Fertility and spermatogenesis are altered in $\alpha 1b$ -adrenergic receptor knockout male mice

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Abstract

To examine whether norepinephrine, through activation of $\alpha 1b$ -adrenergic receptor, regulates male fertility and testicular functions, we used $\alpha 1b$ -adrenergic receptor knockout ($\alpha 1b$ -AR-KO) mice. In the adult stage (3–8 months), 73% of the homozygous males were hypofertile with relatively preserved spermatogenesis. Of the remaining males, 27% exhibited a complete infertility with a drastic reduction in testicular weight and spermatogenesis defect with germ cells entering a cell death pathway at meiotic stage. In both phenotypes, circulating levels of testosterone were highly reduced (–55 and –81% in hypofertile and infertile males respectively versus wild-type males). Consequently, circulating levels of LH were significantly elevated in $\alpha 1b$ -AR-KO infertile mice. When incubated *in vitro*, the whole testes from infertile KO mice released significantly lower levels of testosterone (–40%). This, together with the fact

that the mean absolute volume of Leydig cells per testis was not changed, suggests a compromised steroidogenic capacity of Leydig cells in infertile animals. In addition, RNA *in situ* hybridization study indicated an apparent higher expression of inhibin α - and β B-subunits in Sertoli cells of infertile $\alpha 1b$ -AR-KO mice. This was correlated with a higher intra-testicular content of inhibin B (+220% above wild-type mice). Using specific primers, mRNA encoding $\alpha 1b$ -AR was localized in early spermatocytes of wild-type testes. Our results indicate, for the first time, that $\alpha 1b$ -AR signaling plays a critical role in the control of male fertility, spermatogenesis, and steroidogenic capacity of Leydig cells. It is thus hypothesized that the absence of $\alpha 1b$ -AR alters either directly germ cells or indirectly Sertoli cell/Leydig cell communications in infertile $\alpha 1b$ -AR-KO mice. *Journal of Endocrinology* (2007) **195**, 281–292

Introduction

Noradrenergic innervation of the mammalian testis has been shown by immunocytochemical and ultrastructural studies (Mayerhofer *et al.* 1999, Frungieri *et al.* 2000). Adrenergic nerve varicosities were located mainly in proximity of the Leydig cells, lamina propria of seminiferous tubules, and perivascular wall (Prince 1992, 1996), suggesting that the norepinephrine released from sympathetic nerves has multiple sites of action in the control of testicular functions. Disruption of the neuronal input (Chow *et al.* 2000), electrical stimulation of the spermatic nerves (Chiocchio *et al.* 1999) as well as chemical sympathectomy with guanethidine (Rosa-e-Silva *et al.* 1995) or 6-hydroxydopamine (Mayerhofer *et al.* 1990) demonstrated that norepinephrine modulates luteinizing hormone (LH) receptors expression, testosterone output, spermatogenesis, and testicular blood flow. Interestingly, incubation of dispersed testicular cells with norepinephrine or epinephrine significantly enhanced the viability of spermatogenetic cells (Nagao 1989).

Norepinephrine is implicated in a wide range of physiological processes through activation of nine different G-protein-coupled receptors ($\alpha 1a$, $\alpha 1b$, $\alpha 1d$, $\alpha 2a$, $\alpha 2b$, $\alpha 2c$, $\beta 1$, $\beta 2$, $\beta 3$). *In vitro* studies using selective adrenergic agonists or antagonists indicated that both β - and α -adrenergic receptor (AR) might be involved in the neuroendocrine control of testicular functions (Verhoeven *et al.* 1979, Cooke *et al.* 1982, Anakwe & Moger 1986, Mayerhofer *et al.* 1989, Wanderley *et al.* 1989). However, whereas $\beta 1$ -/ $\beta 2$ -subtypes were shown to be predominantly expressed in Leydig and Sertoli cells (Tolszczuk *et al.* 1988, Eikvar *et al.* 1993, Troispoux *et al.* 1998, Hellgren *et al.* 2000), the localization of $\alpha 1$ -AR subtypes within the testis is less documented and no data are presently available to assign functional role to specific $\alpha 1$ -AR subtypes.

In recent years, much knowledge about the functions of defined genes in spermatogenesis has been gained by making use of mouse transgenic and gene knockout (KO) models. Indeed, spermatogenesis is under the complex control of many molecular and cellular events. This involves gene expression in the developing germ cells, cell–cell interactions of germ cells with Sertoli cells, communication between

tubular and Leydig cell compartments that are, in turn, regulated by gonadotropins and androgens (Skinner 1991, Saez & Lejeune 1996). Failure of any of these events leads to disturbances of male fertility. Therefore, to investigate the role of $\alpha 1b$ -AR in testicular physiology, we used KO mice lacking the $\alpha 1b$ -AR subtype (Cavalli *et al.* 1997). In the latter study, it was briefly reported that disruption of the $\alpha 1b$ -AR gene does not seem to have any major effect on fertility since homozygous mice were capable of giving progeny and initiating the breeding colony. The present study was then designed to determine more accurately the consequences of the $\alpha 1b$ -AR-KO on male reproductive processes. For this purpose, we used appropriate experiments that addressed the effect of $\alpha 1b$ -AR absence on testicular morphology, male fertility and the level of gonadotropins, testosterone, and inhibin B in adult mice. The obtained findings underscore the role of $\alpha 1b$ -AR signaling in the regulation of Leydig cell homeostasis and spermatogenesis processes.

Materials and Methods

Mice and tissue collection

The founder animals used to initiate our colony were wild-type (+/+) mice and $\alpha 1b$ -AR-KO mice with 129/Sv $\times$ C57BL/6J genetic background, kindly provided by Pr S Cotechia (Cavalli *et al.* 1997). Adult males and females with different genotypes and from different litters were randomly intercrossed to obtain $\alpha 1b$ -AR +/+, +/- and -/- progeny. Only the resulting male $\alpha 1b$ -AR-KO mice (-/-) and wild-type littermates (+/+) were used in the present experiments. Mice were housed in a room with a controlled photoperiod (lights on from 0900 to 1700 h) and temperature (22–24 °C) and were given free access to a nutritionally balanced diet (UAR B03) and water. Animals 3- to 8-month-old belonging to generations F2–F4 issued from the same colony were killed by cervical dislocation, in accordance with the guidelines for care and use of laboratory animals (NIH Guide). For hormone assays, blood was collected immediately by cardiac puncture and plasma was stored at –20 °C.

Fertility studies

Continuous mating studies were performed during a 2-month period to compare the fertility of the wild-type and $\alpha 1b$ -AR-KO male mice. Three 12-week-old wild-type proven fertile females were allowed to mate with one male. Females were checked for post-coital plugs each morning. If a plug was observed, the female was noted as being at day 1 of gestation. Plug-positive females were killed on day 16 of gestation and litters were assessed for the number of embryos. The lack of a copulatory plug within the 2-month period of mating indicated a loss of either fertility or mating performance of male mice. The fertility state was then assessed for the entire group of $\alpha 1b$ -AR-KO mice at the end

of the mating period by evaluating spermatogenesis on testis sections in comparison with wild-type animals.

Assay for mice genotyping

Genotyping was performed by PCR using specific mouse upstream and downstream primers of $\alpha 1b$ -AR (Mhaouty-Kodja *et al.* 2001). The DNA was extracted from 1 cm tail-tip biopsy specimens of animals at 30-day post-natal by overnight incubation at 55 °C in buffer (containing 100 mM Tris pH 8.5, 0.5% SDS, 0.2 mM NaCl, 5 mM EDTA) with proteinase K (100 µg/ml). After a phenol/chloroform extraction, genomic DNA contained in the supernatant was precipitated by the addition of 1 volume of isopropanol, washed twice with alcohol 70%, dried, and resuspended in sterile water.

Reverse transcription (RT)-PCR analysis of $\alpha 1b$ -AR expression

Total RNAs from mice testis and liver were prepared using the RNA-PLUS kit (Bioprobe Systems, Montreuil, France). Five micrograms of total RNA were reversed-transcribed using SuperScript Reverse Transcriptase kit from Gibco BRL Life Technologies and the resulting cDNA was stocked at –80 °C. PCR was performed using specific mouse upstream and downstream primers of $\alpha 1b$ -AR (Mhaouty-Kodja *et al.* 2001) and the internal control hypoxanthine phosphoribosyltransferase (Keller *et al.* 1993). The PCR products were separated by electrophoresis on an ethidium bromide-containing 2% agarose gel. Control PCRs performed on non-transcribed RNA indicated no contamination of the RNA preparations with genomic DNA.

Stereological analysis and immunohistochemistry

The left testis from each animal was fixed overnight by immersion in Bouin's fluid, after incision of the tunica albuginea. The fixed testes were divided into two along a plane lying at right angles to the long axis. One half of each piece was dehydrated and embedded into methacrylate resin (Technovit 7100; Kulzer and Co. GmbH, Friedrichsdorf, Germany) according to the manufacturer's instruction. Sections (3 µm) from each testis block were serially cut on a Reichert Jung 2050 (Nossloch, Germany) supercut microtome and then stained with toluidine blue. For the quantitative assessment of the volumes of testicular compartments (seminiferous tubules, testis interstitium, Leydig cells), stereological methods were performed as previously described using the point counting method (Kim *et al.* 2002). The absolute volume of seminiferous tubules, interstitium, and Leydig cells per testis was determined from the product of the volume fraction and the processed testicular volume. The diameter of the seminiferous tubule was also estimated (30 cross sections per testis). In the case of elliptical profiles, the short axis of the ellipse was measured.

For micrography, testes were placed in Bouin's fixative for 24 h, dehydrated in alcohol, and paraffin embedded using standard

protocols. Serial sections of 5 μm thickness were mounted on glass slides and alternately stained with cresyl fast violet or used for immunocytochemical detection of 3β -hydroxysteroid dehydrogenase (3β -HSD) activity, a marker of Leydig cell status. Immunocytochemical detection of 3β -HSD was performed using a polyclonal anti- 3β -HSD (gift from G Defaye, Grenoble, France) diluted at 1:200 and the avidin–biotin peroxidase complex as previously described (Livera *et al.* 2000). Peroxidase was visualized with 3,3'-diaminobenzidine. The specificity of staining was checked by replacing the anti- 3β -HSD antibody with non-immune mouse IgG. The criteria used to identify the spermatogenic cell types within the seminiferous epithelium were those of Russel *et al.* (1990). Sections were photographed using a Leitz Diaplan photomicroscope.

Determination of apoptosis

Testis was immersion fixed in 4% paraformaldehyde/phosphate buffer at room temperature. Then, the fixed tissues were embedded in paraffin and processed for detection of apoptotic cells (Ben-Sasson *et al.* 1995). Tissue sections were incubated with proteinase K for 8 min at 37 °C to increase signal intensity. The 3' ends of fragmented DNA were labeled with digoxigenin (DIG)-dUTP using the enzyme terminal deoxynucleotidyl transferase (TdT; TUNEL enzyme, Roche). The DIG-dUTP was visualized by incubation with a monoclonal antidigoxigenin-peroxidase antibody (1:500), followed by diaminobenzidine tetrahydrochloride substrate and hydrogen peroxide. The negative control where TdT was omitted from the reaction did not demonstrate nuclear staining (not shown). Other serial sections were also treated with the TUNEL reaction mixture containing terminal transferase to label-free 3'-hydroxy ends of genomic DNA with fluorescein-labeled deoxy-UTP. TUNEL labeling was then observed with an epifluorescence microscope (Carl Zeiss, New York, NY, USA).

Hormone assays

Basal and human chorionic gonadotropin (hCG)-stimulated testosterone concentrations were determined by RIA as previously described (Habert & Picon 1984). The sensitivity of this testosterone assay was 10 pg/ml and the mean intra-assay coefficient of variation was 7%. The *in vivo* testicular response of 4- to 6-month-old $\alpha 1b$ -AR-KO mice was examined 1 h following i.p. injection of 5 IU hCG (Organon S A, Puteaux, France) or saline. For *in vitro* testosterone secretion, each testis from $\alpha 1b$ -AR-KO males was decapsulated and cut into small pieces, which were placed on a Millipore filter (pore size, 0.45 μm) and cultured in Ham's F12/Dulbecco's modified Eagle's medium (1:1; Gibco) containing 0.35% glutamine (Flow Laboratories, Rockville, MD, USA) and 80 $\mu\text{g}/\text{ml}$ gentamicin (Gentalline, Schering-Plough, Levallois-Perret, France) for 3 days at 34 °C in a humidified chamber gassed with 95% O_2 /5% CO_2 . The amount of testosterone released in the incubation medium during the last 4 h of the culture indicated the secretory capacity of Leydig cells per testis.

The plasma levels of LH and follicle-stimulating hormone (FSH) were assayed by RIA using reagents generously supplied by Dr A F Parlow and the NIDDK (Baltimore, MD, USA) respectively. The intra-assay coefficients of variation were 4.2 and 6.5% for LH and FSH respectively. All plasma extracts were included in the same assay to avoid inter-assay variability. The testicular content of inhibin B was measured by ELISA (kit from Oxford-Bioinnovation, Oxford, UK) as previously described (Sharpe *et al.* 1999). The sensitivity of the assay was 5 pg/tube. Intra- and inter-assay coefficients of variation were 4.9 and 12% respectively. This assay system had no significant cross reaction with pro-ac subunit and activins. Cross reaction with inhibin A was about 1%.

In situ hybridization of inhibin subunits and $\alpha 1b$ -AR

Sense and antisense riboprobes for inhibin subunits (Guigon *et al.* 2003) and the $\alpha 1b$ -AR antisense nucleotide with a sequence complementary to bases 1028–1072 in the third intracellular loop (5'-AACTCCTGGGGTTGTGGCCCTTGGCCTTGG TACTGCTGAGGTGT-3') were labeled at the 3' end with DIG-11-dUTP as previously described (Guigon *et al.* 2003).

After prehybridization, hybridization of 8 μm tissue sections was carried out overnight at 55 °C for inhibin or 37 °C for $\alpha 1b$ -AR with labeled probes diluted in prehybridization mix without EDTA and salmon testes DNA. For inhibin subunits detection, sections were then treated with ribonuclease A for 30 min at 37 °C and washed with 30% formamide/0.1 $\times$ SSC at 65 °C for 1 h. Detection of labeled probes was performed using an alkaline phosphate-conjugated sheep anti-DIG antibody (1:500) and the chromogen substrates of alkaline phosphatase as previously described (Guigon *et al.* 2003). In control experiments, sections were treated identically, except that a 100-fold excess of the unlabeled oligonucleotide was added in the hybridization medium. All sections were mounted in glycerol gelatin.

Statistical analysis

Data are expressed as the mean $\pm$ S.E.M. All data were analyzed by ANOVA followed by the Student–Neuman–Keuls test. Student's *t*-test was used when the values of two groups were compared and was applied at the level of 5% ($P < 0.05$).

Results

Fertility studies

Of the $\alpha 1b$ -AR-KO males, 27% are infertile whereas the remaining 73% mice are hypofertile (6.0 ± 0.4 , $n = 155$, vs 8.5 ± 0.3 , $n = 144$ pups/l in wild-type mice, $P < 0.01$). PCR analysis of genomic DNA confirmed that targeted disruption of the $\alpha 1b$ -AR gene was successful in both infertile and hypofertile males (Fig. 1A). Moreover, RT-PCR analysis confirmed the presence of a specific signal of 470 bp corresponding to $\alpha 1b$ -AR

Figure 1 (A) PCR analysis of genomic DNA from wild-type female (No. 461) and male (No. 383), as compared with knockout female (No. 432) and hypofertile (No. 424) and infertile (No. 426) males. The DNA 100 bp size markers are shown on the left. Negative template sample is included as control PCRs. (B) Representative gel for RT-PCR detection of $\alpha 1b$ -AR (470-bp) and the internal control Hprt (249-bp) mRNA from the testis of wild-type (+/+), hypofertile (hypf), and infertile (inf) knockout (-/-) mice. Wild-type and knockout mice livers were used as positive controls. The DNA 50 bp size markers are shown on the left. (C) Dissection of urogenital tracts of wild-type (wt) and infertile (inf) knockout (-/-) male mice at the age of 4 months. dd, ductus deferens; sv, seminal vesicles; t, testis; e, epididymis.

transcript in the testis of wild-type mice as well as in mouse liver, which was used as a positive control. In contrast, no signal was detected in hypofertile or infertile $\alpha 1b$ -AR-KO mice (Fig. 1B). The infertile phenotype is not due to a progressive degenerative process that could be more extensive in older males since, in the same litter, some males were hypofertile, while others were infertile. We did not observe the development of infertility throughout the 2-month tested period.

Testis weights, histology and volumes of testicular compartments

The male urogenital tract of infertile $\alpha 1b$ -AR-KO mice was normally developed (Fig. 1C). The size of the testes and seminal vesicles was, however, reduced (Figs 1C and 2B). The testicular weight was reduced to 16% of that in wild-type males (Fig. 2A).

The reduction of the testis volume (-96%) was associated with a 99% decline of the absolute volume of seminiferous tubules (Table 1) and a decreased diameter of tubules ($114 \pm 22 \mu\text{m}$ in infertile $\alpha 1b$ -AR-KO mice versus $228 \pm 10 \mu\text{m}$ in wild-type mice). These changes mainly resulted from the large reduction of spermatogenic cell number as evidenced by the examination of stained testis sections (Figs 3 and 4A). The identity of the spermatogenic cells was defined based on their general size, shape, and location within the seminiferous tubules. At the light microscopic level, spermatogonia, some of which in mitosis, and a few spermatocytes were still observed (Fig. 4A). A population of early meiotic cells has entered an apoptotic pathway as indicated by the TUNEL methods (Fig. 4A insert and B). There was no evidence of normal progression of spermatogenesis beyond the differentiating spermatocytes stage. Spermatogenesis

Figure 2 Consequences of $\alpha 1b$ -AR-knockout on testicular morphology. (A) Weights of testes obtained from 5 to 15 mice at the indicated ages. ^a $P < 0.001$ versus wild-type (+/+) mice, ^b $P < 0.001$ versus hypofertile knockout (-/-) mice. (B) Representative photomicrographs of transversal cross sections from the testis of infertile and hypofertile $\alpha 1b$ -AR-KO mice and wild-type mice at 4 months of age. magnification $\times 25$ for all micrographs.

was arrested before early spermiogenic stages as characterized by the absence of round and elongated spermatids in the seminiferous tubules of infertile mice (Fig. 4A). The seminiferous tubules appeared to consist mainly of Sertoli cells, easily identified on the basis of their morphology and location within the tubules of the infertile phenotype. They showed a high

degree of vacuolization inside their cytoplasm which filled the tubule lumen (Figs 3A and 4). Another observation was the cluster formation of the Sertoli cells (Fig. 4A). Examination of serial sections of testis showed that the presence of Sertoli cell clusters in the tubule lumen results from the unfolding of the seminiferous tubule wall.

In the hypofertile $\alpha 1b$ -AR-KO mice, the testes weight and volume were also significantly decreased ($P < 0.05$) but less drastically compared with infertile mice (Table 1 and Fig. 2). Testis weight was reduced to 73% of that in wild-type males (Fig. 2). This reduction in testis size was associated with reduced seminiferous tubule volume. However, the seminiferous tubules displayed an apparently normal histological structure (Fig. 3B) as compared with the wild-type mice (Fig. 3C), with no evident disruption or alteration of spermatogenesis. Further, tubular diameter of the cross sections of seminiferous tubules was not significantly reduced compared with that of wild-type mice ($207 \pm 12 \mu\text{m}$ versus $228 \pm 10 \mu\text{m}$ respectively). For both $\alpha 1b$ -AR-KO mice phenotypes, as well as for wild-type mice, we have not denoted any degenerative process during the studied period (from 3 to 8 months of age).

In addition to these histomorphometric parameters, $\alpha 1b$ -AR-KO males displayed a smaller absolute volume of interstitium compared with the wild-type mice (-50 and -80% in hypofertile and infertile animals respectively; Table 1). However, in this compartment, the mean absolute volume of the 3β -HSD positive cells did not change significantly in either group of mice (Table 1). Consequently, these cells represented 27, 17, and 7% of the absolute volume of the interstitium in the infertile, hypofertile, and wild-type males respectively. The immunostained Leydig cells were arranged in characteristic clusters in the peritubular space (Fig. 3). Differences were observed in the intensity of immunostaining for 3β -HSD, suggesting that Leydig cells may display different activity levels in $\alpha 1b$ -AR-KO males. In contrast, the morphology of Leydig cells was not adversely affected and no difference in Leydig cell size was noted among the experimental groups of mice. Further, for all the three mice groups, the Leydig cell compartment remained unchanged within the studied period (3–8 months).

Levels of testosterone, LH and FSH

To determine whether the disruption of spermatogenesis in infertile $\alpha 1b$ -AR-KO male was accompanied with an alteration of hormone levels, we measured circulating levels of testosterone, LH, and FSH. The results illustrated in Fig. 5A indicate a significant reduction of basal levels of plasma testosterone in $\alpha 1b$ -AR-KO males (-81% in infertile males and -55% in hypofertile males, $P < 0.05$) in comparison with the concentrations determined in control wild-type males. In the hypofertile mice, the range of plasma testosterone values was intermediate between that of wild-type mice and infertile $\alpha 1b$ -AR-KO mice (Fig. 5A).

Table 1 Mean testis volume (mm³) and mean absolute volume of testicular components (mm³) in wild-type and α1b-adrenergic receptor knockout (α1b-AR-KO) mice. Values are mean ± s.e.m. (n=14–27).

	Wild type mice (+/+)	α1b–/– hypofertile	α1b–/– infertile
Testis volume	69.4 ± 5.4	17.2 ± 1.1*	2.6 ± 0.3*,†
Mean absolute volume			
Seminiferous tubules	59.9 ± 2.7	12.5 ± 0.1*	0.7 ± 0.1*,†
Interstitial	9.4 ± 0.2	4.7 ± 0.1*	1.89 ± 0.04*,†
Leydig cells (3β-HSD staining)	0.65 ± 0.06	0.82 ± 0.10	0.52 ± 0.09

*P<0.05 compared with wild-type (+/+) mice. †P<0.05 compared with hypofertile α1b-AR-KO (–/–) mice.

Administration of hCG at an appropriate dose and time point produced normal increases in plasma testosterone levels in both hypofertile and infertile α1b-AR-KO males (Fig. 5A). This indicates the ability of Leydig cells to respond to exogenous gonadotropins. Nevertheless, although our results clearly showed a smaller range of stimulated plasma testosterone levels in infertile α1b-AR-KO males, no statistically significant differences for hCG-stimulated plasma testosterone concentrations were established within the three examined groups (Fig. 5A). This could be due to the large fluctuations in plasma testosterone levels with values ranging from <1 ng/ml to over 6.5 ng/ml in mice of the same age and treated under identical conditions. When incubated *in vitro*, testes from infertile α1b-AR-KO mice released significantly lower levels of testosterone (2.9 ± 0.6 pg/testis per h versus 4.9 ± 0.7 pg/testis per h in hypofertile males, P<0.05). Since the mean absolute volume of Leydig cells per testis was not changed in the infertile α1b-AR-KO mice, we suggest that the steroidogenic capacity of Leydig cells is compromised in infertile α1b-AR-KO males.

Interestingly, plasma LH levels measured in α1b-AR-KO infertile males were significantly higher than those observed in hypofertile and wild-type mice (Fig. 5B). In both the latter groups of males, LH values remained essentially similar (Fig. 5B). In contrast, basal plasma FSH concentrations were not affected in α1b-AR-KO mice (Fig. 5B).

Testicular content and expression of inhibin

Testicular content of inhibin B was highly increased (P<0.01) in infertile α1b-AR-KO mice (190 ± 30 pg/testis) in comparison with hypofertile and control mice (93 ± 17 and 53 ± 14 pg/testis respectively, values not significantly different). *In situ* analysis performed on testes from wild-type and α1b-AR-KO mice with an inhibin α riboprobe showed that the expression of inhibin α-subunit was restricted to the basal cytoplasm of Sertoli cells (Fig. 6). A similar cellular localization was observed for βB-subunit transcripts (data not shown). Further, data illustrated in Fig. 6C strongly suggested that the level of inhibin α-subunit expression was substantially higher in individual Sertoli cells of infertile α1b-AR-KO testes compared with control mice (Fig. 6A). This finding that Sertoli cells of infertile deficient mice do express inhibin α- and βB-subunits mRNA is an indication that these cells have kept some of their functions despite disruption of spermatogenesis.

Localization of α1b-AR expression in the testis

To determine the site(s) of α1b-AR expression in the testis, we hybridized testes sections of adult mice with specific DIG-labeled oligonucleotide antisense probes. α1b-AR-transcripts were predominantly detected in the cytoplasm of early spermatocytes (Fig. 7A) in a stage-specific manner as seen in the seminiferous epithelium of adjacent sections of tubules from wild-type testis (Fig. 7B). No reaction was observed in the presence of an excess of unlabeled probe (Fig. 7C).

Discussion

In the present study, we investigated the effect of α1b-AR invalidation on male reproductive performances, spermatogenic processes, and related endocrine parameters. Our findings show that 27% of α1b-AR KO males are infertile. The ability of a high percentage (73%) of α1b-AR-KO males to produce offspring probably explains why Cavalli *et al.* (1997) concluded on an unaltered fertility of their breeding colony. Furthermore, the α1b-AR KO females have follicular development, rate of pregnancy, and number of live pups per litter not notably disturbed in comparison with wild-type mice (S Mhaouty-Kodja unpublished data).

Fundamental perturbations that affect fertility of the 27% of mutant male mice include an extensive damage of testicular morphology, spermatogenesis arrest, and alterations of endocrine parameters. In contrast, a significant number of the homozygous males (about 73%) showed a relatively well-preserved spermatogenesis and minor endocrine defects. Nevertheless, these males produced fewer copulatory plugs and litter sizes than wild-type males. We ascribe this hypofertility, first, to a low sperm production in relation to the reduced volume of the seminiferous tubules compartment. Secondly, disturbances of ejaculatory competence cannot be excluded if we consider the effects of α1-AR blocking agents on rat ejaculatory dysfunction (Ratnasoorija & Wadsworth 1990, 1994). Alternatively, this hypofertile phenotype could also be a side effect of the invalidation approach.

By comparing the histological appearance of testes between 3 and 8 months of age, we have not denoted any extensive disruption of seminiferous epithelium in older hypofertile or

Figure 3 Comparison of the general appearance of the testis between infertile (A) and hypofertile (B) $\alpha 1b$ -AR-deficient mice and wild-type animals (C). All mice were at 4 months of age. Leydig cells (L) were immunolocalized in the interstitium by staining with a specific antibody for 3β -HSD and testis sections were counter-stained with hematoxylin. Roman numerals indicate the stages of the seminiferous epithelium. In the infertile $\alpha 1b$ -AR-KO male, note the reduced diameter of the seminiferous tubules, the few spermatogenic cells, and the vacuolization (va) of Sertoli cells. Bars = 50 μ m, magnification $\times 180$ for all micrographs.

infertile males. Further, there was no coexistence of normal and dysmorphic seminiferous tubules in the testes with disrupted spermatogenesis as reported in the infertile estrogen receptor KO (ERKO) mice model (Eddy *et al.* 1996).

Consequently, the percentage 27% infertile versus 73% hypofertile males remained nearly constant throughout all our study. Partial penetrance of infertility was also reported in other models of genetically modified mice. For instance, in mice lacking a functional aromatase (Robertson *et al.* 1999) or in transgenic mice overexpressing insulin-like growth factor-binding protein-1 (IGFBP-1) in the liver (Froment *et al.* 2004), ~25–30% of 3- to 6-month-old males showed impaired reproduction and spermatogenesis, whereas the other males produced offspring. The causes of partial penetrance of the phenotype are often attributed to the mixed genetic background of mice used in KO studies, although the involvement of additional nongenetic factors cannot be excluded.

In the infertile $\alpha 1b$ -AR-KO male mice, histological examinations clearly identified the step at which spermatogenesis becomes arrested. Early pachytene-like spermatocytes were the most evident apoptotic cells, suggesting that germ cells were entering a cell death pathway during meiosis. The remaining spermatogenic cells observed in the testes were spermatogonia and rare preleptotene/leptotene spermatocytes. Concomitantly, Sertoli cells appeared as masses of vacuolated cells. Similar morphological alterations of Sertoli cells were described in other germ cell-depleted situations as in *jsd/jsd* mice (Tohda *et al.* 2001), ERKO male mice (Eddy *et al.* 1996), or in rats treated with Sertoli cell toxicants (Hild *et al.* 2001). The early arrest in spermatogenesis, as a consequence of the formation of the vacuolated structures in the Sertoli cells, was also emphasized in mice deficient in inositol polyphosphate 5-phosphatase (Hellsten *et al.* 2002), or in mice lacking connexin 43 (Roscoe *et al.* 2001). In these models, it was suggested that massive vacuolization of Sertoli cells impairs the functional interactions between maturing germ cells and Sertoli cells, thus causing the germ cell apoptosis. In the present study, defective cell adhesion between Sertoli cells and germ cells could explain why cells reaching the prophase of meiosis have stopped developing before completion of the pachytene stage.

As assessed by low levels of testosterone, Leydig cell steroidogenesis seems to be deficient in all $\alpha 1b$ -AR-KO male mice. Indeed, Leydig cells, deprived of their normal environment, were unable to assume their normal functional capacities. The resulting dramatic depletion of testosterone in infertile mice is consistent with the failure of spermatocyte/spermatid development. Nevertheless, our present data in hypofertile males clearly evoke a critical threshold of testicular androgens to allow progression of spermatocytes into their mature state. In line with this observation, Zhang *et al.* (2003) reported that spermatogenesis in mice is possible without a high level of intra-testicular testosterone, thus contradicting the dogma of the past years. In the $\alpha 1b$ -AR-KO males, hCG owing to its high transducing efficiency upon LH receptor binding was able to stimulate testosterone secretion, suggesting that LH receptor expression and function may be rather well preserved in Leydig cells. This hypothesis was validated by a pilot study

Figure 4 Disruption of spermatogenesis and detection of apoptotic cells in the seminiferous tubules of infertile $\alpha 1b$ -AR-KO mice testis (A). Sections from animals of 4 months of age were stained with cresyl fast violet, $\times 330$. Seminiferous tubules contain spermatogonia (sg), preleptotene/leptotene spermatocytes (sp; upper panel), and pachytene spermatocytes (P) many of which in apoptosis germ cells (thick arrow; middle panel). Thin arrows show mitotic spermatogonia (lower panel). Sertoli cells (S) are present at the periphery of the tubules, containing vacuoles (va). Asterisk indicates Sertoli cell clusters. White arrows show peritubular myoid cells. Bars = $50\ \mu\text{m}$, same magnification for all micrographs. The inset in the middle panel ($\times 560$) shows the brown DAB precipitate reaction in the nucleus of early meiotic cells. (B) Labeling for the detection of apoptotic cells in the seminiferous tubules by the TUNEL method using fluorescein-labeled deoxy-UTP. Representative fluorescence of three experiments indicates apoptotic spermatocytes in the tubules of $\alpha 1b$ -AR-KO mice (upper panel). In wild-type mice, the seminiferous epithelium shows rare or no TUNEL-positive apoptotic cells (lower panel). Magnification $\times 200$.

designed, in collaboration with Schumacher *et al.* (Bicêtre, France), to identify and quantify the output of testicular progesterone as a precursor of testosterone production using gas chromatographic-mass spectrometric techniques (Liere *et al.* 2000). We found that progesterone was ~ 3.5 times lower in infertile KO mice than in wild-type mice, indicating the inability of Leydig cell population to adequately convert $\Delta 5$ -pregnenolone into progesterone and, thence, to assume testosterone pathway. This together with the differences noted in the intensity of their 3β -HSD immunostaining in the

KO males led us to conclude that the deficiency of steroidogenesis is probably due to an inability of newly formed Leydig cells to acquire normal levels of enzyme activity. In addition, the infertile males, which present the highest reduction in testosterone production, exhibited an expected increased level of circulating LH due to the absence of negative feedback exerted by testosterone on hypothalamic gonadotrophin-releasing hormone and pituitary LH secretion. The normal range of LH levels found in plasma of hypofertile mice indicated no potential alteration of this

Figure 5 Levels of testosterone and gonadotropins in wild-type and $\alpha 1b$ -AR-KO mice. (A) Plasma testosterone levels of untreated (basal) or hCG-administered wild-type and $\alpha 1b$ -AR-KO mice (4 months of age). Values are means $\pm$ S.E.M. of 5–15 animals. ^a $P < 0.05$ versus untreated wild-type (+/+) mice, ^b $P < 0.05$ versus untreated hypofertile knockout (-/-) mice, and ^c $P < 0.05$ versus untreated infertile knockout (-/-) mice. (B) Circulating LH and FSH levels of wild-type and $\alpha 1b$ -AR-KO males (4 months of age). Data are means $\pm$ S.E.M. of 6–15 animals. ^a $P < 0.05$ versus wild-type (+/+) mice, and ^b $P < 0.05$ versus hypofertile knockout (-/-) mice.

axis. Interestingly, similar alterations of testicular morphology and endocrine parameters as well as male reproductive performance with different degree of alteration were recently described in IGFBP-1 transgenic mice (Froment *et al.* 2004). However, a relationship between the $\alpha 1b$ -AR-signaling in spermatocytes and the IGF system components of the different testicular compartments would be highly speculative.

In infertile $\alpha 1b$ -AR-deficient mice, *in situ* hybridization experiments localized inhibin α - and β B-subunit transcripts in Sertoli cells. No appreciable signals were detected over Leydig/interstitial cells, in accordance with previous observations in male mice (Tone *et al.* 1990). The finding that Sertoli cells in $\alpha 1b$ -AR-KO males keep their ability to give rise to the seminiferous epithelium and express inhibin-subunits mRNA, at least as much as in wild-type testis, is an indication that Sertoli cells are probably functionally competent. Such observation, which is consistent with the detection of inhibin in the testis, indicates that Sertoli cells remain responsive to stimuli responsible for inhibin production. Sertoli cell production of inhibin B may be sufficient to exercise a normal degree of negative feedback control on pituitary FSH secretion (Hayes *et al.* 2001, Meachem *et al.* 2001). Indeed, plasma levels of FSH

Figure 6 Distribution of inhibin α -subunit in testicular sections of wild-type mice (A) and hypofertile (B) and infertile (C) $\alpha 1b$ -AR-KO mice. *In situ* hybridization, representative of three independent experiments, shows that mRNA expression is found predominantly within basal cytoplasm of Sertoli cells. i indicates interstitium. Bars = 50 μ m, magnification $\times 180$ for all micrographs.

in the $\alpha 1b$ -AR-KO male mice are unchanged compared with normal mice. This demonstrates that no intimate relationship exists between testosterone concentrations and pituitary FSH secretion. It is thus unlikely that this gonadotropin favorably influences the completion of meiosis and the initiation of spermiogenesis via Sertoli cells in the infertile $\alpha 1b$ -AR-KO males. This contrasts with data obtained in other transgenic models (Krishnamurthy *et al.* 2000, Allan *et al.* 2001). Besides the drastic deficit in testosterone production in the $\alpha 1b$ -AR infertile male mice, a deleterious effect of high testicular concentrations of inhibin B on spermatogenesis (van Dissel-Emiliani *et al.* 1989) cannot be excluded.

Figure 7 Localization of $\alpha 1b$ -AR mRNA expression in wild-type testis. *In situ* hybridization is representative of three independent experiments. $\alpha 1b$ -AR-mRNA is restricted to the perinuclear cytoplasm of early spermatocytes at stages II/III of seminiferous epithelium (A), $\times 400$. Transcripts were expressed in a stage-specific manner (B), $\times 120$. The negative control was performed in the presence of a 100-fold excess of unlabeled oligonucleotide (C), $\times 120$. Bars = 50 μ m.

Our results show, for the first time, that the ubiquitous deletion of $\alpha 1b$ -AR expression alters fertility, spermatogenesis, Leydig cell response to LH, and testosterone production in the adult male mutants. Since we detected $\alpha 1b$ -AR transcripts in germ cells during early meiotic prophase stages, one possibility is that catecholamines act directly on maturing spermatocytes to maintain spermatogenesis. Spermatogenesis arrest in KO males would then indirectly affect Sertoli cell/Leydig cell communications (Onoda *et al.* 1991), thereby reducing testosterone production. Another possibility is that germ cell $\alpha 1b$ -AR signaling is involved in the production of paracrine factors, which regulate Leydig cell homeostasis. The absence of $\alpha 1b$ -AR germ cell in KO males would then have a deleterious effect on testosterone production. This could, in turn, result in deficient spermatogenesis as known in androgen-deficient models or more recently in mice where the androgen receptor was selectively invalidated in Sertoli cells (De Gendt *et al.* 2004). Future studies need to be addressed to identify which of spermatocytes or

Leydig cells in $\alpha 1b$ -AR-KO males are the primary affected cells. For this, administration of testosterone to infertile KO males could be performed. The absence of $\alpha 1b$ -AR at the hypothalamic level could also contribute to the infertile phenotype of $\alpha 1b$ -AR-KO mice. Indeed, this receptor is expressed in the hypothalamus, in areas related to reproductive functions such as the preoptic nucleus (Papay *et al.* 2004). In contrast to females where the facilitating role of nor-epinephrine via $\alpha 1b$ -AR in lordosis behavior and preovulatory LH surge is well established (reviewed by Etgen 2003), the importance of the hypothalamic $\alpha 1b$ -AR in male reproductive physiology is less documented. However, it was recently described that transgenic mice overexpressing the $\alpha 1b$ -AR in the central nervous system exhibit an altered fertility (Zuscik *et al.* 2000). Transplantation of germ cells from $\alpha 1b$ -AR-KO males into spermatogenesis-depleted wild-type testes as reported by Dobrinski *et al.* (Honaramooz *et al.* 2005) could help to evaluate the importance of the cerebral $\alpha 1b$ -AR in the regulation of male spermatogenesis and fertility.

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