Nitric Oxide-cGMP Signaling: Its Role in Cell Junction Dynamics During Spermatogenesis[#]

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Abstract: During spermatogenesis, development of spermatogonia into elongated spermatids takes place in the seminiferous epithelium of the adult mammalian testis. Specifically, post-meiotic germ cell maturation occurs in a unique microenvironment sequestered from the systemic circulation by the blood-testis barrier (BTB), which is formed by adjacent Sertoli cells. Therefore, an intact BTB, as well as stable Sertoli-germ cell adhesion, are important criteria for successful spermatogenesis. To date, numerous factors have been shown to influence spermatogenesis, and among them is the well-studied nitric oxide (NO)/guanosine 3',5'-cyclic monophosphate (cGMP) signaling cascade. The enzymes of this pathway, namely nitric oxide synthase, soluble guanylate cyclase and cGMP-dependent protein kinase, have all been shown to regulate cell junctions in the testis. Likewise, recent findings have shown that this signaling cascade also plays a critical role in the regulation of Sertoli-germ cell adhesion. In this mini-review, we briefly discuss the regulatory role of each protein component of the NO/cGMP pathway in the context of testicular junction dynamics, as well as their importance in fertility and male contraception.

Key Words: Testis, tight junctions, adherens junctions, nitric oxide signaling pathway.

I. INTRODUCTION

Spermatogenesis, a process that takes place in the seminiferous epithelium of the testis, refers to the development of spermatogonia (diploid, 2n) into mature spermatids (haploid, 1n). For this process to proceed to completion, it must take place in an environment isolated from the systemic circulation. Thus, germ cell development requires an array of proteins, steroids and glycans, which are needed for nutrition, growth/development, orientation/polarization and protection of maturing germ cells [1]. Virtually all of the above-mentioned tasks are performed by nurse-like Sertoli cells, which create an impermeable barrier near the basement membrane known as the blood-testis barrier (BTB). While this barrier is one of the tightest tissue barriers in the mammalian body, the BTB is also very dynamic in nature because it must allow the migration of leptotene spermatocytes across at stages VIII-XI of the seminiferous epithelial cycle [2]. Once germ cells enter the adluminal compartment, they continue to traverse the epithelium while remaining attached to Sertoli cells *via* specialized adhesive contacts, which are crucial for germ cell development and survival. Thus, cell junctions in the testis are required for successful spermatogenesis and the maintenance of fertility.

While the different types of junctions present between adjacent Sertoli cells and between Sertoli and germ cells have been identified and characterized, much less is known about how these junctions are regulated during spermatogenesis, especially during the movement of germ cells across the epithelium. Results obtained from *in vitro* experiments have begun to address some of these issues, but they have only done so partially because the use of cell cultures cannot substitute for the complexity of spermatogenesis *in vivo*. Despite the ubiquitous expression of cyclic nucleotides and their associated signaling molecules, recent studies have shown that cyclic guanosine 3',5'-monophosphate-mediated signaling participates in the regulation of cell junctions in the testis [3], in particular Sertoli cell tight junctions and Sertoli-germ cell adherens junctions [4]. Equally important, we have previously reported that this signaling pathway is stimulated during the assembly and disassembly of these junctions [5]. In this review, we discuss the key players involved in the NOS signaling pathway, including known protein-protein interactions. We also consider the biological significance of these interactions in light of developing innovative approaches for male contraception. For additional information relating to the role of cyclic nucleotides in spermatogenesis, readers are encouraged to refer to the following informative reviews: [6-10].

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II. THE NO/cGMP SIGNAL TRANSDUCTION PATHWAY

The nitric oxide and guanosine 3',5'-cyclic monophosphate (NO/cGMP) signaling pathway, one of the best characterized signaling cascades, is found in most mammalian cells including Sertoli and germ cells in the testis. It has been shown to play a central role in several physiological processes such as in the regulation of the vasculatory, as well as the urogenital and reproductive systems [11]. In short, the amino acid L-arginine is converted to NO and L-citrulline by nitric oxide synthase (NOS) in the presence of various co-factors (e.g., NAPDH, BH_4, O_2, heme, Ca^{2+}/calmodulin) (Fig. **1**). Once NO is produced, it diffuses out of the target cell to either act on this (autocrine regulation) or neighboring (paracrine regulation) cells before being rapidly decomposed into nitrite (NO^{2-}) and nitrate (NO^{3-}). Upon diffusion, NO can also bind to iron in the active site of soluble guanylate cyclase (sGC). In the presence of Mg^{2+}, this activated form of sGC dephosphorylates GTP to cGMP, in turn activating cGMP-dependent protein kinases (cGKs). This induces phosphorylation of target proteins. Alternatively, cGMP can bind to other target proteins such as cGMP-gated cation channels and cGMP-regulated phosphodiesterases to regulate cell function [12, 13].

Additionally, NOS signaling is regulated by a combination of factors including cell type, environmental conditions (e.g., fluctuations in temperature, presence of toxicants), changes in intracellular NO concentrations and availability of downstream signaling molecule(s). In the following sections, we briefly describe the function and regulation of individual components of this signaling cascade, and discuss their relevance to spermatogenesis.

(i). NOS

Nitric oxide synthase (NOS) is the enzyme responsible for the production of NO [8, 10]. Three isoforms, namely neuronal NOS (nNOS or NOS-1), inducible NOS (iNOS or NOS-2) and endothelial NOS (eNOS or NOS-3), have been identified. All three isoforms have been shown to be functionally dependent on calmodulin

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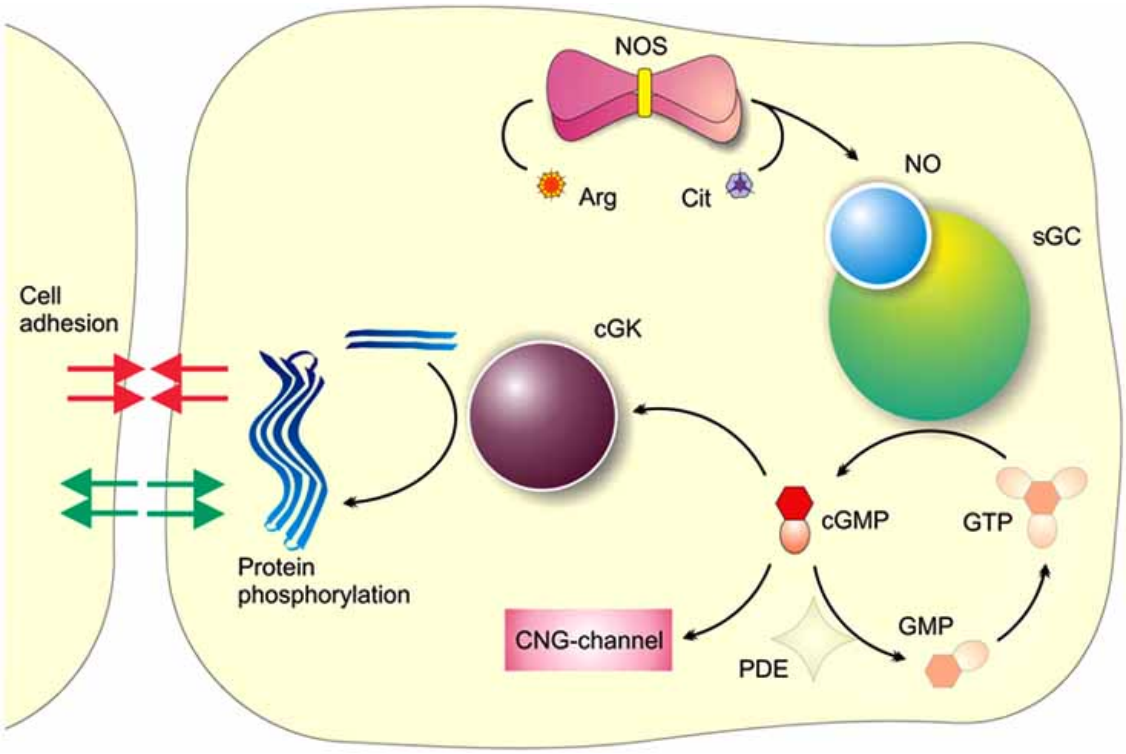

Fig. (1). A schematic drawing illustrating the NO/cGMP signaling pathway studied in different epithelia. Abbreviations: Arg, L-arginine; Cit, L-citrulline; NO, nitric oxide; NOS, nitric oxide synthase; sGC, soluble guanylate cyclase; GTP, guanosine triphosphate; cGMP, cyclic guanosine 3', 5'-monophosphate; GMP, guanosine 5'-monophosphate; cGK, cGMP-dependent protein kinase; PDE, cGMP-specific phosphodiesterase; CNG-channel, cyclic nucleotide gated cation channel. See text for discussion on the NO/cGMP signaling pathway.

[14]; Ca^{2+} is required for the catalytic activity of nNOS and eNOS [8, 15]. Although this appears to be the primary means by which NOS activity is regulated, phosphorylation at specific site(s) is also mandatory for its activity, and several potential phosphorylation sites have been identified in all three isoforms [16, 17].

Extrinsically, NOS function is regulated by proteins which interact with it. For instance, nNOS-interacting DHHC domain-containing protein with dendritic mRNA (NIDD) is a transmembrane protein that associates with nNOS and increases its activity. NIDD has the ability to target nNOS to the plasma membrane and is believed to restrict NO production to a specific site at the plasma membrane [18]. NIDD was shown to be expressed in the testis, but a functional 40 kDa protein corresponding to NIDD could not be detected [18], perhaps due to low antibody specificity and/or titer. On the other hand, a small 10 kDa protein known as protein inhibitor of nNOS (PIN) was reported to inhibit the activity of nNOS in the presence of calcium and thrombin in HEK 293 cells by potentially interfering with redox reactions between different domains of nNOS [19, 20]. Overexpression of PIN resulted in reduced cGMP formation [19, 20]. Screening of various organs revealed that the PIN mRNA level was highest in the testis [19]. Interestingly, recent studies have shown PIN to be dynein light chain 8, which belongs to the dynein family of motor proteins known to provide energy to transport cargo along microtubules [21], revealing a close relationship between NOS function and the cytoskeleton [22].

Conversely, eNOS interacting protein (NOSIP), a 34 kDa protein, regulates NO production in two ways: by (i) detaching eNOS from caveolae (plasma membrane invaginations) and sequestering it into vesicles [23, 24], and (ii) affixing NOS to the actin cytoskeleton [25]. NOSIP is predominantly a cytoplasmic protein that is activated only after translocation into the nucleus, which is facilitated by a nuclear localization sequence (NLS) and importin-α. NOSIP mutants lacking this NLS can neither travel into the nucleus nor bind importin-α, and essentially are inactive. Additionally, overexpression of NOSIP in CHO-eNOS cells was shown to inhibit eNOS activity [25].

Nitric oxide synthase traffic inducer (NOSTRIN), another NOS interacting protein, was demonstrated to negatively regulate eNOS localization [26]. Specifically, overexpression of NOSTRIN was shown to remove eNOS from the plasma membrane, concomitant with a decrease in eNOS activity. Because NOSTRIN is enriched at caveolae, it has been suggested that it can sequester eNOS while recruiting dynamin (a large GTPase) and N-WASP (Wiskott-Aldrich Syndrome Protein) to aid in the transport of eNOS to the trans-Golgi network [27-29]. These results illustrate that NOS may be participating in the endocytic recycling of proteins, thereby affecting the steady-state level of integral membrane proteins at the cell surface. In this context, it is interesting to note that an increase in cell-cell contact was shown to stimulate NOSTRIN and inhibit eNOS [30]. When the actin cytoskeleton was disrupted by cytochalasin D, the eNOS/dynamin-2/N-WASP complex was unable to move away from the plasma membrane and resulted in elevated NO levels [27].

Lastly, eNOS was demonstrated to exist in a complex with a 90 kDa protein referred to as eNOS-associated protein (eNAP, later shown to be HSP90) which when tyrosine phosphorylated, targets eNOS to the cytoskeleton and increases eNOS activity [31]. Because eNAP binding to eNOS leaves its N- and C-termini free, eNOS can be regulated by an array of known (e.g., NOSTRIN, NOSIP, NIDD, eNAP-1, caveolin-1, caveolin-3, dynamin-2, N-WASP) and yet-to-be identified proteins [32] (Table **1**).

In the testis, all three NOS isoforms were shown to localize to the basal compartment of the seminiferous epithelium, suggestive of their presence in Sertoli cells, as well as in Leydig cells in the interstitium. Germ cells, on the other hand, express eNOS and iNOS isoforms only [3]. NOS was also found to associate with cytoskeletal and tight junction proteins in the testis [33], which supports studies from other laboratories suggesting NOS involve-

Table 1. NOS Interacting Proteins*

Protein	Function(s)	M_r (kDa)	Reference(s)
NIDD	*n*NOS-*i*nteracting *D*HHC *d*omain-containing protein with dendritic mRNA Targets nNOS to the plasma membrane and increases its activity, resulting in localized NO production mRNA present in the testis, but protein is not detectable	40	[18]
PIN or LC8	*P*rotein *i*nhibitor of *nNOS* Decreases cGMP production by interfering with electron transfer between various domains of nNOS Requires calcium and thrombin for activity mRNA level is highest in testis Also known as dynein light chain 8	10	[19, 20, 22]
NOSIP	*N*itric *o*xide *s*ynthase *i*nteracting *p*rotein Decreases eNOS activity by uncoupling eNOS from the plasma membrane	34	[24, 25, 88]
NOSTRIN	*N*itric *o*xide *s*ynthase *tr*affic *in*ducer Inactivates eNOS by internalization in caveolae Complexes eNOS with dynamin, N-WASP and caveolin-1	58	[27-30]
eNAP-1 or Hsp90	*eN*OS *a*ssociating *p*rotein Increases the activity of eNOS Targets eNOS to the cytoskeleton where it may act locallly May recruit eNOS to other proteins to form a multi-protein complex Identified as heat shock protein 90	90	[31, 32, 89]

*This table is not meant to be comprehensive. Readers are encouraged to refer to the following review articles for additional references [6, 90].

ment in the dynamics of the cytoskeleton (see discussion above). Furthermore, NOS has been shown to associate with the cadherin–catenin complex present at the adherens junction, although it was not part of the nectin–afadin complex [4]. Given that NOS functions at tight and adherens junctions in the testis, we cautiously speculate that NOS may be facilitating cross-talk between these two cellular structures at the BTB [5].

(ii). sGC

The soluble form of guanylate cyclase (sGC) is a receptor for NO whose function is to dephosphorylate GTP to cGMP upon activation. sGC is a heterodimer composed of an α and a β subunit, both of which contribute to its enzymatic activity. While dimerization of α (α1, α2, α3) and β (β1, β2, β3) subunits into various combinations has been described, maximal biological activity was reported for α1β1 [34-36]. In addition to the dimerization of α and β subunits, sGC also requires a heme moiety for activation [36]. As a sensor for NO, sGC has been implicated in a wide array of physiological processes such as smooth muscle relaxation, regulation of the cardiovascular system, renal fluid retention, bone marrow progenitor cell proliferation and sperm capacitation and motility [37-40]. As mentioned above, sGC activity is stimulated by NO, but it can also be activated by NO-donors, metal ions and protein-protein interactions. Nevertheless, 1*H*-(1, 2, 4) oxadiazolol-(4, 3-α) quinoxalin-1-one (ODQ) (Table **2**) remains the primary choice of inhibitor for studying sGC function *in vitro* [41].

The 95 kDa postsynaptic density-95 (PSD95) protein recruits sGC from the cytosol to the plasma membrane. PSD95 is important since it brings sGC closer to NOS, facilitating the signal transduction cascade [42]. Recent findings of a sGC-inhibiting protein, chaperonin containing t-complex polypeptide subunit η (CCTη), have shown that sGC may also be inhibited by protein-protein interactions. CCTη is believed to affect the NO-binding site and act on sGC after it is activated by NO. Although CCTη has been shown to be expressed primarily in the brain, its presence in other organs, including the testis, has been demonstrated [43]. While it has become increasingly clear that CCTη inhibition is mediated by additional factors, their significance remains unknown [43] (Table **2**).

Localization of sGC in the testis has been reported in human Sertoli cells, spermatids and residual bodies [44], yet relatively weak expression was reported in rat germ cells by another group of investigators [45]. Additionally, the expression of sGC by Sertoli cells was not demonstrated by this group [45]. Equally important, this latter study showed that sGCβ1 was absent in stages I-VI of the seminiferous epithelial cycle but present at all other stages. However, experiments from this laboratory could not reproduce these observations; we could detect the presence of sGCβ1 in stages I-VIII of the seminiferous epithelial cycle with its level being highest in stages VI-VII but not in stages IX-XIV [5, 45]. Thus, we speculate that sGC may have a role in Sertoli cell-spermatid attachment [5]. sGC was also shown to localize at the site of the BTB, further supporting the notion that the NO/cGMP signaling pathway is critical to the function and regulation of cell junctions in the testis [5]. Since sGC is an important regulator of many cellular events (e.g., Sertoli cell-spermatid adhesion, sperm motility), it would be an ideal target to disrupt spermatogenesis, that is, if its function was restricted to the testis. While several years of research are clearly needed, it will be interesting to determine if the testis expresses a unique guanylate cyclase, one with a specific role in spermatogenesis.

(iii). cGMP

A biochemically significant step of NOS signaling is the production of cGMP from GTP (Fig. **1**). cGMP-mediated signaling is ubiquitous and has been reported to function in smooth muscle relaxation, neurotransmission, vasodilation and platelet aggregation [11, 46, 47]. cGMP signaling is mediated *via* three distinct targets. First, it modulates the concentration of cAMP by activating or inhibiting cAMP-specific phosphodiesterases (PDEs) [48]. Second, it opens cyclic-nucleotide-gated cation channels (CNGs), which are essential for signal generation in systems such as the retina and olfactory neurons [49]. Third, cGMP activates cGMP-dependent protein kinases (cGKs) [49].

In the testis, cGMP concentrated predominantly to endothelial and smooth muscle cells in blood vessels, but also to Leydig and Sertoli cells [44]. The presence of cGMP in blood vessels has been ascribed to a role in vasodilation [44]; whereas that in Leydig cells suggests a role in testosterone production, although there is little experimental evidence to support this claim. Nevertheless, testicular cGMP production by sGC is regulated, at least in part, by heme oxygenase-1 (HO-1) *via* its end-product carbon monoxide (CO)

Table 2. Activators and Inhibitors of sGC*

Activators	Function(s)	Reference(s)
YC-1	3-(5'-hydroxymethyl-2'-furyl)-1-benzyl indazole NO-independent effect Binds to allosteric sites other than the heme moeity/NO binding site Independent of ODQ	[91]
A-350619	3-[2-(4-chlorophenylthio) phenyl]-N-(4-dimethylaminobutyl) acrylamide hydrochloride NO-independent effect Binds to the same allosteric site as YC-1 in a competitive manner Susceptible to ODQ	[92]
BAY41-2272	5-cyclopropyl-2-[1-(2-fluoro-benzyl)-1H-pyrazolo[3,4-b]pyridine-3-yl]pyrimidin-4ylamine NO-independent effect	[93]
NONOates	Nitric oxide/nucleophile adducts (e.g., azide, sodium nitroprusside, s-nitrosothiols)	[94, 95]
Metal ions	Fe^{2+}, Mn^{2+}, Mg^{2+}	[41, 96]
PSD95	*P*ost*s*ynaptic-*d*ensity-95 M_r 95 kDa Recruits sGC from the cytosol to the plasma membrane Brings sGC into the vicinity of NOS Restricts sGC effects to a localized area within the cytosol	[42]
CO	Carbon monoxide Activation of signaling pathway is less efficient than NO CO is the primary activator of sGC in the brain and spleen	[50]
Inhibitors		
ODQ	1*H*-(1, 2, 4) oxadiazolol-(4, 3-a) quinoxalin-1-one Oxidizes the heme moiety and inhibits sGC Ineffective against the inhibition of YC-1	[97]
CCTη	*C*haperonin *c*ontaining *t*-complex polypeptide subunit η Present in the brain, heart, kidney, lung and testis Binds to sGC, thereby preventing NO from binding to the heme moiety Counters NO-induced activation of sGC	[43]
Copper	Cu^{+} ion has inhibitory action, which is countered by the Cu^{2+} ion Inhibition independent of heme moiety/NO binding site	[98]

* This table is not intended to be exhaustive. It presents some representative compounds that have been used by investigators to study sGC function in different systems. Readers are encouraged to refer to review articles for additional references [46, 47, 99].

[50]. In the seminiferous tubule, HO-1 is found in the apical compartment. Interestingly, equimolar concentrations of CO and NO were both shown to cause an increase in cGMP production in pure sGC extracts obtained from bovine lung, but in cellular environments, NO is significantly more potent in its effects [51]. What this essentially reveals is that multiple factors regulate cGMP production. Additionally, heat shock can also induce testicular HO-1 [52], and the ensuing increase in cGMP may have a role in protecting Sertoli and Leydig cells from stress [53]. In this context, it should be noted that a recent study has reported that cGMP has a biphasic effect on Sertoli cell tight junction barrier function [33]. At low doses, cGMP facilitated the formation of the permeability barrier, yet at high concentrations it disrupted the function of Sertoli cell tight junctions *in vitro* [33]. While the mechanism(s) by which cGMP mediates these effects is presently unknown, it may be related to cGK downstream since this kinase can directly alter the phosphorylation status of tight junction proteins [54], in turn affecting cell adhesion.

(iv). cGK

The physiological end-point of NOS signaling is the phosphorylation of target proteins (Table **3**), resulting in an array of cellular and physiological changes. This phosphorylation is brought about by a family of cGMP-dependent protein kinases (PKG, alternatively known as cGK) that are stimulated by cGMP [48, 55]. Two major isoforms have been identified: a cytosolic form known as cGKI, (types α and β exist with cGKIα having ten times more affinity for cGMP than cGKIβ), and a membrane-bound form known as cGKII [48, 56]. Both enzymes are homodimers composed of two identical subunits having an apparent M_r of ~76 kDa. The N-terminal domain of cGK is crucial for targeting the enzyme to specific subcellular locations, dimerization and activation by cGMP [57]. Presently, several cGK-mediated phosphorylated proteins have been identified (Table **3**). These include protein phosphatase 2A [58], telokin [59, 60], vasodilator stimulated phosphoprotein (VASP) [61, 62], cystic fibrosis transmembrane conductance regulator (CFTR) [63], β-catenin [54] and the small GTPase RhoA [64]. Additional proteins such as inositol 1,4,5-triphosphate receptor-associated cGMP kinase substrate (IRAG) and 42 kDa cGMP-dependent protein kinase anchoring protein (GKAP42) are also putative binding partners of cGK [65, 66].

cGK plays an important regulatory role in cardiovascular, neurological and gastrointestinal systems, as well as in renal homeostasis and bone metabolism [55]. For instance, cGK mediates smooth muscle relaxation by interacting with many proteins. It phosphorylates and activates the myosin binding domain of myosin phosphatase 1 (PP1M) [55], and thereby participates in smooth muscle relaxation. cGK also associates with IRAG, a regulator of the cGK-mediated intracellular calcium level [67], and was shown to be present in the heart, brain, kidney, uterus and testis [65, 66]. Phos-

Table 3. Known Targets of cGK*

Protein	Function(s)	Reference(s)
PP2A	*P*rotein *p*hosphatase *2A* Membrane-bound protein target of cGK phosphorylated specifically by cGK Phosphorylation of PP2A activates maxi K_{ca} channels, allowing for less calcium influx Role in smooth muscle relaxation	[58]
Telokin	M_r 17 kDa Phosphorylated telokin stabilizes the actomyosin cytoskeleton by dephosphorylating myosin light chain Mediates cGMP-dependent smooth muscle relaxation	[59, 60]
VASP	*Va*sodilator *s*timulated *p*hosphoprotein M_r 46-50 kDa Phosphorylated by both cAMP-dependent adenylate kinase and cGK Role in cytoskeletal organization	[61, 62]
CFTR	*C*ystic *f*ibrosis *t*ransmembrane conductance *r*egulator Phosphorylation does not activate the protein nor affect chloride channel permeability	[63]
IRAG	*I*nositol 1,4,5-triphosphate *r*eceptor-*a*ssociated c*G*MP kinase substrate (or IP_3RI) M_r 125 kDa cGK/IRAG–IP_3RI interactions are essential for cGMP-mediated smooth muscle relaxation	[65, 66, 100]
RhoA	Small GTPase M_r 21 kDa Phosphorylation results in the inactivation of RhoA No effects on RhoA/ROCK-mediated actin reorganization cGMP/cGK pathway counteracts the RhoA/ROCK signaling pathway	[64]
β-catenin	A cell adhesion protein Phosphorylation allows b-catenin to remain in the cytosol Down regulates transcription factor activity	[54]
GKAP42	42 kDa c*G*MP-dependent protein *k*inase *a*nchoring *p*rotein Germ cell-specific protein Stage-specific expression that was highest in spermatocytes and round spermatids Not found in spermatogonia, late spermatids, Sertoli and Leydig cells Present in the Golgi complex and acrosome of developing spermatozoa	[69]

* This table is not meant to be comprehensive. Readers are encouraged to refer to the following review articles [48, 55].

phorylation of IRAG by cGKI inhibits inositol 1,4,5-phosphate-induced calcium release and is essential for cGMP-mediated smooth muscle relaxation [65, 67]. However, the significance of this interaction in the testis is not known.

Moreover, cGK was reported to be present in Sertoli cells and to localize at the BTB [4]. These results were corroborated when cGK was demonstrated to interact with tight junction proteins occludin and ZO-1, as well as with adherens junction proteins N-cadherin and β-catenin [5]. As important, cGK was shown to associate with a germ cell-specific protein called GKAP42 [68]. This protein was absent in Sertoli and Leydig cells, but was expressed in germ cells in a stage-specific manner with its level being highest in spermatocytes and round spermatids. Spermatogonia and late spermatids were not immunoreactive for GKAP42. Specifically, GKAP42 localized to the acrosome of developing germ cells and was shown to phosphorylate Golgi-associated proteins [69]. Taken collectively, these data seemingly suggest that cGK has a role in BTB dynamics because of its association with proteins that constitute tight and adherens junctions.

III. THE NOS SIGNALING PATHWAY IN THE REGULATION OF CELL JUNCTIONS IN THE TESTIS

(i). Effects on Sertoli-Sertoli Cell Tight Junctions

As previously discussed, the BTB creates a unique microenvironment in that it segregates post-meiotic germ cell development from the systemic circulation. The BTB is found between adjacent Sertoli cells near the basement membrane and is composed of tight and adherens junctions, desmosome-like junctions, ectoplasmic specializations and tubulobulbar complexes [1]. Tight and adherens junctions are formed by hetero- and homotypic interactions between transmembrane proteins (occludin, claudin, JAM-1 and cadherin) on the plasma membrane of adjacent Sertoli cells, whereas peripheral membrane adaptors (ZO-1, α-catenin, β-catenin) link these transmembrane proteins to the actin cytoskeleton [1, 70, 71]. These structures are very dynamic since they are constantly being assembled and disassembled to facilitate germ cell migration, and a number of signaling molecules, among them cGMP, regulate the opening and closing of these junctions. This signaling is not an on-off type of signaling, but one which involves numerous changes in the steady-state protein levels of signaling molecules, their cellular distribution, phosphorylation status and protein-protein interactions. Understanding the role of NOS signaling during junction restructuring in the testis will allow us to better understand the process of spermatogenesis.

An increase in NOS (iNOS and eNOS) expression was detected during the assembly of the Sertoli cell tight junction permeability barrier *in vitro*. However, after the barrier was established, NOS levels decreased significantly [33]. Moreover, the effects of cGMP on Sertoli cell tight junction barrier function were biphasic. For instance, the use of a phosphodiesterase resistant cGMP analog, 8-bromo cGMP, was shown to have an adverse effect on barrier assembly and maintenance [33]. When this experiment was repeated but significantly lower doses of 8-bromo cGMP were used, tight junction barrier function was enhanced. Alternatively, when cGMP levels were reduced by inhibiting cGK with the use of KT-5823, the tight junction barrier became tighter [33]. These results are consis-

tent with an earlier report on the biphasic effects of cyclic nucleotides, such as cAMP, on Sertoli cell tight junction function *in vitro* [72]. Taken collectively, these data clearly demonstrate that Sertoli cell tight junctions are sensitive to fluctuations in the level of cGMP. They also validate the *in vivo* model in that a "localized" increase in cGMP can perturb the integrity of tight junctions and compromise BTB function.

(ii). Effects on Sertoli-Germ Cell Adherens Junctions

In the seminiferous epithelium, developing germ cells rely on Sertoli cells for sustenance, and throughout their development they must remain attached to Sertoli cells *via* specialized cell junctions. Thus, formation and maintenance of stable adherens junctions is critical for germ cell survival and spermatogenesis. The best studied type of adherens junction in the testis is the ectoplasmic specialization, which is formed by homotypic (N-cadherin/N-cadherin) or heterotypic (nectin 2/nectin 3, laminin $\alpha 3\beta 3\gamma 3$/integrin $\alpha 6\beta 1$) interactions between transmembrane proteins [1, 73, 74]. A host of peripheral membrane adaptors including α-catenin, β-catenin, γ-catenin, $p120^{ctn}$, afadin and ponsin link these transmembrane anchors to the actin cytoskeleton [1]. These multi-protein complexes also co-function with many kinases/phosphatases and GTPases, which allow for the rapid attachment and detachment of germ cells during germ cell movement [75, 76]. In this context, it is interesting to note that assembly and disassembly of adherens junctions is regulated, at least in part, by the cGMP signaling cascade [4, 5, 33].

During Sertoli-germ cell adherens junction formation *in vitro*, the intracellular level of NOS (iNOS and eNOS) increased steadily with a concomitant increase in cGMP. This was mirrored by an increase in the level of cGK [4]. After the establishment of stable adherens junctions, however, a significant decline in the cGMP level was noted. This suggests that signaling may only be required to mediate adherens junction formation. Nevertheless, it remains to be determined if an inhibition of NOS, sGC, cGMP or cGK can prevent the attachment of germ cells to Sertoli cells *in vitro*, which could be performed by using a specific inhibitor(s) and/or gene silencing.

The role of the NOS pathway in adherens junction disassembly was also demonstrated in experiments using Adjudin [5]. Adjudin (formerly known as AF-2364 or 1-(2,4)-dichlorobenzyl-*1H*-indazole-3-carbohydrazide) is a drug that disrupts adherens junctions between Sertoli and germ cells (e.g., spermatids, spermatocytes but not spermatogonia) without perturbing tight junctions at the BTB during the depletion of germ cells from the seminiferous epithelium [1, 77]. Using this *in vivo* model of junction disassembly, iNOS, sGC and cGK levels were shown to be induced [4, 5]. sGC disassociated from the tight junction and relocalized to the adherens junction, seemingly in an attempt to "rescue" the adherens junction from Adjudin-induced disruption which leads to germ cell loss from the epithelium [5]. Moreover, this study is significant in that it illustrates the existence of cross-talk between tight and adherens junctions in the testis and that the cGMP pathway is involved in these signaling events.

(iii). Effects on the Cytoskeleton

The cytoskeleton maintains cell shape and functions in the adhesion of cells. It also participates in an array of cellular and biochemical processes ranging from apoptosis to endocytosis [78]. Interestingly, various proteins of the NOS pathway have been shown to interact with the actin cytoskeleton [4, 5]. For example, it has been reported that eNOS can interact directly with actin microfilaments, and actin polymerization is believed to contribute significantly to eNOS activity [79]. When intestinal epithelial cells were exposed to an elevated NO level, the actin cytoskeleton was adversely affected, and the permeability of the tight junction barrier increased, illustrating a loss of tight junction function [80]. In Sertoli cells, eNOS and sGC were both shown to bind to actin [4, 5]. However, it remains to be determined if the NO/cGMP pathway can affect microtubule- and intermediate filament-based cytoskeletons.

IV. ANIMAL KNOCKOUT MODELS

Recent studies using animal knockout models have provided interesting results on the role of NOS. In the nNOS knockout mouse (nNOSKO), animals did not exhibit the expected decrease in the NO level. This seemingly suggests that iNOS and eNOS can take over the function of deleted nNOS. Interestingly, even "double knockout" (nNOSKO and eNOSKO) mice failed to completely inhibit NO synthesis [81]. Of the NOS knockout models, only "triple knockout" mice were sub-fertile with additional symptoms of diabetes insipidus and cardiovascular abnormalities [82]. Furthermore, deletion of sGC (sGCKO) resulted in mortality [46]. In a more recent study, knockout of sGCβ1 resulted in a loss of enzyme activity. While these mice were only viable for ~3-4 weeks post-partum due to abnormalities in intestinal peristalsis, administration of a fiber-free diet appeared to rescue them [83]. Similarly, cGK knockout mice were hypertensive at ~4-6 weeks post-partum, and displayed impaired intestinal peristalsis and retarded intestinal food passage [84], perhaps due to compromised smooth muscle activity surrounding the intestine. Nevertheless, under conditions of complete NOS silencing *in vivo*, knockout animals were sub-fertile [82], but additional biochemical studies are needed in order to investigate the effects of gene deletion on spermatogenesis *per se*.

V. CONCLUDING REMARKS AND FUTURE PERSPECTIVES

As reviewed herein, cyclic nucleotides, particularly cGMP and its associated signaling molecules sGC and cGK, are important regulators of integral membrane proteins and/or their adaptors at the BTB because they can affect Sertoli cell tight junction barrier function and Sertoli-germ cell adhesion *in vitro* and *in viv*o. However, additional studies are needed in the testis, especially on the other known components of this signaling pathway, such as the NOS interacting proteins (Table **1**) and target proteins of cGK [Table 3]. Fortunately, several specific inhibitors that can be used to study different signaling molecules in this pathway have been identified (Table **2**), some of which can be used to study Sertoli-Sertoli and Sertoli-germ cell interactions. While it may be somewhat naïve to suggest that these proteins can be targets for contraceptive development, recent developments in the field are beginning to report otherwise. For instance, soluble adenylyl cyclase (sAC), the corresponding homologue of sGC in epithelial cells, is a bicarbonate-regulated and calcium-responsive adenylate cyclase that is found in spermatids and spermatozoa. It was shown to be a potential druggable target for male contraceptive development [85, 86] because $sAC^{-/-}$ mice were reported to be oligozoospermic, coinciding with a loss of sperm motility and spontaneous acrosome reaction [85]. Furthermore, sAC function was restricted to the early phases of sperm capacitation, namely the induction of protein tyrosine phosphorylation and motility [87]. These results suggest that sAC can be a potential target for male contraceptive development since it would disrupt sperm function without compromising the hypothalamic-pituitary-testicular axis. Perhaps this approach can also be used to target sGC in Sertoli cells, as this would likely maintain the BTB in a "closed" state, thereby denying access of leptotene spermatocytes into the adluminal compartment and causing azoospermia.

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