent demonstration that the testis contains exceptionally high levels of ET-converting enzyme (725) and that this enzyme is localized in Leydig cells and not in the seminiferous tubules (735) suggests that Leydig cells could be the principal site of testicular ET-1 synthesis.

The expression of a specific transcript for ET-1, and ET-1-like immunoreactivity, has been found in human testis (736). Positive staining was confined to the Sertoli cells and a small number of peritubular and interstitial cells (736).

2. Receptors. Specific high-affinity ET receptors have been demonstrated in mouse Leydig tumor cells (737), in normal immature rat Leydig cells (733), and PMC (738, 739), while the receptors were expressed at very low concentrations in Sertoli cells and spermatogenic cells. The immunochemical characterization of the ET receptors in the bovine testis revealed that the ET receptor A subtype is the major form in this tissue (740), in agreement with the report by Sakurai *et al.* (728), who showed that no mRNA for ET receptor B occurs in rat testis (728). Northern analysis of mRNA extracts from marmoset testis revealed the presence of both ET receptors with the prevalence of the ET receptor A mRNA; interestingly, the animals maintained on a high-fat/high-cholesterol diet exhibited a significant increase of both receptor mRNAs in all tissues and in the testis (741). In the rat testicular PMC, ET induces a rapid production of inositol triphosphate and mobilization of intracellular calcium in a concentration-dependent fashion (739, 742). Autoradiographic studies have confirmed the presence of abundant ET-binding sites both in the interstitial space, containing Leydig cells, and in the peritubular myoid layer of the adult rat testis (743).

Specific ET receptor A and B transcripts have been identified in human testis, with a more abundant expression of ET A subtype (736). The binding sites were mostly concentrated into the seminiferous tubules, although interstitial cells and PMC were also positive. Within the seminiferous

tubules ET-1 binding was confined to spermatocytes and spermatids, whereas the Sertoli cells were negative (736).

3. Local functions. ET-1 stimulates progesterone production and transient expression of the protooncogenes c-*jun* and c-*myc* in mouse Leydig tumor cells (737). In purified adult rat Leydig cells, ET positively regulates basal and hCG-induced steroidogenesis (743, 744). This effect on testosterone production in Leydig cells could be suppressed by a selective ETA receptor antagonist, but not by ETB receptor antagonists, confirming that rat Leydig cells are mainly provided with the ETA receptor subtype (743). PMC treatment with ET induces a phenotypic response involving the rearrangement of the cytoskeleton with a switch from a round to a flat and elongated morphology (739). Moreover, ET-1 stimulates DNA synthesis and contraction of rat PMC in culture (742). Although ET receptors have not been found in rat Sertoli cells isolated from 20-day-old rats (739) and in adult human Sertoli cells (736), Sharma *et al.* (745) have reported that in Sertoli cells isolated from 9-day-old animals, ET-1 activates an intracellular signaling pathway involving [Ca^{2+}] and attenuates FSH-stimulated cAMP and estradiol accumulation (745).

Local intratesticular injections of physiological and pharmacological doses of ET-1 cause a dose-related decrease in testicular blood flow that can be blocked by concomitant injection of an ET receptor A antagonist (734). Since hCG treatment in high doses causes a major decrease in intratesticular blood flow at 4–6 h after treatment and the intratesticular levels of ET-1 are increased after hCG treatment, the possibility that ET-1 could be a mediator of the vascular effects of hCG has been suggested.

Studies of ET-1 gene-targeting mice have provided direct evidence for an unsuspected developmental role in neural crest-derived tissues for ET-1 in addition to the previously recognized regulatory effect on vascular tone in the adult context (746, 747). Also ET-3 and ET receptor B gene-targeted neonates display a phenotype that suggests an important role of ET-3/ET receptor B in neural crest formation (748, 749). The phenotype resulting from the homozygous ET-1 mutations is lethal within 15–30 min from birth while the heterozygotes are phenotypically normal and fertile, but develop elevated blood pressure. The viability of the ET-3 and ETB receptor varied greatly with a mean life span of 21 days. Also in this case the heterozygous mice were phenotypically normal and fertile. The limited viability of the homozygous mutants does not allow the study of the reproductive effects of these genotypes.

In conclusion, despite the evidence for production, receptor expression, and *in vitro* action of ET, the physiological role of this peptide in the testis remains unknown.

B. Angiotensin-II (AT-II)

The renin-AT system is one of the major control systems of the blood pressure and water and electrolyte balance. The octapeptide AT-II is the biologically active product generated by a cascade of proteolytic cleavages. Renin from the juxtaglomerular cells of the kidney cleaves angiotensinogen, a glycoprotein precursor from the liver, to form the decapeptide AT-I, and AT-converting enzyme (ACE) removes a dipeptide from the C-terminal end of AT-I to form AT-II (750). AT-II cell surface receptors mediate the action of the hormone (751). Other evidence suggests the existence of two AT receptor subtypes. The structure of the AT1 receptor subtype has seven-membrane-spanning domains and is coupled through G-binding proteins to pathways involving PI hydrolysis, PKC, and Ca^{2+}. The AT1 receptor is widely distributed and is the receptor that mediates signal transduction in the cardiovascular system, adrenal, and kidney. The signaling pathway and function of the AT2 receptor are not clear, but tissue distribution of the receptor suggests a role in brain function (751).

Here the evidence of the testicular expression of the various components of the renin-AT system is described.

1. Expression and localization. Renin immunoreactivity has been found by means of immunohistochemistry in adult rat Leydig cells; some heterogeneous staining was observed in the interstitial cells of animals between 30 and 40 days of age, and no immunoreactivity was detected in animals younger than 30 days (752). Staining was suppressed or abolished by hypophysectomy and estrogen treatment and was reduced by gonadotropin stimulation, leading to the speculation that in gonadotropin-treated rats the increased secretion of the renin-like substance, not compensated for by a parallel increase in synthesis, might lead to decreased storage, explaining the weaker specific staining of stimulated Leydig cells (752). Subsequently, the renin-specific immunohistochemical staining exclusively localized in the Leydig cell was confirmed, and the biochemical determination of renin activity in the rat testis was also reported together with a decrease after hypophysectomy and a remarkable increase after gonadotropin treatment (753). The existence of renin enzyme activity in Leydig cells purified from adult rat testis has been demonstrated together with an inactive form of latent renin that is activated by sulfhydryl reagents (754). A renin mRNA has been detected in adult mouse (755) and rat testis (756) and in the testis of a transgenic mice carrying the human renin gene (757). The immunocytochemistry and *in situ* hybridization of renin and its mRNA in adult rat testis confirmed the Leydig cell localization of the enzyme (758).

LH and cAMP elicit a dose- and time-dependent induction of renin activity in a cultured mouse Leydig tumor cell line, which parallels the increase in steroid biosynthesis (759). Further studies have demonstrated that the LH-induced renin activity in these cells is retained within the cells while about 15% of AT-I and the majority of AT-II are secreted (760).

ACE has been implicated in at least two other areas, both of major physiological and pathophysiological interest, which are not related to the control of blood pressure and fluid electrolyte homeostasis: reproduction and immunology (761). Leydig cells isolated from adult rat testes were found to contain renin, ACE, and AT-I, AT-II, and AT-III as determined by HPLC and RIA, while in germinal cells only AT-II and AT-III were found at significant levels (762). Subsequent studies showed that testicular ACE activity is associated primarily with germinal cells of the mature rat and rabbit (763, 764). The testicular transcripts encode for the ancestral, nonduplicated form of the enzyme in both rodents and men

(765–767). This form appears in the rat testis at puberty and is testosterone dependent (763, 768, 769). The transcription of germinal ACE occurs via a testis-specific promoter located within the intron 12 of the ACE gene (770–774), which is completely extinguished in somatic tissues (775). Characteristically, testicular ACE is smaller than its pulmonary counterpart, has different N- and C-terminal sequences and mRNA, yet arises from a common gene (776, 777). It probably represents an internal portion of pulmonary ACE and has similar enzymatic properties (778, 779). By using immunoperoxidase detection and *in situ* hybridization, the temporal expression and cell distribution of the germinal isoform of ACE have been studied in the testis of normal mice and rats (780). In both species, specific testicular ACE mRNA and its gene product are present only after completion of meiosis. The study of the pattern of expression of germinal ACE during spermatogenesis in mouse and rat showed that ACE mRNA and its corresponding protein are first synthesized during stages IV-VII, the maximum expression occurs at stages VIII-XII, and ACE mRNA is no longer detectable in spermatids beyond stage XIV, whereas its gene product is expressed until the end of spermatid maturation (780, 781). By using specific isoenzyme antisera, immunofluorescent staining of sections of adult, but not immature, pig and rabbit testes revealed specific staining to testicular ACE within the lumen of seminiferous tubules in the cytoplasm of spermatocytes, whereas the antiserum specific for endothelial ACE showed the presence of this isoenzyme only in blood vessels (782, 783).

Although no angiotensinogen mRNA has been detected in the adult rat testis (784), AT-II has been found in adult rat Leydig cells and germinal cells (762) and is produced by mouse Leydig tumor cells treated with LH (760).

In human testis, renin immunoreactivity was found in the Leydig cells (785). Studies conducted assaying prorenin, renin, and angiotensinogen in internal spermatic venous blood from young men suggested that the only form of renin secreted by the human testis is prorenin (786). In other studies it has been reported that under basal conditions very low levels of renin and AT-II are released into internal spermatic vein blood, while after treatment with hCG the secretion of both renin and AT-II are significantly higher (787, 788).

In man the results of genomic DNA analysis are consistent with the presence of a single gene for ACE in the aploid human genome. According to the results obtained in different animal species, the ACE gene is transcribed as a 4.3-kb mRNA in vascular endothelial cells whereas a 3.0-kb transcript was detected in the testis, where a shorter form of ACE is synthesized (777). Immunolocalization of ACE in the human male genital tract has shown an intracellular positivity in spermatids on the acrosomal cap (789).

2. Receptors. High-affinity receptors for AT-II have been reported in the Leydig cells in rat and primate testis, including human (790). Studies on the AT-II receptors in rat testis revealed the presence of high receptor density in newborn rats, which decreased toward the adult age parallel to the fall in the ratio of interstitial to tubular tissue (790–792).

3. Local functions. AT-II inhibits adenylate cyclase activity in Leydig cell membranes and reduces basal and hCG-stimulated cAMP pools and testosterone production in intact cells from adult rats (791). Male but no female mice homozygous for a disrupted ACE gene have impaired fertility despite being potent and having sperm of normal appearance, suggesting that sperm produced by homozygous male mutants have a reduced fertilizing ability (793). The mechanisms responsible for the reduced fertility in these male mutants is currently unknown. A direct involvement of AT-II seems unlikely. Thus far, three laboratories have studied the phenotype of homozygous mice carrying null mutation of the angiotensinogen gene primarily focusing on the blood pressure control of the mutant. While one study does not document structural abnormalities in major organs (794), the others report kidney abnormalities similar to those observed in ACE-deficient mice (795, 796). The absence of a functional angiotensinogen gene is compatible with survival to birth, but postnatal survival is severely compromised. However, an occasional survival to adulthood of angiotensinogen $-/-$ animals, tested for fertility, produced three litters of normal size, demonstrating that AT is not essential for male fertility in mice. Accordingly, although gene targeting of the AT1 receptor (797) and AT2 receptor (798, 799) in mice has marked effects on blood pressure and central nervous system and cardiovascular function, it does not impair the animals' fertility. Moreover, in transgenic mice carrying both human renin and human angiotensinogen genes leading to overproduction of AT-II, no change in reproductive performance was reported (800).

In conclusion, several components of renin-AT system are expressed within the testis, but the complete system has not yet been identified. For example, in the rat testis, renin, ACE, AT-I, and AT-II have been found but angiotensinogen could not be detected. The evidence for AT-II production, receptor expression, and *in vitro* action on Leydig cell testosterone production are convincing. However, the physiological role of AT-II in testicular functional control is unclear. The reported impaired fertility in male mice ACE knock-out is interesting but probably involves pathophysiological mechanisms that do not relate to AT-II generation.

C. Atrial natriuretic peptide (ANP)

ANP is a peptide hormone released from the cardiac atrial myocytes that regulates blood pressure and fluid volume (801). In addition to its natriuretic, diuretic, and vasorelaxant activities, ANP inhibits the release of aldosterone, renin, and AVP while stimulating the release of androgen, progesterone, and GH. ANP selectively activates particulate guanylate cyclase and inhibits adenylate cyclase, thereby increasing the intracellular accumulation of cGMP and decreasing cAMP (802). The biological actions of ANP are mediated by specific cell surface receptors. The diverse cellular responsiveness to ANP action is thought to be due to the heterogeneity in the ANP-binding sites. Two types of ANP-specific receptors have been identified in target tissues: two different guanylate cyclase-linked receptors that appear to mediate most of the ANP biological effects, and a clearance receptor (803).

1. Expression and localization. Low levels of an immunologically recognized ANP prohormone together with ANP

mRNA transcripts have been found in the adult rat testis (804–806), and immunoreactive ANP has been localized in mouse and rat spermatids and elongating spermatozoa (807).

2. Receptors. Pandey *et al.* (808–810) have characterized ANP receptors in cultured mouse Leydig tumor cells that contain predominantly the guanylate cyclase-coupled form of the ANP receptors, and ANP receptors have been detected also in purified mouse Leydig cells (811). The radioautographic localization of ANP-binding sites in the adult rat testis demonstrated specific binding only in interstitial cells (812). An ANP-dependent membrane guanylate cyclase has been demonstrated in mouse and rat testis (813), as well as three immunologically and biochemically similar but distinct ANP receptors (809, 814). Kinetic analysis of the fate of the ANP receptors on Leydig cells demonstrates internalization, recycling, and redistribution of the receptors (815). Recently, evidence that both Gs and Gi subunits of G proteins seem to be involved in the regulation of Gs catalytic activity of the ANP receptors in the plasma membrane of a murine Leydig tumor cells line (MA-10) has been provided (816).

3. Local functions. ANP has been demonstrated to stimulate testosterone and cGMP production in isolated mouse Leydig cells (817–820) and a murine Leydig tumor cell line (810), while in rat Leydig cells a lower stimulation of cGMP formation and a lack of testosterone production enhancement, probably due to an insufficient formation of cGMP in these cells, was observed (818). However, subsequent studies showed that ANP exerts stimulatory effects on steroidogenesis and calcium flux in rat Leydig cells (821). ANP increases the levels of cGMP, inhibits cAMP accumulation and progesterone secretion in response to hCG, and negatively regulates the phosphorylation of PKC in cultured Leydig tumor cells (822, 823). More recently it has been reported that a specific antagonist inhibits the testosterone production and cGMP accumulation stimulated by ANP in purified mouse Leydig cells (824). Moreover ANP enhances basal and LH-stimulated testosterone production of purified mouse Leydig cells involving both the Δ^4 and Δ^5 pathways of steroidogenesis (811). Although ANP receptors have not been found in Sertoli cells, treatment of cultured Sertoli cells with ANP resulted in both a dose-dependent and time-dependent elevation of cGMP levels (392).

In humans the intravenous administration of 100 μg of ANP induced a rapid significant increase of spermatic vein testosterone levels without affecting peripheral testosterone and LH concentrations, suggesting that ANP may directly influence the Leydig cell function in man (825).

Mice with either proANP or ANP receptor-disrupted gene have been generated (826, 827). Genetically reduced production of ANP leads to salt-sensitive hypertension (826) while mice lacking the guanylyl cyclase-A receptor for ANP develop salt-resistant hypertension (827). Both homozygous mutants, except for the blood pressure, appear normal and fertile.

A transgenic mouse line expressing ANP fusion genes has been also produced (828). These mice have large amounts of pro-ANP in the liver, and their plasma ANP levels are significantly elevated. The chronic endogenous hypersecretion of ANP results in sustained perturbations in cardiovascular homeostasis not accompained by significant changes in several other physiological parameters including fertility.

VII. Conclusions and Perspectives

In this article, we have summarized the information acquired over the past two decades on the locally produced polypeptide factors with supposed regulatory roles in the testis. They can be viewed as one of the elements involved in the control of the male gonad.

Similar to other endocrine-regulated tissues, the testis is subject to a hierarchy of controls. In this light, the systemic hormones represent the first-step regulators. In addition, the local factors synthesized by the cellular components of the testis may act as paracrine or autocrine/intracrine hormones, or, as a further variation of this theme, as juxtacrine factors, regulatory molecules that remain exposed on the cell surface and modulate adjacent cells by contact. Polypeptide factors are among the most widely studied of the second-step local modulators of testicular function, and other molecular classes may serve such functions as well. The second-step substances act through cell surface or intracellular receptors (third step) that trigger the cascade of intracellular events (fourth step) from the early signal transduction to the final cell response. The interactions of all these elements within the structural context of the testis regulate its function. This hierarchical vision, while acknowledging FSH, LH, and testosterone as prime regulators, recognizes that an imbalance of each of the substances involved in the subsequent steps may potentially impair organ function. Moreover, the actors of the intratesticular control mechanisms may not be the same during testicular ontogenesis. Also in the mature organ, the environment to which each cellular component is exposed varies cyclically, due to the marked morphological and functional changes of Sertoli cells and germ cells throughout the seminiferous epithelium cycle. This is clear from the data summarized in Tables 6 and 7.

Why, one might wonder, should such a complex network of regulators be required for the organization of testicular function? Why wouldn't a simple feedback relationship suffice? The answer to this questions seems to be that a classic feedback mechanism might be enough if the testis were only devoted to the secretion of testosterone, but in any case such a vision would be too simplistic since all the endocrine glands possess a complex network of intraglandular regulators. Furthermore, the testis is the organ in which spermatogenesis takes place, and this is a dynamic process organized so that, at a given time point, adjacent tubules are at different stages of the cycle of the seminiferous epithelium (Fig. 1). This structural complexity implies that to furnish the optimal focal intratesticular environment, local regulators should be produced to generate the unique microchemical context required for germinal cell development.

Thus, the next question is, to what extent do local regulatory peptides play a role in the control of testicular function? Coming back to the classic definition, the basic requisites for a regulatory peptide to be considered a potential local modulator are: 1) the substance should be produced in

the target tissue; 2) the cell/tissue should contain specific functional receptors; 3) the substance should affect at least one single function of the target cell. Evidence for the presence of numerous regulatory peptides in the testis that fulfil these criteria has been provided. However, despite the enormous amount of data, they can hardly fit into a physiological picture. As noted in the introduction, this is mainly due to the difficulty in distinguishing between effects that are restricted to the artificial conditions of the *in vitro* systems and effects that are relevant to testicular control *in vivo*. Notwithstanding, evidence from *in vivo* experiments suffers from some intrinsic limitations as well. For example, the pharmacological doses employed and the ubiquity of the regulatory substances make it difficult to distinguish between direct effects and effects mediated by interference on other systems. Another limit is the redundancy of the actions of the regulatory peptides. This concept is readily apparent if one looks at the *in vitro* effects on the cellular components of the testis as listed in Table 4. This redundancy is probably less active *in vivo* because of three conditions: 1) it is presumably the interaction between several factors that ultimately determines the net response of the cell to the stimulus and, given the complexity of such interactions, it is conceivable that even if two different stimuli induce the same response *in vitro*, small environmental variations might result in qualitatively different patterns of subsequent effects *in vivo*; 2) the expression of some of these substances and of their receptors is developmentally regulated; thus there are time windows in their potential actions (Table 6); 3) the expression of substances and receptors may also vary depending on the stages of the seminiferous epithelium (Table 7). Again, these variables can be hardly evaluated *in vitro*. Another important element that should be considered is cross-regulation among the regulatory factors. For example, the production of IGF-I by Sertoli cells is stimulated by FGF and EGF (401–403, 415), and IL-1α and IL-1β increase Sertoli cell production of IL-6 (665) and Leydig cell secretion of NPY (318).

Once released, other mechanisms are involved in the control of availability and domain of influence of the testicular regulatory peptides, such as proteolytic processes and interactions with extracellular matrix and binding proteins. These events can function in two directions either to increase or to decrease the availability of polypeptide regulators. For example, studies of TGFβ1 processing show that the growth factor is secreted as an inactive molecule and that extracellular proteolytic cleavage is required to release the biologically active dimer from the precursor molecule (829). Complexes formed by matrix proteins with regulatory factors might have either higher affinity for specific receptors or remain sequestered in the extracellular matrix before release by enzymes such as heparinase and plasmin to function as diffusible regulators. In this way the extracellular matrix can provide a further mechanism for regulating the cytokine's sphere of influence.

Thus, at present, there is a great need for more information about how combinations of signaling molecules are used to organize the control of the testis *in vivo.*

Despite the experimental evidence reported in this chapter, for many of the locally produced regulatory peptides, their relevance in the control of testicular function is still speculative. On the other hand, the need to provide answers to the open questions on the control of the gonadal function is becoming increasingly important in light of the prevalence of male idiopathic infertility. In this context, the suspicion that regulatory peptide pathways are involved in the control of testicular function and in the pathogenesis of some cases of male infertility deserves much further investigation.

Promising new information has been obtained from studies that employ molecular genetic approaches, using targeted gene inactivation or overexpression in transgenic animals. However, in interpreting the results from these models one must be conscious of the limits of the technique. A major consideration concerns the ability of the biological system to adapt or respond to the alterations. Such physiological adaptations or compensations may obscure other important functions of the gene under investigation. For example, this is particularly evident if one considers the defects generated by single knockouts of receptors or substances belonging to a family, which can be milder than expected because of functional redundancy. As a matter of fact, the deficiency of any one of the family members may be compensated for by the action of other members of the same group. Furthermore, many of the presently available mutations cannot reveal all of the functions of a given gene within a given organ because they may result in a lethal phenotype. Mutant mice represent *in vivo* models that involve the entire organism, and thus the effects produced within an organ cannot be easily ascribed to a direct effect of the gene product on that organ. The gene expression *in vivo* is spatially and temporally regulated while transgenic animals are chronically exposed to the alteration of the gene.

Nevertheless, given all these major premises, significant advances have been made over the last few years in defining the local requirements for normal testicular development and function. Among the substances described in this review, particular mention should be made of INH, IGF-I, TGFβ, SLF/c-*kit*, and ENK. The studies on IGF-I and SLF/c-*kit* have defined the important role of IGF-I in Leydig cell differentiation and of SLF/c-*kit* in PGC and spermatogonia migration and survival. INH has been shown to be a tumor suppressor gene with gonadal specificity. The testicular overexpression of pENK has been shown to lead to histological abnormalities of the seminiferous tubule. Persistent overexpression of TGFβ1 elicits a cellular overresponse that may lead to serious fibrotic or proliferative disease in various organs and in the testis. In the future, for many of the substances described here, their testicular actions will be more clearly revealed, limiting gene overexpression or gene deletion spatially and temporally and allowing their circulating or endocrine functions to remain intact. New technologies that provide a mechanism to limit gene disruption to specific tissues have recently emerged (830).

There are two more lines of evidence that deserve mention here because of their potential relevance in revealing the influence of locally produced regulatory substances in the control of testicular function.

Very recently, mutant mice lacking the cAMP-responsive element modulator (CREM) gene have been generated and shown to have spermatogenic arrest at the spermatidic stage (831, 832). These animals may constitute a tool for studying

some cases of altered physiological processes leading to infertility because spermatogenic arrest is also a feature of many cases of infertility in human males. The CREM gene encodes multiple regulators of the cAMP-transcriptional response by alternative splicing (833). A developmental switch in CREM expression occurs during spermatogenesis, whereby CREM function is converted from an antagonist to an activator, CREMτ, which accumulates from the premeiotic spermatocyte stage onward (834) and is particularly abundant in round spermatids, predominantly at stage VII-VIII of spermatogenesis (835). Mutant mice lacking the CREM gene are sterile, as their developing spermatids fail to differentiate into sperm (831, 832). In these animals the inhibition of sperm development is not accompanied by decreases in the levels of FSH or circulating and intratesticular levels of testosterone (831, 832). This provides an interesting experimental model that resembles the frequent condition found in infertile men in which the defect in the production of mature spermatozoa is not accompanied by a concomitant modification of the gonadotrophic hormones. It has been established that FSH is responsible for the CREM switch (836). The effect of FSH is not direct, however, because germ cells do not have FSH receptors. Thus, it has been suggested that another hormonal message, originating from the Sertoli cells upon FSH stimulation, mediates CREM activation in germ cells (837).

Another example that seems to indicate a role for locally produced regulatory peptides in controlling testicular function comes from a study conducted to investigate the role of the bone morphogenetic protein 8B (BMP8B) during spermatogenesis (838). BMPs are members of the TGFβ superfamily synthesized as large precursors and processed and proteolytically cleaved to yield carboxy-terminal mature protein dimers (839). BMPs are multifunctional regulators of embryonic development required for the organogenesis of kidney, lung, heart, teeth, gut, and skin. In particular, BMPs may help to mediate inductive interactions between mesenchymal and epithelial cells in these tissues. Zhao *et al.* (838) have generated mice with a targeted mutation in BMP8B. The homozygous BMP8B mutant males exhibit variable degrees of germ cell deficiency and infertility. Interestingly, two different defects can be observed in the mutant testes. During early puberty, the germ cells of the homozygous mutants either fail to proliferate or show a marked reduction in proliferation and delayed differentiation. In adults there is a significant increase in apoptosis of spermatocytes that leads to germ cell depletion and sterility. Although an endocrine pathway influencing gonadotropin production in the pituitary gland by BMP8B cannot be completely ruled out, the lack of any significant changes in morphology, proliferation, or apoptosis in Leydig cells and Sertoli cells does not support a gonadotropin-mediated BMP8B function. Also, androgen production by Leydig cells appears to be normal since the morphology of the androgen-dependent tissues in all homozygous mutant males is unaffected. Thus the BMP8B proteins function locally within the seminiferous tubules, either through an autocrine effect on germ cells and/or through a short-range paracrine effect on Sertoli cells. This hypothetical model of action is further reinforced by the notion that BMPs bind ACT receptors (840), which are expressed in germ cells and Sertoli cells at specific stages of the seminiferous cycle (350, 361).

Finally, a promising new technique has been developed that in the near future may allow the investigation of the interactions of germ cells with Sertoli cells and other cellular components of the testis. The procedure consists in the transfer of spermatogonial stem cells from a donor mouse (841, 842) or rat (843) testis into individual seminiferous tubules of an infertile recipient mouse. The donor cells are able to establish complete spermatogenesis in the seminiferous tubules of the host and to produce normal spermatozoa. Moreover, cryopreserved mouse spermatogonia can be successfully transferred to recipient host testis where they produce viable and morphologically normal sperm (844). The transfer of spermatogonia between species will represent a radical contribution to reproductive biology and promises benefits in areas such as animal conservation and cryopreservation of spermatogonia of patients before radical chemotherapy, since stem cells from males with azoospermia may be capable of generating spermatogenesis in a surrogate. The ability to manipulate the donor or the host could represent a further opportunity to study the local control mechanisms of the testis. For example, the use of genetically sterile mice as recipients should help in determining the role played by locally produced regulatory substances in the engrafting and development of donor spermatogonia.

In conclusion, the testis is a totally integrated cellular system. Regulation at a local, intragonadal level is an integral part of the overall regulation of development and functional control of the male gonad. Interaction between cells within the testis extends beyond the same cell type to include germ cell-somatic cell interactions as well. Gonadal peptides are among the signals that are used to communicate within the testis, and it is conceivable that an alteration in their function either during development or in adult life may be responsible for some cases of idiopathic male infertility.

Hopefully, the knowledge and experimental tools generated during more than 20 yr of research and summarized in this review will help to elucidate the role of gonadal peptides in the pathophysiology of the testis.

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