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Stage- and Cell-Specific Expression of Soluble Guanylyl Cyclase Alpha and Beta Subunits, cGMP-Dependent Protein Kinase I Alpha and Beta, and Cyclic Nucleotide–Gated Channel Subunit 1 in the Rat Testis

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ABSTRACT: Several studies suggest that nitric oxide (NO) and cyclic guanosine monophosphate (cGMP) modulate testicular function. In this study, we examined the expression of cGMP-dependent protein kinase G-I (PKG-I), and cyclic nucleotide–gated channel 1 (CNG-1), 2 known mediators of cGMP action, and the expression of soluble guanylyl cyclase (sGC) subunits in the rat testis. Immunohistochemical analysis revealed that the alpha subunit of sGC was expressed in the blood vessels and Leydig cells of adult rat testes. In addition, the sGC alpha subunit was observed in the acrosomal structures of spermatids undergoing the middle and later stages of spermiogenesis, but not in mature spermatozoa. Similar localization and expression patterns were seen for the sGC beta subunit, indicating coexpression of the sGC subunits. PKG-I was expressed in blood vessels and in the acrosomal region of spermatids during the early and middle stages of spermiogenesis but was not observed in Leydig cells or in mature spermatozoa. In contrast to sGC and PKG-I, CNG-1 was expressed only in cytoplasm and the residual bodies of late-stage (17–19) spermatids, with no staining observed in blood vessels and Leydig cells. These results demonstrate that sGC, PKG-I, and CNG-1 are expressed in a stage- and cell-specific manner in the rat testis. The distinct temporal patterns of expression of these components of cGMP signaling pathways suggest different physiological roles for sGC, PKG-I, and CNG-1 in spermiogenesis and steroidogenesis.

Key words: Cyclic guanosine monophosphate, sGC, PKG-I, CNG-1.

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Spermatogenesis, the production of functional sperm cells in the testis, represents a complex process involving specific interaction between the developing germ cells and their supporting Sertoli cells, which is regulated by androgen-producing Leydig cells (for review, see de Kretser, 1995). The process can be separated into 3 distinct phases that, in contrast to oogenesis, occur without interruption: 1) spermacytogenesis, during which spermatogonia undergo mitotic cell division and generate a pool of spermatocytes; 2) meiosis, yielding the haploid spermatids; and 3) spermiogenesis, during which the spermatids undergo an elaborate process of cytodifferentiation before being released as viable sperm into the lumen of the seminiferous tubules. During this cytodifferentiation process, condensation of the nucleus, tail formation, and redistribution of cytoplasmic organelles occur, and the spermatids shed a major part of the cytoplasm. These cytoplasmic remnants, the residual bodies, are eventually phagocytosed by the Sertoli cells. The molecular mechanisms regulating spermacytogenesis and meiosis have been relatively well characterized (Sassone-Corsi, 1997); the molecular basis of spermiogenesis, however, is largely unknown.

The soluble form of guanylyl cyclase (sGC), a dimeric protein consisting of an alpha and a beta subunit, is the main receptor for the signaling agent nitric oxide (NO; Ignarro, 1991; Hobbs, 1997; Mikami et al, 1998; Shi et al, 2004). NO diffuses into the target cells and binds to and activates sGC, resulting in increased levels of the second messenger, cyclic guanosine monophosphate (cGMP; Koesling et al, 1991; Bredt and Snyder, 1994; Inagami et al, 1995). cGMP is known to act on target cells by regulating phosphodiesterase (PDE) activity, by activating cGMP-dependent protein kinase G (PKG), and by regulating cyclic nucleotide–gated channels (CNGs; Hanafy et al, 2001). PDEs metabolize cyclic adenosine monophosphate (cAMP) and cGMP, which are second messengers regulating multiple functions in various cells and tissues (Revelli et al, 2002). PKG is a member of a family of cyclic nucleotide-dependent protein kinases that

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also includes cAMP-dependent protein kinase (PKA). Previous studies have identified 2 forms of PKG (I and II) that are encoded by distinct genes, as well as 2 different isoforms of PKG-I (designated alpha and beta) that are produced by alternative splicing (Wernet et al, 1989; Lohmann et al, 1997; Lincoln et al, 2001). CNG channel activation leads to depolarization of the membrane voltage and to a concomitant increase of cytosolic Ca^{2+}. Native CNG channels are heteromeric complexes consisting of the principal alpha subunits (CNG-1 through -3), which can form functional channels by themselves, and modulatory beta subunits (CNG-4 and -5; Gerstner et al, 2000).

Although much has been learned about the regulation of NO synthase (Boissel et al, 1998; Seo et al, 1999; Igarashi et al, 2001; Revelli et al, 2002), there is scarce data on sGC regulation, despite its critical role in actions such as those mediated by endogenous or exogenous NO (Koesling and Friebe, 1999; Andreopoulos and Papapetropoulos, 2000). Compared with many reports on PDE in the mammalian testis (Manganiello et al, 1995; Fawcett et al, 2000; Lefievre et al, 2000; Middendorff et al, 2000; Yan et al, 2001; Fournier et al, 2003; McCullough, 2003), few studies on PKG and CNG have been analyzed in the testis. Although previous reports demonstrate expression of sGC, PKG-I, and CNG-3 in the adult mammalian testis (Davidoff et al, 1997; Middendorff et al, 1997b; Wiesner et al, 1998), information regarding the regulation of sGC alpha and beta subunits and of PKG-I during spermiogenesis is very limited. Furthermore, expression of CNG-1 in the mammalian testis has not been described.

Therefore, we used immunoblot and immunohistochemical techniques to examine the expression of sGC and 2 known mediators of cGMP signaling, PKG-I and CNG-1, in testicular compartments of rat testes.

Materials and Methods

Antisera

Anti-rabbit sGC alpha 1 serum (Product G4280, Lot 011K4888) and beta 1 serum (Product G4405, Lot 011k4880) were purchased from Sigma-Aldrich Inc (St Louis, Mo). Anti-rabbit PKG-I alpha and beta sera (Product 370652, Lot B41856) were purchased from Calbiochem Inc (Darmstadt, Germany). Anti-rabbit CNG-1 antiserum (Product CNG11-A, Lot 398384A1) was purchased from Alpha Diagnositic International Inc (San Antonio, Tex).

Animals

Intact and young (4-month-old) male rats of the Long-Evans strain were used. Animals were maintained under a 14-hour light, 10-hour dark schedule with food and water available ad libitum. The experimental protocol was approved in accordance with the Guide for the Care and Use of Laboratory Animals prepared by the Institutional Animal Care and Use Committee, Nanjing Agricultural University.

Testes were collected at approximately 1000 hours from 4 male adult rats. One testis of each rat was fixed in 4% paraformaldehyde and processed for immunohistochemical analysis of sGC, PKG-I, and CNG-1. The remaining testis from each rat was snap-frozen and used for protein extraction and subsequent immunoblot analysis.

Immunohistochemistry

After fixation, testes were embedded in paraffin, and 8-μm sections were cut and mounted on slides. The stages of the cycle of seminiferous epithelium and the cell types of spermatids were identified according to Russell et al (1990). Sections were prepared for immunohistochemical analysis similar to our previous report (Shi et al, 2000; Shi and LaPolt, 2003). Briefly, sections were deparaffinized with xylene and rehydrated in graded ethanol before being washed with twice-distilled water. To increase epitope exposure, sections were heated for 15 minutes in sodium citrate buffer (0.01 M, pH 6.0) in a microwave oven. The sections were cooled and washed with 0.01 M phosphate buffered saline (PBS), pH 7.2, and then blocked with 5% bovine serum albumin [BSA] in TBST (20 mM Tris-buffered saline, 0.05% Tween 20, pH 7.5) for 1 hour at room temperature. The sections were incubated overnight at room temperature with a diluted polyclonal antibody against sGC alpha 1 (1:5000) or beta 1 (1:2000) subunit, PKG-I alpha and beta subunit (1:100), or CNG-1 (1:200) developed in rabbits. The binding sites of antibodies were visualized with an ABC Kit Elite and 0.05% 3,3′-diaminobenzidine tetrachloride (Sigma) in 0.01 M PBS, pH 7.2, containing 0.01% H_2O_2 for 10 minutes. Specificity of the antibody was examined with the use of normal rabbit serum instead of primary antibody. The sections were counterstained with hematoxylin and mounted with coverslips. Relative levels of immunostaining between animals and cell types were evaluated by 3 independent observers and repeated at least 4 times. Results described represent consistently observed patterns of immunostaining.

Immunoblot Detection of sGC Alpha and Beta, PKG-I Alpha and Beta, and CNG-1 Subunits

To confirm the presence of sGC alpha and beta subunits, as well as PKG-I and CNG-1 subunits, in rat testes, protein was extracted from whole frozen testes with RIPA lysis buffer (50 mM Tris-HCl, pH 8, 150 mM NaCl, 0.5% NP-40, 20% glycerol, 25 mM benzamidine, 0.5 μg/mL leupeptine, 0.7 μg/mL pepstanin A, 2 μg/mL aprotinin, 10 μg/mL trypsin inhibitor) in a Dounce homogenizer (FLUKO Co Ltd, Shanghai, China). After homogenization, samples were centrifuged for 20 minutes at 14 000 × *g*. The supernatant was separated, and protein concentration was determined by a modification of the Bradford method (Biorad Laboratories, Hercules, Calif) with BSA standards, and microplate absorbance readings were made at 595 nm. A homogenized protein sample (20 μg) was run on a 7.5% sodium dodecyl sulfate polyacrylamide gel, followed by transfer to nitrocellulose blots. Then blots were cut into individual lanes and blocked with 5% BSA in TBST buffer, followed by washing (Shi et al, 2004). Blots were then incubated with a diluted primary antibody (the

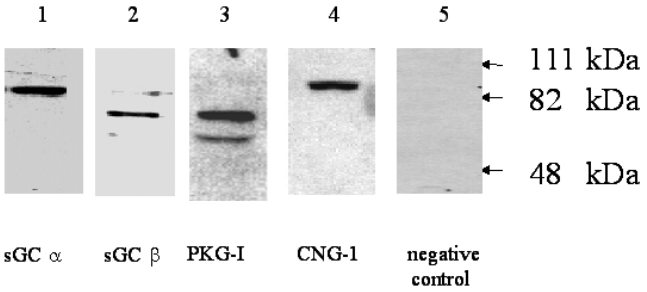

Figure 1. Expression of soluble guanylyl cyclase (sGC) alpha and beta, cGMP-dependent protein kinase G-I (PKG-I) alpha and beta, and cyclic nucleotide–gated channel 1 (CNG-1) subunit proteins in whole rat testis. Protein homogenates from testis (20 μg/lane) were fractionated on sodium dodecyl sulfate polyacrylamide gel electrophoresis gels and transferred to nitrocellulose, followed by immunoblot analysis with antisera specific to sGC alpha and beta, PKG-I alpha and beta, and CNG-1 subunits. For the negative control, immunoblot analysis was performed without the primary antisera. The migrating positions of the molecular mass standards are shown on the right.

same antibodies as previously described in the immunohistochemistry) that detects sGC alpha (1:10 000), sGC beta (1:5000), PKG-I alpha and beta (1:750), or CNG-1 (1:500) subunits in 1% BSA in TBST. Blots were washed again and incubated with horseradish-peroxidase conjugated goat anti-rabbit IgG, followed by washing and detection of immunoreactivity by chemiluminescence detection methods (Pierce Biotechnology, Rockford, Ill). Blots were then used to expose x-ray film to visualize immunoreactive signals.

Results

Expression of sGC Alpha and Beta, PKG-I Alpha and Beta, and CNG-1 Subunit Proteins in Rat Testes

Immunoblot analysis of protein extracts from adult rat testes with a polyclonal antibody against alpha or beta subunits of sGC detected a main immunoreactive band of 82 or 72 to 76 kd (Figure 1, lanes 1 and 2), which corresponded to the reported molecular mass of these subunits (Koesling et al, 1991). These findings are consistent with our previous report on ovary and granulosa cells (Shi et al, 2004). When blots were incubated with primary antisera against PKG-I alpha and beta, 2 immunoreactive bands were observed (Figure 1, lane 3). The lower molecular mass band (~76 kd) corresponded to the PKG-I alpha (Kumar et al, 1999), whereas the higher band (~80 kd) corresponded to the larger, PKG-I beta subunit (Wolfe et al, 1989). When blots were incubated with primary antisera against CNG-1, one immunoreactive band with an apparent molecular mass of approximately 90 kd (Figure 1, lane 4) was detected. This band was larger than the reported molecular band of this subunit in brain (Bradley et al, 1997). Specificity of immunoreactivity was confirmed by omission of the primary antisera, which resulted in the absence of any bands (Figure 1, lane 5).

Localization of sGC in the Testis

Immunohistochemical analysis revealed that the alpha subunit of sGC was first observed in the acrosome of spermatid only later than stage 7. This pattern is normally followed in stages 8 through 19 by an increased and high expression in acrosomes of spermatids. Leydig cells and blood vessels are moderately stained with the sGC alpha subunit, but mature spermatozoa are not stained (Figure 2A; Table). Similar localization and expression patterns were seen for the sGC beta subunit. This finding indicates that the sGC subunits are coexpressed (Figure 2B; Table).

Localization of PKG-I in the Testis

Immunohistochemical analysis revealed that PKG-I was first observed in the small proacrosomal vesicles of spermatids in stages 2 through 3. This pattern was followed in stages 4 through 6 by increased expression in the acrosome vesicle of spermatids, with the highest expression levels observed in the acrosomal in stages 7 and 8. From stages 9 through 16, expression decreased, and most acrosomes showed negative staining by stages 17 and 18. Expression ceased by stage 19. Blood vessels in rat testis continued to stain, but Leydig cells and mature spermatozoa showed negative staining (Figure 2C; Table).

Localization of CNG-1 in the Testis

In contrast to sGC and PKG-I, CNG-1 was first observed in the cytoplasm at stage 17 and stained strongly at cytoplasmic droplets or residual bodies of spermatid 19 (Figure 2D and E; Table). Mature spermatids left some irregular residual bodies that stained intensively with CNG-1 in tubes IX, demonstrating that CNG-1 remained in the rumen of the tubes (Figure 2E). No positive staining for CNG-1 was observed in blood vessels, Leydig cells, or mature spermatozoa (Figure 2D and E).

Discussion

This study demonstrates that the testicular expression of the sGC alpha and beta, PKG-I alpha and beta, and CNG-1 subunits is stage- and cell-type–specific. The distinct temporal expression patterns of these components of the cGMP signaling pathways suggest different physiologic roles for these subunits in spermiogenesis and steroidogenesis. PKG-I is expressed in the acrosomal region of spermatids during the early and middle stages of spermiogenesis, whereas sGC alpha is expressed in the acrosomal structures of spermatids undergoing the middle and later stages of spermiogenesis. These findings indicate that both PKG-I and sGC alpha and beta subunits play important and different functions during acrosomal structure formation in rats. The expression of sGC subunits in Leydig cells is consistent with previous reports

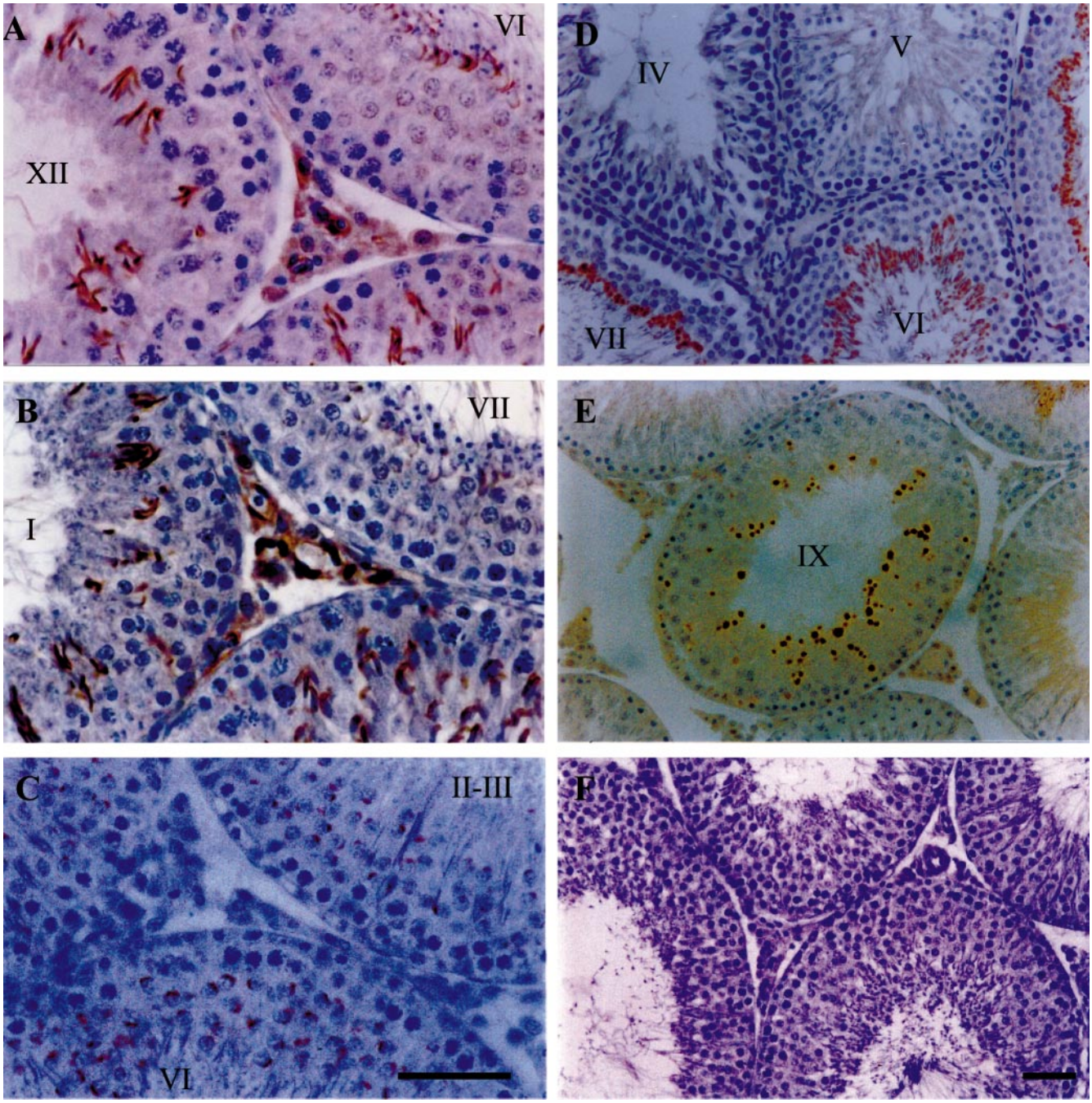

Figure 2. Representative microphotographs of testis sections from adult rats incubated with antisera against soluble guanylyl cyclase (sGC) alpha **(A)**, beta **(B)**, cGMP-dependent protein kinase G-I (PKG-I) alpha and beta **(C)**, cyclic nucleotide–gated channel 1 (CNG-1) **(D, E)** subunits, and normal rabbit serum (NRS) **(F)**. The binding sites of antibodies were visualized with an ABC Kit Elite (Sigma) and 0.05% 3,3′-diaminobenzidine tetrachloride in 0.01 M, pH 7.2, phosphate-buffered saline containing 0.01% H_2O_2. Positive immunostaining was indicted by brown reaction products. The sections were counterstained with hematoxylin and mounted with coverslips. Roman numerals indicate the stages of the spermatogenic cycle. Bar = 50 μm.

(Middendorff et al, 1997a,b) and supports a role for NO and cGMP in modulating testosterone synthesis.

Yuasa et al (2000) reported that PKG-I, also called cGK-I, directly interacts with and phosphorylates the novel male germ cell–specific protein GKAP42 in vitro and in vivo. In Yuasa's study, the interaction of cGK-I with GKAP42 facilitated the translocation of cGK-I to the Golgi complex. cGK-I was released in response to intracellular cGMP accumulation. In the male germ cells, the Golgi complex is important for the formation of the acrosomic system and the chromatoid body. The results of this study demonstrate that PKG-I stained intensively in

*Expression of soluble guanylyl cyclase (sGC) alpha and beta subunits, cAMP-dependent protein kinase G-I (PKG-I), and cyclic nucleotide–gated channel 1 (CNG-1) in the stages of spermatogenesis in rat testis**

	Stage											
	1	2–3	4–5	6	7	8	9	10	11–15	16	17	18–19
sGC α and β	−	−	−	−	+	++	++	+++	+++	+++	+++	+++
PKG-I	±	+	++	++	+++	+++	++	+	+	±	−	−
CNG-1	−	−	−	−	−	−	−	−	−	−	+	+++

* Staining intensity: − indicates no staining detected; ±, doubt; +, weak; ++, moderate; and +++, strong.

the acrosomic structure of the early stages of spermatogenesis. This finding suggests that PKG-I might function via interaction with Golgi-associated proteins during spermatogenesis. Spruill et al (1981) showed that PKG is observed in several cell types adjacent to the seminiferous tubular wall, including Sertoli cells and spermatogonia. The same study also found PKG in association with the meiotic chromosomes of pachytene spermatocytes in rat testis with the use of an antiserum produced against purified soluble cGMP-dependent protein kinase (ATP: protein phosphotransferase EC 2.7.1.37) isolated from bovine lung. The differences between our data and Spruill's results could be a result of the use of different antibodies in the 2 studies.

Several reports on the components of the NO system referred to local production of NO in the blood vessels and seminiferous tubules of human testis (Davidoff et al, 1997; Middendorff et al, 1997a,b). Every report showed positive staining for sGC in Leydig cells. However, data concerning sGC immunoreactivity in Sertoli cells has been inconsistent. Davidoff et al (1997) found sGC in Sertoli cells, in some atypically situated spermatids, and in the residual bodies of seminiferous tubules. In this study, we did not find any positive staining for sGC alpha and beta subunits in Sertoli cells. This difference in findings could be a result of the differences in species (rat vs human) and different antisera.

A CNG channel has been identified in mammalian sperm (Weyand et al, 1994). Wiesner et al (1998) examined CNG-3 in the testis. CNG-4 transcripts have been found to be present in retina, testis, kidney, heart, and brain (Biel et al, 1996). In this study, unlike CNG-3, CNG-1 was not expressed in the mature sperm. The different expressions of CNG isoforms in the mature sperm suggests that different isoforms play different roles in spermiogenesis. Iida et al (2001) found that Iba1 protein (ionized calcium-binding adapter molecule 1) is specifically expressed in the cytoplasm of elongate spermatids, which seems localized to the same area as CNG. This finding implies that CNG and Iba1 could be involved in the final stage of spermiogenesis (ie, in elimination of the residual cytoplasm from spermatids).

Our findings demonstrate that sGC, PKG-I, and CNG-1 are expressed in a stage- and cell-specific manner in the testis. The distinct temporal patterns of expression of these components of cGMP signaling pathways suggest differing physiological roles in spermiogenesis and steroidogenesis.

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