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MINI-REVIEW

Neuropeptides of the Pituitary Adenylate Cyclase–Activating Polypeptide/Vasoactive Intestinal Polypeptide/Growth Hormone–Releasing Hormone/Secretin Family in Testis

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Mammalian testicular development and the maintenance of spermatogenesis are hormone-dependent processes that are controlled by the pituitary gonadotropins and testosterone. Recent studies have demonstrated the presence of many neuropeptides and their receptors in the testis, suggesting that these peptides operate as local regulators of testicular germ cell development and function. Among these testicular neuropeptides, the peptides that belong to the pituitary adenylate cyclase–activating polypeptide (PACAP) family, particularly growth hormone–releasing hormone and secretin, appear to show some unique common features in terms of intratesticular localization and the time of expression during the spermatogenic cycle. However, their precise physiologic roles and mechanisms of action remain unknown. This review analyzes the available information on the functional interactions among the testicular cells that appear to be mediated by locally produced neuropeptides, with a special emphasis on the peptides of the PACAP family.

Key Words: Neuropeptide; testis; pituitary adenylate cyclase–activating polypeptide; vasoactive intestinal polypeptide; growth hormone–releasing hormone; secretin.

Introduction

The testis contains many "neuropeptides" that were discovered in the central nervous system (CNS). Testis is a complex organ that serves two crucial functions, spermatogenesis and steroidogenesis, that depend on normal stimulation by the pituitary gonadotropins, follicle-stimulating hormone (FSH), and luteinizing hormone (LH), whose release is stimulated by gonadotropin-releasing hormone (GnRH) *(1)*. Spermatogenesis is a complex morphogenetic process that is largely under the control of endocrine, paracrine, and autocrine factors. In this aspect, several similarities have been observed between the testis and the brain. First, both sperm cells and neurons are highly differentiated, nonproliferative cells *(2)*. Second, there is a structural and functional similarity between the germ cell/Sertoli cell and the neuron/glia organization *(3)*. Finally, the brain and the testis share in common the tissue-specific expression of mRNAs for "signaling" proteins, ranging from protooncogenes *(4,5)*, G proteins *(6)*, and cyclic nucleotide–gated channels *(7)* to molecular adapters of signaling proteins *(8)*. Thus, even if considerably less studied in the testis than in the brain—mainly because of the lack of long-term germ cell and somatic cell co-cultures that are required for the classical research on cell to cell interaction—testicular germ cells appear to utilize an armamentarium *(3)* of cell signaling molecules reminiscent of that of neuronal cells.

The testis is functionally compartmentalized into the gamete and endocrine segments, where spermatogenesis and testicular steroid hormone biosynthesis take place, respectively. Testosterone functions as a male hormone but also regulates the maintenance of spermatogenesis *(9,10)*. The spermatogenic compartment is formed by the seminiferous epithelium, which is composed of Sertoli cells and the various stages of germ cells. The blood–testis barrier is formed by tight junctions between Sertoli cells near their basal aspect, plus the basal laminae and peritubular myoid cells that render the seminiferous tubule an immunologically privileged site that is sequestered from the peripheral circulation. The endocrine compartment comprises the constituents of the testicular interstitium: Leydig cells, the site of testosterone biosynthesis; resident testicular macrophages, which are closely associated with Leydig cells; the endothelium, which is the distal aspect of the testicular circulation; and the lymphatic space that surrounds the cellular components of the interstitium. The Leydig, peritubular, and Sertoli cells of the testis provide the milieu essential for germ cell development and steroidogenesis. Testosterone, which is synthesized and

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secreted by Leydig cells, serves as the primary paracrine stimulus for germ cell development. Its actions are aided by a variety of peptide hormones and growth factors. Many of these are eutopically produced by the testis and are involved in paracrine, autocrine, and/or juxtacrine processes *(11)*. Spermatogenesis is a continuous process that takes place sequestered within the blood–testis barrier, suggesting a need for regulation by locally produced factors. Recent studies have demonstrated high local concentrations of pituitary adenylate cyclase–activating polypeptide (PACAP) *(12)*, growth hormone–releasing hormone (GHRH) *(13)*, and secretin *(14)*, suggesting their potential effects on germ cell development and functional control of testis involved in spermatogenesis and/or functional maturation of the germ cells.

The testis is a source of many neuropeptides including GHRH, PACAP, GnRH *(15)*, corticotropin-releasing hormone (CRH) *(16,17)*, natriuretic peptides *(18–21)*, vasoactive intestinal polypeptide (VIP) *(22)*, oxytocin *(23)*, arginine vasopressin (AVP) *(24)*, thyrotropin-releasing hormone (TRH) *(25)*, somatostatin (SRIF) *(26)*, opioid peptides *(27–30)*, substance P *(31)*, neuropeptide Y (NPY) *(32)*, and secretin. Among these, PACAP, VIP, GHRH, and secretin constitute a family of peptides with significant similarities in their amino acid sequences *(33)*. The roles of these neurohormones/neuropeptides in testicular physiology are largely unknown. The advent of transgenic and gene-targeting techniques has allowed the unique opportunity to test the functions of the specific genes for these peptides 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, several groups have reported the results generated by these powerful technologies, which may help to clarify the complex functions of the regulatory peptides in the testis *(34–37)*. The function of testicular cells is primarily controlled by the hypothalamic-pituitary-testicular axis that directs both steroidogenesis and spermatogenesis. The pituitary gonadotropins also appear to affect the expression of testicular neuropeptides via paracrine factors produced by Sertoli and/or Leydig cells *(38–42)*.

The aim of the present review is to outline and analyze the intratesticular sites of expression of these peptides and their receptors and their functions and the possible physiologic events, with a special emphasis on the peptides of the PACAP/GHRH family.

Testicular Neuropeptides

Secretin was the first peptide hormone to be characterized; it is a 27 amino acid peptide that was originally isolated from the duodenum *(43)*. Secretin mRNA has a wide tissue distribution in animals, including the small intestine, kidney, heart, lung, brain, and testis *(14)*. Secretin is now known to act via specific G protein–coupled receptors with seven-transmembrane moieties that are coupled to Gsα and activate adenylate cyclase *(44)*. Secretin receptor mRNA has been found in rat heart, stomach, pancreas, and testis *(44)*.

The hypothalamic neurohormone GHRH contains 44 amino acid residues that stimulate the synthesis and secretion of GH from the somatotropes of the anterior pituitary *(45,46)*. Additional proteolysis in vivo can yield peptides of 37 and 40 amino acids that possess full biologic activity. The rat testis contains a GHRH-like immunoreactivity (LI) and mRNA *(47)*. The receptor of GHRH belongs to the family of the G protein–coupled receptors containing seven potential membrane-spanning domains *(46,48)*.

VIP was originally isolated from porcine duodenum and considered to be a gut hormone involved in smooth muscle relaxation and vasodilation *(49)*. VIP is widely distributed throughout the central and peripheral nervous systems *(50)* and is present in the nerves of both male and female reproductive tracts *(22)*. Interestingly, in the male rat, VIP-LI was exclusively localized to neural fibers derived from the inferior spermatic nerve *(51)*. On the other hand, no report indicating the presence of VIP in the testicular cells has appeared.

During an attempt to discover a novel hypothalamic hypophysiotropic hormone based on its ability to stimulate adenylate cyclase in pituitary cell cultures, an amidated 38 residue basic peptide was isolated and identified from ovine hypothalamic extracts and named it PACAP38 *(52)*. Subsequently, a truncated amidated form with 27 residues was isolated from the side fractions of the initial purification and named PACAP27 *(53)*. PACAP was found to be a new member of the VIP/glucagon/GHRH/secretin family, being most similar in amino acid sequence to VIP (68%) *(52,54)*. However, the adenylate cyclase–activating potencies of PACAP38 and PACAP27 were 1000–10,000 times greater than VIP in pituitary, neuronal, and astrocyte cell cultures *(52,54)*. A heterologous radioimmunoassay (RIA) method for PACAP38 and a homologous RIA for PACAP27 were established to determine the PACAP content in the CNS and peripheral tissues *(12)*. The highest concentration of radioimmunoassayable PACAP38 was found in the hypothalamus, but other brain regions also contained considerable amounts *(12)*. Unexpectedly, adult rat testis contained a high abundance of PACAP38, and the total amount of PACAP in both testes exceeded its content in the whole brain *(12)*. Reverse-phase high-performance liquid chromatography (RP-HPLC) combined with RIAs for PACAP suggested that the major species of testicular PACAP-LI is PACAP38. In all tissues, PACAP38 represented the major form of total PACAP, and its concentration was at least 10 times greater than PACAP27 *(12)*.

Expression and Localization of PACAP, VIP, GHRH, Secretin, and Their Receptors in Testis

The intratesticular localizations of the neuropeptides and their receptors during developmental and in adult testis are presented in Table 1. It is noteworthy that PACAP, GHRH, and secretin, but not VIP, in this peptide family are expressed

Table 1
Developmental Expression and Localization of Neuropeptides of PACAP/VIP/GHRH/Secretin Family and Their Receptors in Mammalian Testis[a]

	Peptides		Receptors	
Neuropeptide	Immunoreactivity	mRNA	Binding	mRNA
GHRH	Testis (P4) Round SpT/SpZ Acrosome (A) Leydig cells (A)	Testis (E19) (P2→P21⇑→P40⇑⇑⇑) SpC/SpT (A)	Leydig cells (A)	Germ cells (A) Sertoli cells (A) Leydig cells (A)
PACAP	Testis (E) Germ cells (P21→P28⇑→P56⇑⇑⇑) Round/elongated SpT acrosome (A)	Round SpT (P28→P42⇑→P56⇓) SpG/SpC (A) Round/elongated SpT (A)	SpG/SpC/SpT/SpZ Epididymis (A) Sertoli cells (A) Leydig cells (A)	Testis (A) Germ cells (A) SpT acrosome (P & A)[c] Sertoli cells (A)
Secretin	SpC (A) Round and elongated SpT (A)	SpC/SpT (A)		Round SpT (A)
VIP	Cauda epididymis and penis (P & A)[b] Genital system (A)	ND	SpZ (A) Leydig cells (P & A)	Testis (E15.5) (P1→P7⇑→P14⇑⇑⇑) ST (A)/SpZ (A)

[a]⇑, increases; ⇓, decreases; E, embryo; P, postnatal; A, adult; ND, not detectable; ST, seminiferous tubuli; SpG, spermatogonia; SpC, spermatocytes; SpT, spermatids; SpZ, spermatozoa.
[b]Nerves.
[c]Immunoreactivity.

in haploid germ cells, apparently stage specifically. The immunoreactivity of GHRH was also detected in spermatozoa, but its mRNA was found only in spermatocytes and spermatids. VIP is found only in the nerve fibers distributed in the epididymis and interstitial area. The patterns of protein and gene expression for both the peptide and its cognate receptors in the germ cells are usually found to overlap with stage-specific expression during spermatogenesis *(42,55–59)*.

As in the brain, PACAP38 is the major molecular form in the testis *(12)*. PACAP-LI is abundant in germ cells of the testis and is expressed in a stage-specific manner during spermatogenesis *(56,60)*. In fact, the concentration of PACAP in the testis appears to exceed the concentration of any other known testicular peptide *(12)*. A study of the ontogeny of testicular PACAP transcripts and its immunoreactivities has been performed in the developing rat testis. The PACAP mRNA in the testis was first detectable on postnatal d 28 (P28) immediately after the first appearance of round spermatids. The mRNA levels increased progressively thereafter, peaking on P42, and then slightly decreased on P56 (unpublished observation). An RIA of the testicular extract from developing rats showed that PACAP-LI was detectable at low levels in the fetal and neonatal testis. The levels of PACAP-LI reached the nearly nondetectable level on P21, began to increase on P28, and reached the highest levels on P56. The results suggest that the expression of PACAP mRNA is developmentally regulated and that the changes in PACAP mRNA levels are followed by the changes in PACAP-LI. The low, but detectable, levels of PACAP-LI in the testis of the fetuses and the neonates could be accounted for by PACAP originating from extratesticular tissues, such as the placenta.

In adult rat testis, *in situ* hybridization (ISH) indicated that the signals for PACAP mRNA were detected in early developing germ cells, especially in spermatogonia and primary spermatocytes *(56)*, indicating that transcription of the PACAP gene occurs during these early stages of germ cell differentiation *(61,62)*. A more detailed analysis showed that in round and elongated spermatids, PACAP mRNA was detected at stages III–VII of the seminiferous cycle. Northern blot analysis showed that the highest amount of PACAP mRNA was detected at stages V to early VII of the cycle, whereas very low levels of the mRNA were present at stages I to II and IX–XIV *(63)*. However, using reverse transcriptase polymerase chain reaction (RT-PCR) analysis of consecutive seminiferous tubule segments, Daniel and Habener *(64)* reported that the PACAP transcript is expressed in a highly stage-specific manner, in stages I–III slightly preceding a sharp drop in the message for the FSH receptor during stages II–VI. The stage-specific expression of PACAP transcripts has also been confirmed in the mouse testis *(65)*.

These findings suggest that testicular PACAP participates in sperm maturation, in particular when spermatids are about to begin their dramatic morphologic changes. The localization of PACAP-LI in the rat testis was studied with

immunohistochemistry (IHC) using antisera against PACAP. PACAP was detected in spermatids situated near the lumen of the seminiferous tubules. In rat spermatids, PACAP was expressed during the cap and acrosome phases, but not in earlier stages of spermatogenesis nor in mature spermatozoa, Sertoli cells, or Leydig cells *(56,59)*. At the ultrastructural level, PACAP-LI was found in the acrosomal granules and acrosomal caps of spermatids, and weak immunoreactive PACAP was also found in the Golgi apparatus *(56)*. However, neither the cytoplasmic matrix nor structures other than the acrosomes in spermatids showed immunoreactivity for PACAP.

Cloning of PACAP38 cDNAs has confirmed the expression of the corresponding mRNAs and the presence of this neuropeptide in human testis *(66)*. It can be concluded that the expression of both PACAP transcripts and PACAP-LI in testicular germ cells is stage specific during spermatogenesis, being highest in the spermatids.

Three PACAP receptors have been cloned so far and are called the PAC_1, $VPAC_1$, and $VPAC_2$ receptors according to their relative affinities for PACAP and VIP. The PAC_1-R is PACAP specific, while $VPAC_1$-R and $VPAC_2$-R have similar affinities for PACAP and VIP *(67)*. In the adult testis, PACAP-binding sites have been characterized in germ cells *(68)*, Sertoli cells *(69)*, and Leydig cells *(70)*. Although RT-PCR apparently detected the transcripts of PAC_1-R, neither Northern blot nor RNase protection assay detected the PAC_1-R mRNA. The location and characteristics of PACAP-binding sites were studied using ^{125}I-PACAP27 with conventional methods of receptor autoradiography. Autoradiography with ^{125}I-PACAP27 on rat testes exhibited abundant specific binding sites for PACAP in the spermatogonia, spermatocytes, late spermatids, and mature spermatozoa of seminiferous tubules, displaying the highest density of labeling over the tail portion of the spermatozoa *(68)*. PAC_1-Rs were also observed in the lumen of the epididymal ducts. Few or no PACAP-binding sites were present in the Leydig cells *(68)*. Binding specificity was established by displacement with unlabeled PACAP27 or VIP. PACAP27-binding sites were localized autoradiographically in the testis, epididymis, adrenal gland, lung, liver, prostate gland, and seminal vesicles *(68)*. In the testis and epididymis, a PACAP27-binding site was localized on germ cells *(68)*. Excess VIP did not displace most of the PACAP27 binding, indicating that these binding sites are mainly PACAP-specific binding sites (PAC_1-R). However, a conventional radioreceptor assay did not identify significant amounts of specific binding to *(68,71–75)* PACAP27 in the membrane preparations of the rat testis *(68,73)*. A polyclonal antiserum was generated against a 25 amino acid peptide corresponding to the intracellular domain of the rat PAC_1-R and used for IHC for the PAC_1-R *(74)* (Fig. 1). IHC using the PAC_1-R antiserum showed a distribution in the brain of PAC_1-R that was similar to the distribution of the mRNA for PAC_1-R *(62)*. Thus, the PAC_1-R antibody has been thought to be sufficiently specific to be used as a tool for studying the distribution of PAC_1-R in the testis. PAC_1-R-LI was demonstrated in spermatids during early developmental stages when the expression of PACAP reaches its peak *(57)*. Intense immunostaining was demonstrated in the acrosome of spermatids, but not in spermatogonia or primary spermatocytes *(57)*.

The studies on VIP transcripts did not reveal the expression of VIP mRNA in the fetal or newborn testis using RT-PCR or Northern blot analysis *(76,77)*. The distribution and relative density of VIP-containing nerves were studied to assess the progression of neuronal changes during the postnatal development of the male genital system from the prepubertal age to adulthood of the rat. Numerous VIP-containing nerve fibers were observed in the cauda epididymis, ductus deferens, accessory glands, and penis of 20-d-old rats *(51,78–80)*. The number of nerves increased in 35-d-old rats whereas no further changes were observed in older adult rats *(80)*. The testicular parenchyma and caput epididymis did not show any immunoreactivity *(81)*. Others have reported that the epididymis was densely innervated by nerve fibers immunoreactive for VIP *(82)*. These data suggest that VIP-containing nerves appear in the genital system after birth and reach full development before the completion of puberty. VIP-containing nerve fibers are visible first in the interstitial area and then spread into the muscular coat of the ducts, glands, and blood vessels of the subepithelial regions *(80)*, suggesting a role for this neuropeptide in the control of epithelial functions. On the other hand, VIP-LI in the adult testicular tissue is very low and unmeasurable with the RIA method *(76,77)*.

In both pubertal and adult rats, VIP-binding sites with high and low affinity were detected in Leydig cells, but the binding sites with low affinity were considerably fewer in the pubertal animals. They increased 10-fold during development, whereas the high-affinity binding sites remained unchanged. It is possible that the low-affinity VIP-binding sites initially identified represented PAC_1-R-binding sites, while the high-affinity binding sites, probably $VPAC_1$-R or $VPAC_2$-R, do not change drastically *(83)*. VIP at low concentrations does not activate PAC_1-R but activates VPAC-Rs, and it stimulates cyclic adenosine monophosphate (cAMP) and testosterone production in fetal testicular cells *(76,77)*. The human VIP-R gene is expressed selectively in various human tissues including the testis *(84)*. Using RT-PCR and Northern blot analysis, El-Gehani et al. *(76,77)* could not detect any mRNA for $VPAC_1$-R in the fetal or newborn testis, although it was expressed in the adult brain and liver. By contrast, $VPAC_2$-R was detected by RT-PCR in the fetal and newborn testes as early as embryonic d 15.5 (E15.5) *(76,77)*. These results suggest that VIP, from intrinsic and extratesticular sources, may regulate fetal testicular steroidogenesis through $VPAC_2$-R as early as E15.5, which may have a physiologic significance because the onset of fetal testicular steroidogenesis occurs at an age (E15.5–19.5) before the onset of pituitary LH secretion. Northern blot analysis

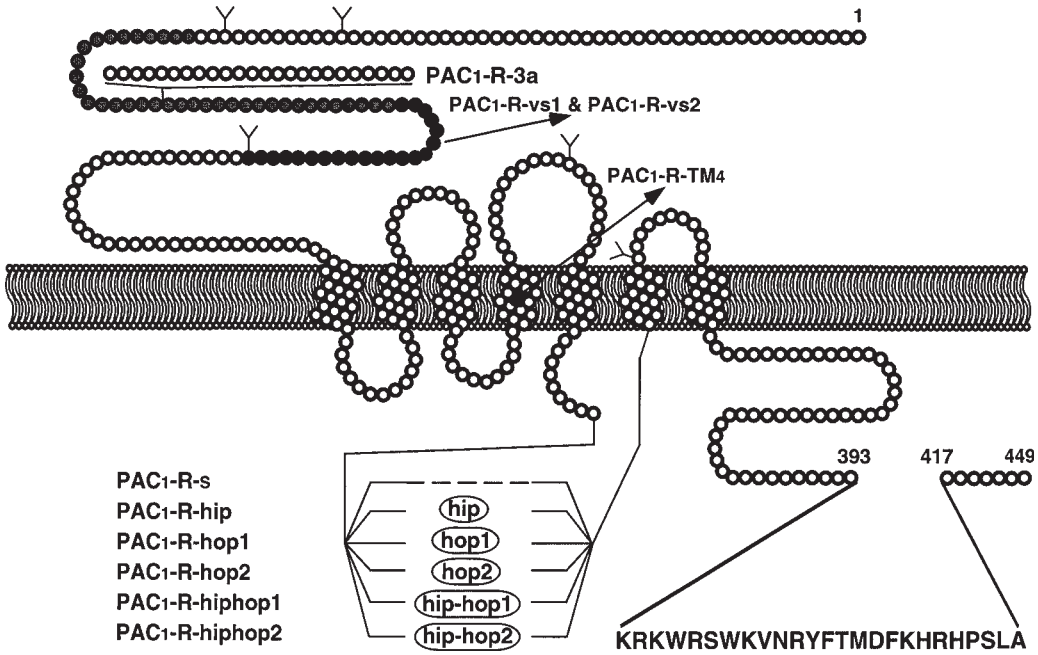

Fig. 1. Schematic illustration of the structure of PAC_1-R. Ten variants of PAC_1-R resulting from alternate splicing have been identified. Six variants differ from one another by the presence or absence of the hip, hop1, or hop2 cassette inserted at the C-terminal region of the third intracellular domain. Each of these six variants is coupled to different signaling pathways. The subtype PAC_1-R-vs lacks 21 amino acids in the N-terminal extracellular domain, as shown by the dark circles. In contrast to PAC_1-R-s, which has a greater affinity for PACAP38 than PACAP27, PAC_1-R-vs binds to PACAP38 and PACAP27 with a similar affinity. The efficacy of adenylate cyclase and phospholipase C (PLC) activations by interaction with PAC_1-R-vs is also similar for PACAP38 and PACAP27. Another subtype of PAC_1-R, PAC_1-R-TM4, differs from the other PAC_1-R subtypes by two amino acid substitutions and deletions (CVTV to SA) in the fourth transmembrane domain, as shown by the arrow. Two additional amino acid substitutions, D136N and N190D, have also been found (not shown). PAC_1-R-TM4 is coupled to an increase in $[Ca^{2+}]_i$ by enhancing Ca^{2+} influx through L-type Ca^{2+} channels, but not with activation of adenylate cyclase or PLC. A testis-specific splice variant with the insertion of 24 amino acids (exon 3) in the N-terminal extracellular domain was recently cloned and named PAC_1-R(3a). The PAC_1-R-specific antiserum 93093-3 was generated against a synthetic peptide corresponding to 25 amino acids in the C-terminal intracellular domain.

of total testicular RNA in pre- and postnatal rats of different ages using a cRNA probe for the $VPAC_2$-R showed a very low signal in fetal (E17.5), neonatal (1-d-old), and infantile (7-d-old) testes *(83)*. The level of expression increased clearly between d 7 and 14 postpartum and stayed high thereafter. In adult rats, ISH with a $VPAC_2$-R probe showed strong labeling within some cross-sections of the seminiferous tubuli, whereas other tissues were not labeled *(85)*. The presence of both high- and low-affinity binding sites for VIP was identified on isolated Leydig cells from the adult rat testis *(83)*. The low-affinity VIP-binding sites initially identified probably represented PAC_1-binding sites. Consequently, the approx 10-fold increase in the low-affinity binding sites during development probably reflects an increase in PAC_1-R expression, while the high-affinity binding sites ($VPAC_2$-R) did not change drastically.

The testicular GHRH transcript and peptide are developmentally regulated. No GHRH mRNA is detected in testes from E19 rats, but it is present in low amounts on P2 and increases gradually to P21 *(86)*. The expression of the GHRH gene increases more dramatically beginning on P21 and reaches adult levels by P40 *(86)*. IHC studies have confirmed that GHRH-LI was found from P4 and the number of the positively stained cells increased with age *(42)*. In adult rat, high levels of a GHRH-LI are present at both the level of the gene transcript and protein product, with the mRNA being substantially larger than the GHRH transcript from the hypothalamus *(47)*. GHRH mRNA has been localized in developing spermatogenic cells by ISH and Northern blot analysis *(87)*. GHRH-LI was also demonstrated in the rat spermatozoa by IHC *(55)*. More recently, Fabbri et al. *(42)* reported that GHRH is present in the interstitium and the germ cells of the rat testis. GHRH-LI was demonstrated in the acrosomal region of early and intermediate spermatids at stages III–VI of the seminiferous epithelium cycle, but not in Sertoli cells *(42)*. There seems to be some discordance in the immunohistochemical localization of GHRH among reports.

GHRH could significantly accelerate maturation in oocytes from both estrogen-primed and unprimed rats *(88)*, and

PACAP was also able to affect granulosa cells and oocyte meiotic maturation *(89)*. These findings suggest a cooperative function in maturation of ova by both GHRH and PACAP. Along with the supporting data on the impaired fertility of PC4-null spermatozoa *(90)*, it is also possible that both GHRH and PACAP regulate sperm maturation cooperatively. However, it is unlikely that biologically active neuropeptides are released during the acrosomal reaction, because PACAP-LI is only transiently expressed in round and elongated spermatids, and not in spermatozoa. Although a report indicated that GHRH-LI was demonstrated in spermatozoa *(55)*, the acrosome is well known for nonspecific staining in IHC. It is necessary to reconfirm that GHRH is indeed expressed in the acrosome of spermatozoa. Although no direct evidence is available at this time, it is possible that a regulatory molecule(s) generated or activated by these peptides in the acrosome, including various enzymes, is released during the acrosome reaction.

Moretti et al. *(91)* also found GHRH-LI in human testis. They noted intense staining of the interstitial compartment with localization to the Leydig cells. The presence of GHRH-LI in human testicular tissue has been further analyzed by means of an enzyme-linked immunosorbent assay and Northern blot analysis of adult testicular extracts *(92)*. Both methodologies confirmed that the human testis is an extrahypothalamic site of expression for both GHRH-LI and the GHRH gene. Recently, the GHRH-R 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 expression of the GHRH-R in a dose-dependent manner *(93)*. It has also been shown that GHRH acts on the Leydig cells of adult rat via a VIP receptor, stimulating cAMP production *(71)*. A novel peptide, the putative 30 amino acid C-terminal peptide of the GHRH precursor, called GHRH-related peptide, has been found in abundance in adult rat germ cells with IHC *(94)*. Specific staining predominated in stage IV seminiferous tubules, in pachytene spermatocytes, and in the acrosomes of spermatids *(94)*. Other reports indicate that GHRH or GHRH-related peptide may, in fact, act on Sertoli and Leydig cells through specific receptors *(95,96)*.

The production of secretin and secretin-R transcripts was localized within adult mouse and rat testis by comparative RT-PCR analysis of RNA from whole testis, and germ, Sertoli, and Leydig cells *(14,58)*. RT-PCR analysis indicated that both secretin and secretin-R mRNA are primarily expressed in germ cells *(58)*. Sequence comparisons of cloned secretin and secretin-R PCR products showed 100% identity with the reported sequences. ISH histochemistry to determine the cellular localization of the secretin and secretin-R transcripts was performed within the testis *(58)*. Secretin transcripts were observed in low abundance in many germ cell types but were most numerous during step 19 over elongating spermatids in stages VII and VIII tubules *(58)*. Low levels of secretin transcripts were expressed over developing spermatids within tubules of all stages and late advanced spermatocytes. The transcripts for the secretin-R were also stage specifically expressed in approximately the same cell types as were the secretin transcripts, but the labeling was greatest in round spermatids (steps 6–8) *(58)*. No transcript signal was discerned in Sertoli or interstitial cells. Spermatogenic cells contain the most abundant transcripts for stage-specific expression of both secretin and the secretin-R. The transcripts for secretin are expressed in more mature spermatogenic cells than those that express the secretin-R mRNA. These results indicate that the sites and the stages of expression of testicular PACAP, GHRH, and secretin overlap (Fig. 2).

Regulation of Transcriptional and Posttranscriptional Processes of Testicular PACAP, VIP, GHRH, Secretin, and Their Receptors

Many proteins and peptides are synthesized initially as a large precursor molecule that requires highly specific intracellular proteolysis to generate the biologically active end products. In rats, PACAP is synthesized as a 175 amino acid precursor *(97)*. Proteolytic processing of the precursor is initiated by endoproteolytic cleavage at sites marked by basic amino acids. A novel family of proteases has been identified that appears to represent these processing endoproteases. The proteases found thus far are all related to the yeast dibasic-specific endoprotease kex2 and include prohormone convertase 2 (PC2), PC3/PC1, PC4, furin/PACE, PACE4, PC5/PC6, and PC7/PC8/LPC *(98)*. All are Ca^{2+}-dependent serine/threonine proteases with catalytic domains organized similarly to bacterial subtilisin. The emerging characteristics of these endoproteases, including their tissue-specific expression, subcellular localization, and cleavage site selectivity, indicate that members of this family arose during evolution to process a diverse group of functionally distinct precursors in a highly specific, compartmentalized, and regulated fashion. PACAP is a neuropeptide that is synthesized in neuroendocrine cells. In the regulated processing pathway characteristic of neuroendocrine cell types, precursors are packaged into granules where they are proteolytically processed and stored for later release in response to the appropriate extracellular signals. Studies with PC2 and PC3/PC1 indicate that these proteases function specifically within this pathway. Both enzymes are expressed only in neuroendocrine tissues, and PC2 has been localized intracellularly to the secretory granules *(98)*.

However, the testicular cells do not express either PC1 or PC2. With ISH, furin and another kex2 homolog, PC4, were found to be expressed in the germ cells of both mouse and rat testis *(99,100)*. Among the specific PCs for various peptides in mammals, PC4 is a precursor-processing endoprotease specifically expressed in round spermatids *(99, 101)*. It is noteworthy that the expression of PACAP mRNA

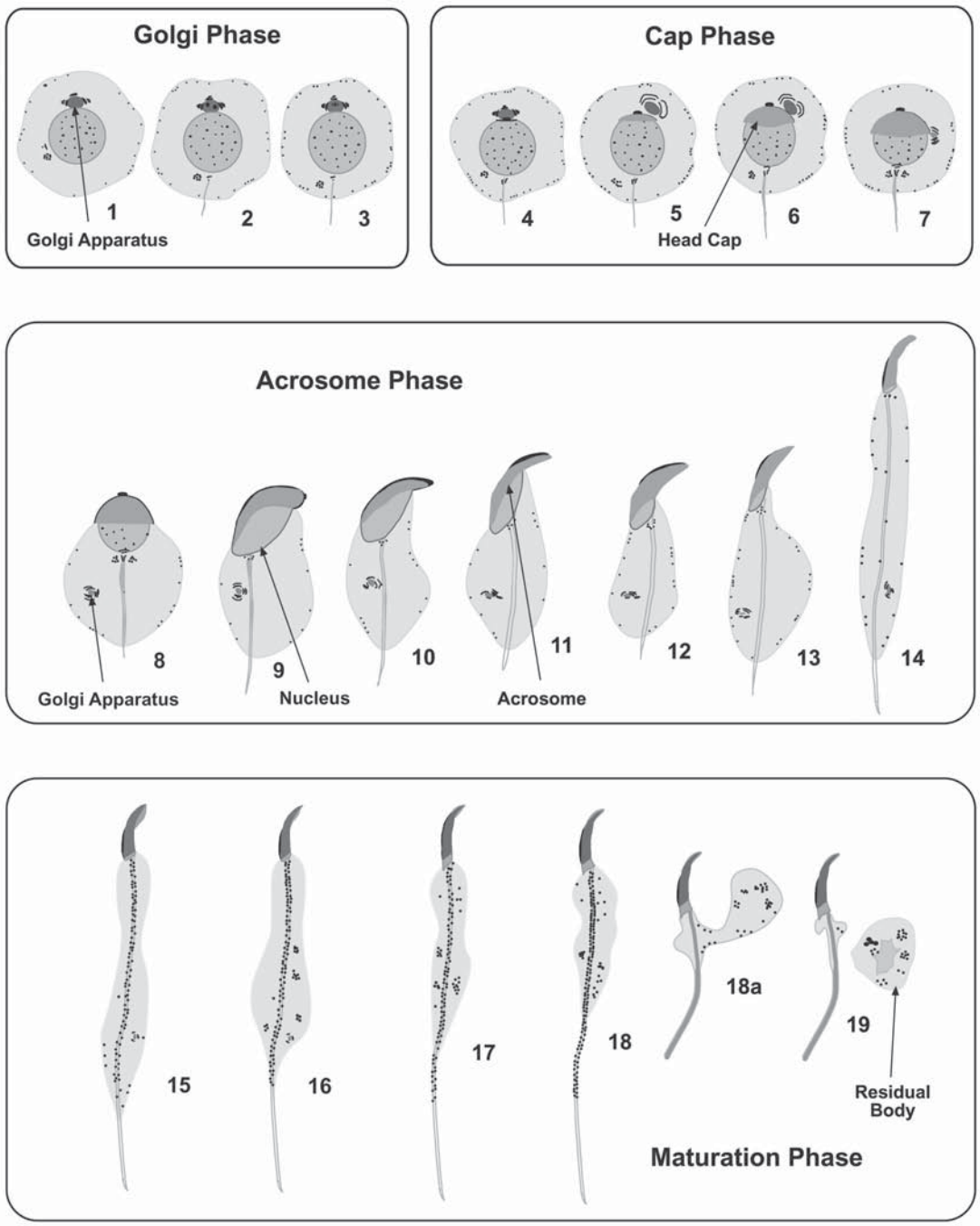

Fig. 2. Nineteen developmental stages (1–19) of spermatids in rat seminiferous tubule. Approximately 12 to 13 d are required for the appearance of the first 14 stages. PACAP and GHRH, and most likely also secretin, as well as their cognate receptors are expressed in round and elongated spermatids, especially during the cap and acrosome phases. Note that the sites and the stages of expression of testicular PACAP, GHRH, and secretin overlap.

is developmentally regulated and reaches its highest level in round spermatids *(102)*. In view of the nearly simultaneous expression of PC4 transcripts and PACAP-LI in spermatids during spermatogenesis *(99)*, these observations led to the speculation that PC4 is involved in the processing of PACAP precursors in the testis. To investigate this possibility, rat pituitary GH_4C_1 cells were stably transfected with human PACAP cDNA, and some of these cells were cotransfected with mouse PC4 cDNA. The acid extracts of the cells were fractionated by RP-HPLC. Each fraction was examined for PACAP-LI using three antisera that recognize the PACAP precursor, PACAP38 and/or PACAP27. PACAP-LI in the cells transfected with only PACAP cDNA did not coelute with synthetic PACAP38 or PACAP27. However, PC4-cotransfected cells showed marked peaks of PACAP-LI that eluted with the retention times appropriate for either PACAP38 or PACAP27 *(103,104)*. Moreover, Western blot analysis revealed immunostained bands, corresponding to

the M_r for PACAP38 and PACAP27, in the PC4-cotransfected cells *(103)*. Bioactivity, as determined by stimulation of cAMP production in pituitary cell cultures, was found only in the extracts of PC4 cotransfected cells *(103)*. These results indicate that the PACAP precursor in the testis is a substrate for PC4 *(105)*.

Mbikay et al. *(90)* reported that PC4 knockout (KO) male mice exhibited severely impaired fertility, although spermatogenesis appeared to be morphologically normal. To determine whether PC4 is the sole processing enzyme for the PACAP precursor in the testis, testicular extracts from the wild-type (WT) ($Pcsk^{+/+}$) and PC4-null ($Pcsk^{-/-}$) mice were analyzed for PACAP38 and PACAP27, and PACAP mRNA using RP-HPLC combined with specific RIAs and RNase protection assays, respectively. Neither PACAP38 nor PACAP27 was expressed in the testis of PC4-null mice *(106)*. On the other hand, PACAP transcripts in the PC4-null mice were considerably increased as compared with the testis of the WT mice *(106)*. This indicates that PC4 is the sole processing enzyme for the precursor of PACAP in the testis. The dramatic increase in PACAP mRNA levels may indicate a compensatory response to the absence of bioactive PACAP. This is similar to the case in hypophysectomized rats in which the PACAP levels in the testes decreased but its message was upregulated *(107)*. It could be speculated that the absence of bioactive PACAP and/or other related peptides in the testis of PC4-null mice could cause the severely impaired fertility without significant changes in spermatogenesis *(106)*. It has been reported that the in vivo fertility of PC4 homozygous null ($Pcsk^{-/-}$) mutant males was also severely impaired in the absence of any evident spermatogenic abnormality *(90)*. In vitro, the fertilizing ability of PC4-null spermatozoa was also considerably reduced. Moreover, eggs fertilized by these spermatozoa failed to grow to a blastocyst, causing early embryonic death *(90)*. These results suggest that PC4 in the male is important for achieving fertilization and for supporting early embryonic development in mice. Since the PACAP precursor remains unprocessed in PC4 KO mice, the impairment of fertilizing ability, rather than spermatogenesis, could be related to the absence of bioactive PACAP in germ cells. It is also possible that the precursors of other testicular peptides, including GHRH and secretin, remain unprocessed in the PC4-deficient animals, and these peptides together with PACAP play critical roles in the control of the regulatory molecules essential for maturation and/or function of spermatozoa.

The effects of hypophysectomy on PACAP-LI and PACAP mRNA expression were investigated in the rat testis to determine whether the expression of testicular PACAP is under the control of the pituitary hormones. The PACAP-LI levels in the testis decreased 2 wk after hypophysectomy *(107)*. IHC also showed a marked reduction of PACAP immunopositive cells without an apparent change in their distribution *(107)*. On the other hand, the PACAP mRNA levels increased 2 wk after hypophysectomy, as determined with an RNase protection assay *(107)*. The increased mRNA levels may represent a compensatory response to the reduction of the mature PACAP in the testis. Replacement therapy with FSH, LH, or testosterone restored the levels of PACAP-LI; the rank order of potency was FSH, testosterone, and LH *(107)*. The elevated PACAP mRNA levels in the hypophysectomized rats were also reduced by FSH, testosterone, and LH with the same rank order of potency. The greatest effect was found with the combined administration of FSH and LH *(107)*. These findings suggest that the expression of PACAP in the testis is controlled by the levels of the pituitary hormones, especially by FSH, probably via the Sertoli cells. The decreased levels of PACAP peptide with increased levels of its mRNA in the testis after hypophysectomy resembles the similar changes in PC4-null mice *(106)*. It is possible that hypophysectomy reduced the level of bioactive PACAP peptide by decreasing a regulatory molecule used for translation or posttranslational processing of the PACAP precursor such as a PC. One possibility is that hypophysectomy reduces the expression of bioactive PC4, which is the sole processing enzyme for the PACAP precursor in the testis *(106)*. The mRNA for the PACAP precursor in the testis is approx 1.5 kb shorter than that cloned from the rat hypothalamus and cortex *(108,109)*. During spermatogenesis, a testis-specific promoter in germ cells regulates the expression of the PACAP gene *(102)*. The testicular PACAP transcript has a truncated 3' noncoding region and a unique 5' end region that are not observed in the brain PACAP transcript; this novel mRNA is the result of alternative splicing and posttranscriptional modification (polyadenylation) of the preproPACAP mRNA *(110)*.

These observations imply that the conformation of the testicular PACAP precursor could be different from that of the brain PACAP precursor. This smaller form of PACAP mRNA is also present in human, murine, and bovine testis *(109)*. A very recent report by Cummings et al. *(65)* described four novel PACAP mRNA splice variants in the mouse, with three of these having alternate 5'-untranslated regions (UTRs), whereas the fourth, only found in lymphocytes, was altered within the coding region, resulting in tissue and stage-specific transcripts.

In 1993, six laboratories independently reported the cloning of cDNAs encoding for the PAC_1-R and the deduced amino acid sequences of the receptor and its subtypes *(72, 111–115)*. The PAC_1-R cDNA, which codes for a 495 amino acid G protein–coupled heptahelical receptor, exhibits a high degree of sequence similarity with the VIP, GHRH, glucagon, gastric inhibitory peptide, glucagon-like peptide-1, and secretin receptor cDNAs *(116)*. Unexpectedly, this family also comprises receptors for parathyroid hormone, calcitonin, and corticotropin-releasing factor, with sequence identities ranging between 30 and 50%. By contrast, they display a low overall sequence identity (<20%) with other receptors of the superfamily of G protein–coupled receptors.

Alternative splicing of the PAC_1-R gene also occurs in the UTR and could represent a regulatory mechanism involved in 5'-tissue-selective expression of the gene and/or in mRNA stability. It has been found that PAC_1-R and $VPAC_2$-R transcripts are expressed at significant levels in the adult rat testis, while mature human spermatozoa were shown to express a high level of $VPAC_1$-R mRNA (unpublished observation) *(76,77)*.

At least 10 subtypes of the PAC_1-R resulting from alternate splicing have been cloned so far (Fig. 1). Each subtype is coupled to specific signaling pathways, and its expression is either tissue or cell specific. Six variants resulting from alternative splicing in the third intracellular loop have been identified *(114)*. The splice variants are characterized by the absence of the third intracellular loop (short variant, also named the null variant) or presence of either one or two cassettes of 28 (hip or hop1 variant) or 27 (hop2 variant) amino acids. The presence of the hip cassette impairs adenylate cyclase stimulation and abolishes PLC activation, suggesting that the various cassettes are involved in second-messenger coupling. In the brain and pituitary, the short variant is the most abundant form, whereas the PAC_1-R hop1 variant predominates in the testes and adrenal gland *(114)*. Moreover, two very short splice variants of PAC_1-R (PAC_1-R-vs1 and PAC_1-R-vs2) displayed either a 21 or 57 amino acid deletion in the N-terminal extracellular domain, revealing similar high binding affinities, and adenylate cyclase– and PLC-stimulating potencies for PACAP38 and PACAP27 *(117,118)*. By comparison, PACAP38 had a substantially higher binding affinity for the PAC_1-R short (PAC_1-R-s) variant than PACAP27 and was more potent as an activator of PLC. Another PAC_1-R variant termed PAC_1-R-TM4 has been cloned from the cerebellum *(117)*. This receptor differs from the PAC_1-R-s by discrete sequence substitutions located in transmembrane domains II and IV. Surprisingly, activation of PAC_1-R-TM4 has no effect on adenylate cyclase or PLC activity but causes calcium influx through L-type voltage-sensitive calcium channels. A testis-specific splice variant with the insertion of 24 amino acids (exon 3) in N-terminal extracellular domain was recently cloned and named PAC_1-R-3a *(119)*. The transcripts for PAC_1-R-3a are preferentially expressed at the highest levels in Sertoli cells and at lower levels in round spermatids of the seminiferous tubules and have a sixfold higher binding affinity for PACAP38 than for PACAP27.

The $VPAC_1$-R is present in the genital organ, but not in the testis, while $VPAC_2$-R appears to be present in the testis *(76,77)*. The human $VPAC_2$-R gene spans approx 22 kb and is composed of 13 exons ranging from 42 to 1400 bp and 12 introns ranging from 0.3 to 6.1 kb *(84)*. The $VPAC_2$-R mRNA detected in human tissues by Northern blot analysis was about 3.5 kb, which is in good agreement with a previous report *(120)*.

The partially purified testicular GHRH is capable of stimulating GH secretion from cultured anterior pituitary cells in a dose-dependent manner. The GHRH mRNA found in the rat testis contains an exon 1 sequence that is different from that found in hypothalamus or placenta, and the initiation of GHRH transcription in the testis begins approx 700 bp upstream of that in the placenta and 10.7 kbp upstream of that in the hypothalamus *(121)*. Thus, the GHRH transcripts in each tissue have distinct exon 1 sequences and may use different promoters, suggesting that an as-yet unidentified spermatogenic-specific transcription factor may bind to the promoter region of the testicular GHRH gene to regulate its expression *(121)*. As in other species, the bovine GHRH-R, which has been cloned recently, encodes a 423 amino acid protein containing the seven hydrophobic domains that are characteristic of a G protein–coupled receptor. The expression of the GHRH-R was detected in the testis and ovary by RT-PCR *(122)*.

The nucleotide sequence analysis of the cDNA has revealed that the rat secretin-R consists of 449 amino acids with a calculated M_r of 48,696 *(44)*. The secretin-R also contains seven-transmembrane segments, and belongs to a family of the G protein–coupled receptors. However, the amino acid sequence of the secretin-R has no significant similarity to those of other G protein–coupled receptors. It has been reported that the human secretin-R encodes a 440 amino acid, 50-kDa, G protein–coupled receptor that has 80% sequence identity with the rat secretin-R and 37% sequence identity with the human $VPAC_1$-R. Northern blot analysis of human tissue mRNA found trace levels of a 2.1-kb secretin-R transcript in the human testis *(75)*.

Effects of PACAP, VIP, GHRH, and Secretin on Testis

In the golden hamster, inhibition of PACAP with a specific PACAP receptor antagonist inhibits sperm motility in vitro in a dose-dependent manner *(123)*. In the epididymal epithelium of rat, PACAP stimulates chloride secretion, which is important for sperm activation *(124)*. The occurrence of PACAP-LI in epididymal tubules indicates that PACAP is locally synthesized and thus may act as a paracrine regulator of sperm maturation *(124)*. The epithelium-derived PACAP may also stimulate epididymal spermatozoa that have lost the ability to synthesize PACAP but still possess PACAP-binding sites *(68)*. The effects of PACAP on protein synthesis in both spermatocytes and spermatids are mimicked by dibutyryl cAMP *(125)*. Two hundred nanomolar PACAP38 or VIP, or 1 m*M* dibutyryl cAMP, significantly increased spermatocyte-secreted and intracellular proteins but decreased synthesis of both spermatid-secreted and intracellular proteins *(125)*. However, these doses are pharmacologic and the physiologic significance of this finding is questionable. Major changes in the cAMP-dependent signal transduction pathway take place during cell differentiation. The development of a postmeiotic germ cell into a mature spermatozoon is a striking example of the mor-

phologic and functional changes that occur in a cell during differentiation *(126–133)*. Although several reports have suggested that cAMP-mediated mechanisms are involved in the regulation of gene expression *(134)*, the exact role of the cAMP-dependent pathway during spermiogenesis is largely unknown. Differentiation of the germ cells is accompanied by the expression of a unique adenylate cyclase in the particulate and soluble fractions of spermatids *(135)*.

PACAP stimulates cAMP accumulation in Sertoli cells from 15-d-old rats in vitro, and this property declines with the increasing age of the donor animals. The effect is additive with submaximal, but not maximal, effective doses of FSH *(69)*. In cultured Sertoli cells, PACAP increased the concentration of the cAMP and stimulated lactate, estradiol, and inhibin secretion *(69)*. Since the expression of PACAP is controlled by FSH *(107)*, the effect of FSH on the Sertoli cells could be at least partly mediated by PACAP expressed in the germ cells. The PACAP-mediated stimulation of cAMP accumulation in the Sertoli cells is not altered by a VIP antagonist, suggesting that PACAP is acting via the PAC_1-R on these cells. PACAP also induces a dose-dependent stimulation of testosterone secretion from isolated rat Leydig cells in vitro *(70,136,137)* and activates or inhibits protein synthesis in spermatocytes or spermatids, respectively *(125)*. In fetal Leydig cells, PACAP activates both adenylate cyclase and PLC through an interaction with the PAC_1-R *(70)*. The effect of PACAP on Leydig cells may also be mediated via a novel receptor subtype of PAC_1-R coupled to a sodium channel through a pertussis toxin–sensitive G protein *(136)*. PACAP also affects prostaglandin E_2 levels and steroidogenesis in the fetal testis *(70,138)*. However, PACAP does not affect adenylate cyclase or PLC in adult isolated cells while stimulating testosterone secretion *(70)*. These data suggest the presence of PACAP receptors on Leydig cells at least during development. Initial radioligand binding and activation studies also suggested the presence of such receptors in Leydig cell membranes *(71)*. Despite the distinct effects of PACAP on the testicular cells, direct proof of its in vivo function is still lacking**.** Furthermore, analyses of ligand binding and signaling functions of testis-specific PAC_1-R, the PAC_1-R-3a, in the membrane preparations from stably transected HEK293 cells expressing the two receptor isoforms revealed a sixfold increase in the affinity of the PAC_1-R-3a for PACAP38, and alterations in its coupling to both cAMP and inositol phosphate signaling pathways relative to the WT PAC_1-R *(119)*. The transcript of this subtype of PAC_1-R is mainly expressed in Sertoli cells and germ cells, not in Leydig cells.

Recently, several laboratories reported various findings in PACAP KO mice *(35–37)*. However, their phenotypes differed from each other considerably, possibly owing to different regions of the targeted portion of the PACAP gene. Gray et al. *(35)* reported that most of the homozygous PACAP-null mice (with targeted disruption of both PACAP and the PACAP-related peptide) did not survive beyond 2 wk of age. Genotyping of 270 offspring from heterozygous crosses showed the expected Mendelian ratio of 21% WT, 52% heterozygotes, and 27% homozygotes, indicating that $PACAP^{-/-}$ mice did not die *in utero*. PACAP-null mice appeared normal at birth with no obvious signs of neuropathology or overt developmental abnormalities. Although only 11% of the PACAP-null mice survived when raised at 21ºC, nearly all (79%) of these animals could survive when raised at 24ºC, indicating that thermogenesis of PACAP-deficient animals in the cold environment is impaired *(139)*. Hashimoto et al. *(36)* generated PACAP KO mice in which only PACAP38 (but not the PACAP-related peptide) was deleted from the mouse PACAP gene *(140)*. Mendelian segregation of pup genotypes from heterozygous breeding was observed at birth, with a genotypic distribution of 23.9, 49.6, and 26.5% for $PACAP^{-/-}$, $PACAP^{+/-}$, and WT mice, respectively. However, $PACAP^{-/-}$ pups had a high mortality rate, and the genotypic distribution on weaning was 15.6, 54.8, and 29.6% for $PACAP^{-/-}$, $PACAP^{+/-}$, and WT mice, respectively, suggesting a significant loss of $PACAP^{-/-}$ pups. Homozygous mating of PACAP-deficient mice resulted in fewer successful pregnancies, thereby yielding few pups. However, no systematic study on the fertility of PACAP-deficient mice was reported.

Extensive neonatal mortality was also seen in PAC_1-R KO mice. A Mendelian distribution of pup genotypes from PAC_1-R heterozygous mating was observed at birth *(34)*. However, at weaning, a loss of PAC_1-$R^{-/-}$ mice became very noticeable, with a genotypic distribution of 9, 59, and 32% for PAC_1-$R^{-/-}$, PAC_1-$R^{+/-}$, and WT mice, respectively. This result indicated a 60% loss of PAC_1-$R^{-/-}$ pups during the first 4 wk after birth. This high mortality rate remains to be explained. No significant difference in the death rate among the three genotypes was observed thereafter. PAC_1-R KO females displayed decreased fertility, whereas male fertility was normal *(141)*. IHC on pituitary sections showed that FSH, LH, and prolactin synthesis were not affected in KO mice. Moreover, the pituitary-gonadal axis responded properly to an acute fasting test in these KO mice. These reports suggest that the absence of PACAP or PAC_1-R alone may not severely impair male fertility. On the other hand, PC4 KO mice, which presumably lack not only PACAP but also other peptides in the testis, develop severely impaired fertility, without any apparent morphologic defect in spermatogenesis *(90)*. These findings imply that the testicular peptides processed by PC4 play cooperative roles in the regulation of the functional maturation of spermatozoa, but not of spermatogenesis.

The effects of VIP on Leydig cells are shown in the fetal testis by the stimulation of cAMP production at a dose of 10^{-9} *M*, whereas a dose as low as 10^{-12} *M* stimulated testosterone production *(142,143)*. This suggests that VIP at low doses may stimulate testosterone production using second-

messenger pathways other than the cAMP pathway in the fetal testis. PACAP, VIP, PACAP antiserum, or VIP antiserum was injected intratesticularly to examine the local effects of PACAP and VIP on testicular function in immature and adult rats. In 9-d-old hemicastrated rats, PACAP or VIP decreased basal testosterone secretion. In 22-d-old hemicastrates, VIP but not PACAP reduced compensatory testicular hypertrophy; however, neither PACAP nor VIP altered steroidogenesis *(144)*. The administration of a VIP antiserum to this age group increased testosterone production and enhanced compensatory testicular hypertrophy *(144)*. In adult hemicastrates, neither the peptides nor the antisera influenced steroidogenesis. This finding suggests that VIP inhibits the stimulatory effects of the pituitary gonadotropins on the testis in immature rats, but not in the adult animals. On the other hand, a portion of the atrial natriuretic peptide (ANP) binding sites can be displaced by high concentrations of VIP-related neuropeptides, suggesting that VIP- and ANP-related peptides may interact to control cellular functions *(18–20)*. Moreover, effects of natriuretic peptides on testicular steroidogenesis have recently been reported *(21)*. Furthermore, VIP treatment decreased the testicular histamine levels to baseline when the animals were subjected to immobilization or cold stress, which caused a significant increase in histamine levels in the testis *(145)*. VIP may be involved in the protection of normal testicular function under stress conditions *(146)*.

It has been shown that PACAP dose dependently relaxes noradrenaline- and electrically contracted preparations of the human cavernous tissue, and that VIP and PACAP induce vasodilation and stimulate the testicular blood flow of adult rat *(142,143)*, suggesting that these peptides are involved in the induction and maintenance of penile erection *(147, 148)*. In line with this finding, a stearic acid VIP conjugate has been shown to increase the copulatory activity and penile reflex in castrated, testosterone-treated rats *(149,150)*. Moreover, inhibition of VIP activity with a specific receptor antagonist, or reduction of VIP levels by genetic manipulation, inhibits male sexual behavior/activity in rats *(151)* and mice *(152)*. These results suggest that VIP is not only associated with penile erection, but is involved in sexual behavior/activity as well. On the other hand, the $VPAC_2$-R-null mice have been shown to be normal in general health, social behavior, and breeding patterns *(153)*.

GHRH enhances steroidogenesis and cAMP accumulation induced by gonadotropins in Leydig cells and also enhances FSH-induced cAMP production in Sertoli cells *(42)*. Although GHRH and VIP had no effect on basal steroidogenesis, GHRH acts as a potentiator for the acute gonadotropin stimulation of testosterone production and cAMP generation *(71)*. GHRH has direct actions on the Sertoli cell, including stimulation of cAMP production and c-*fos* mRNA expression *(154)*. While some investigators have suggested that GHRH acts on Leydig cells via a VIP receptor acting through cAMP production, others indicate that GHRH or GHRH-related peptide may act on Sertoli and Leydig cells through specific receptors *(95,96)*. On the other hand, both males and females carrying the GHRH fusion gene are fertile and transmit the gene *(155)*, excluding any severe GHRH-mediated consequences for male reproductive physiology.

The presence of a secretin-R mRNA in round spermatids suggests that secretin acts in these cells and controls the expression of regulatory molecules in these cells *(58)*. Since secretin is also demonstrated in spermatids *(58)*, these findings suggest that both secretin and its receptors are coexpressed in spermatids. This also seems to be the case for PACAP and its receptors in the testicular germ cells *(57)*. However, whether the ligand and its receptors are expressed in the same intracellular site in the same germ cell remains to be investigated.

Conclusion

The testis is a totally integrated cellular system that has an exquisitely interwoven intratesticular network of hormonal regulators. Regulation at an intragonadal level is an integral part of the overall regulation of development and functional control of the male gonad. The interactions among cells within the testis extend beyond the same cell type to also include germ cell–somatic cell interactions. The demonstration of neuropeptides and their receptors in the testicular germ cells and somatic cells suggests that these peptides mediate the communication between the same as well as the different cell types of the testis. The testis also undergoes dramatic changes during its lifetime—both the one-time maturation to puberty and the cycles of spermatogenesis.

Testicular neuropeptides of the PACAP family, except VIP, are expressed in germ cells at a critical time when germ cells are about to undergo their most striking differentiation, which requires numerous regulatory proteins. Therefore, there is a great need for accurate information about how gonadal peptides act as mediators of development and how signaling molecules are used to organize the functional germ cell maturation. Studies using PACAP-deficient, PACAP/VIP receptor–deficient, and GHRH transgenic mice did not specifically address whether testicular functions were altered in those mice. PACAP KO mice did not show either spermatogenic arrest or severe infertility *(35, 36)*. These findings suggest that the absence of PACAP alone may not severely impair male fertility. On the other hand, PC4 KO mice develop severely impaired fertility, without any apparent morphologic defect of spermatogenesis. In PC4-deficient mice, no PACAP is expressed in the germ cells. PC4 is also expressed in the testicular germ cells in which GHRH and secretin are also expressed. It is important to examine whether these peptides are also absent in PC4-deficient animals. VIP expressed in the nerve fibers in the gonads appears to function as a local vasodilator. On

the other hand, the receptors for VIP, GHRH, PACAP, and secretin appear to be expressed in both germ cells and somatic cells, including Sertoli cells and Leydig cells. However, there is still a considerable gap between the sporadic information on these receptors in the testis and their functional significance. PACAP binds both PACAP-specific receptors and VIP receptors, particularly $VPAC_2$-R. GHRH also interacts with VIP receptors in the testis. The findings again suggest an interwoven intratesticular network of hormonal regulators.

In addition to the PACAP/VIP/GHRH/secretin family of related peptides, GnRH, CRH, oxytocin, AVP, TRH, somatostatin, opioid peptides, substance P, and NPY are synthesized in the testis. The list of neuroendocrine hormones and neuropeptides that are produced by the testis continues to grow, but the biologic significance remains largely unknown. It is possible that an alteration in their function during germ cell development or in adult life is responsible for some cases of idiopathic male infertility. In the testis, most genes are transcribed and translated in a manner identical to that identified in other tissues. However, some neuropeptides are produced from testis-specific mRNA transcripts that do not code for neuropeptides and/or are owing to the use of different polyadenylation sites, while others may undergo testis-specific posttranslational modifications. For example, the expression of the gene for PACAP during spermatogenesis is regulated by a testis-specific promoter in germ cells. Additionally, the presence of multiple transcripts of the same gene resulting in receptor splice variants with slightly different structures implies that there may be divergent biologic activities for each translated receptor protein. Variant products often have different specificities and affinities for receptor binding, providing a means to increase the physiologic complexity of the transcription of a single gene. This unique processing of receptor genes may also be owing to the presence of testis-specific processing enzymes.

Overexpression and double/triple targeted disruption of the genes for these neuropeptides, their cognate receptors, and their processing enzymes could help us to determine the physiologic roles of the testicular neuropeptide-mediated signals that are used to communicate within the testis. Further studies will improve our understanding of the physiologic roles of testicular neuropeptides and may eventually guide us toward targeted approaches for the treatment of male infertility.

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