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Follicle-Stimulating Hormone and Luteinizing Hormone Mediate the Androgenic Pathway in Leydig Cells of an Evolutionary Advanced Teleost[1]

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ABSTRACT

The endocrine pathways controlling vertebrate spermatogenesis are well established in mammals where the pituitary gonadotropins follicle-stimulating hormone (FSH) and luteinizing hormone (LH) exclusively activate the FSH receptor (FSHR) in Sertoli cells and the LH/choriogonadotropin receptor (LHCGR) in Leydig cells, respectively. In some teleosts, however, it has been shown that Lh can cross-activate the Fshra ortholog, and that Leydig cells coexpress the Lhcgrba and Fshra paralogs, thus mediating the androgenic function of Fsh in the testis. Here, we investigated whether these proposed mechanisms are conserved in an evolutionary advanced pleuronectiform teleost, the Senegalese sole (*Solea senegalensis*). Transactivation assays using sole Fshra- and Lhcgrba-expressing cells and homologous single-chain recombinant gonadotropins (rFsh and rLh) showed that rFsh exclusively activated Fshra, whereas rLh stimulated both Lhcgrba and Fshra. The latter cross-activation of Fshra by rLh occurred with an EC_{50} 4-fold higher than for rFsh. Both recombinant gonadotropins elicited a significant androgen release response in vitro and in vivo, which was blocked by protein kinase A (PKA) and 3beta-hydroxysteroid dehydrogenase inhibitors, suggesting that activation of steroidogenesis through the cAMP/PKA pathway is the major route for both Lh- and Fsh-stimulated androgen secretion. Combined in situ hybridization and immunocytochemistry using cell-specific molecular markers and antibodies specifically raised against sole Fshra and Lhcgrba demonstrated that both receptors are expressed in Leydig cells, whereas Sertoli cells only express Fshra. These data suggest that Fsh-mediated androgen production through the activation of cognate receptors in Leydig cells is a conserved pathway in Senegalese sole.

flatfish, gonadotropin receptors, gonadotropins, Leydig cells, spermatogenesis, steroidogenesis

INTRODUCTION

Spermatogenesis is essential for the reproductive biology of dioecious and hermaphroditic vertebrates. It is a highly organized and coordinated process, in which diploid spermatogonia proliferate, undergo meiosis, and differentiate to form mature spermatozoa. It is well established that the pituitary gonadotropins follicle-stimulating hormone (FSH) and luteinizing hormone (LH) play major roles in the regulation of spermatogenesis in all vertebrates [1]. These hormones consist of heterodimers formed by one common α-chain (CGA) and one hormone-specific β-chain (FSHβ or LHβ) [2]. Gonadotropins act on the testis through the activation of their cognate receptors, the LH/choriogonadotropin receptor (LHCGR) and the FSH receptor (FSHR), both of which are members of the family of glycoprotein hormone receptors (GpHRs). In mammals, the physiological roles of FSH and LH in spermatogenesis are distinct and determined by the specific interaction of each hormone with its cognate receptor. Thus, FSH regulates several Sertoli cell functions through FSHR, including the structural, nutritional, and regulatory (paracrine) support of germ cell development, whereas LH regulates sex steroid production by LHCGR-expressing Leydig cells [3].

Teleosts have two gonadotropins orthologous to mammalian FSH and LH, as well as orthologous testicular receptors [4, 5]. The teleost-specific *fshra* gene seems to have evolved from a common ancestor, whereas two mutually exclusive *lhcgr* genes, *lhcgrba* and *lhcgrbb*, exist within the teleost lineage but with conserved functions [6]. By using highly purified gonadotropin preparations or homologous recombinant hormones, it has also been shown that the role of gonadotropins in teleost spermatogenesis seems to be more complex than in mammals. Ligand-binding studies indicate that teleost Fshra shows a preference for Fsh, but it can also be activated by Lh [7–14]. However, such receptor promiscuity is apparently not found in the rainbow trout (*Onchorynchus mykiss*) [15], the Manchurian trout (*Brachymystax lenok*) [16], or the European sea bass (*Dicentrarchus labrax*) [17, 18]. Nevertheless, Fsh is a potent stimulator of testicular androgen production in teleosts. This has been demonstrated both in vitro [14, 17–25] and in vivo [26, 27]. In recent studies, it has been shown that Leydig cells also express *fshra* in addition to *lhcgrba* (or the paralogous gene *lhcgrbb*) [14, 22, 27], although the synthesis of the Fshra protein product in testicular Leydig cells has been observed only in the Japanese eel (*Anguilla japonica*) [22]. In the zebrafish (*Danio rerio*), expression of *lhcgrba* in addition to *fshra* in Sertoli cells has been reported, which might be related to the undifferentiated gonochoristic mode of gonadal sex differentiation in this species [27]. It has thus been suggested that the steroidogenic potency of Fsh in the teleost testis can be explained by its direct trophic action on Fshra-expressing Leydig cells [14].

Previous studies on the mechanism of action of gonadotropins on teleost spermatogenesis have been conducted on a limited number of species belonging to ancestral lineages, including eels, catfish, cyprinids, and salmonids [4, 5]. In more

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advanced teleosts, such as Pleuronectiformes (flatfish), it is known that plasma androgens increase during testis recrudescence before spawning [28–35], whereas progestins have been associated with final sperm maturation and hydration [36–38]. However, the role of gonadotropins in the regulation of testicular steroid release in flatfish is poorly understood. A pioneering study by Ng and Idler [39] showed that gonadotropins isolated from pituitaries of the winter flounder (*Pseudopleuronectes americanus*) stimulated androgen release of hypophysectomized mature males. Other studies using human chorionic gonadotropin (hCG) obtained different responses depending on the species. In the common dab (*Limanda limanda*), hCG increased progestin production but had no effect on androgen plasma levels [28], whereas in the Senegalese sole (*Solea senegalensis*), hCG greatly enhanced the circulating levels of androgens [40]. These discrepancies could be related to the specific developmental stage of the testis of the fish used for these experiments, or to a species-specific response of gonadotropin receptors to hCG. In Atlantic halibut (*Hippoglossus hippoglossus*), pituitary Fsh and Lh have been purified and shown to stimulate androgen release in testicular explants of another flatfish, the turbot (*Scophthalmus maximus*) [20]. However, in Atlantic halibut, or in any other flatfish, there is no information on the cellular distribution of gonadotropin receptors in the testis or their steroidogenic functions.

The Senegalese sole exhibits semicystic spermatogenesis, in which the release of germ cells occurs already at the spermatid or spermatocyte stage, and spermiogenesis and meiosis are completed in the tubular lumen [34, 35, 41, 42]. In this species, cDNAs encoding the gonadotropin subunits as well as the *fshra* and *lhcgrba* have been previously isolated and characterized [6, 35]. Therefore, an initial objective of the present study was the production of Senegalese sole recombinant gonadotropins as well as of specific antibodies against Fshra and Lhcgrba. These homologous tools have allowed investigation for the first time into the pattern of gonadotropin signaling in the testis of an evolutionary advanced teleost, including the ligand preferences of the Fsh and Lh receptors, their in vivo cellular localization in the testis, and the biological activities of the gonadotropins in relation to testicular androgen release.

MATERIALS AND METHODS

Animals

Adult Senegalese sole raised in captivity (F1 generation; weight, 1024 ± 61 g, mean ± SEM) were obtained from the Institut de Recerca i Tecnologia Agroalimentàries (IRTA) in San Carles de la Ràpita (Tarragona, Spain), transported to the Institut de Ciències del Mar (CSIC; Barcelona, Spain), and maintained under ambient temperature and photoperiod as described previously [42]. Wild Senegalese sole (weight, 233 ± 9 g, mean ± SEM) were captured in the Ebro delta (Spain) in autumn and acclimated to captivity at the IRTA facilities. Fish were sampled during the natural reproductive cycle in April or November, approximately coinciding with the two annual spawning periods described for Senegalese sole under natural temperature regimes [43]. At all sampling times, fish were sedated with 500 ppm of phenoxyethanol and killed by decapitation. The fish were weighed before removal of the testis in order to determine the gonadosomatic index (GSI; testes weight/fish weight × 100). Different testis samples were then processed for histology as previously described [6], deep-frozen in liquid nitrogen and stored at −80°C, or used for in vitro experiments. Procedures relating to the care and use of animals were approved by the Ethics Committee from IRTA in accordance with the Guiding Principles for the Care and Use of Laboratory Animals.

Expression Constructs

Recombinant single-chain Senegalese sole Fsh and Lh (rFsh and rLh, respectively) cDNAs were synthesized in vitro (Life Technologies Corp.), following the same strategy as described for human FSH [44], and were subcloned into the pcDNA3 expression vector (Life Technologies). The expression constructs comprised the entire coding sequence of Senegalese sole Fsh β subunit (GenBank accession no. ABW81403) or Lh β subunit (GenBank accession no. ABW81404), followed by six His residues, the carboxyl-terminal peptide sequence of hCG β subunit as a linker, and the mature sequence of the Senegalese sole glycoprotein hormone α subunit (GenBank accession no. ABW81405). The Senegalese sole cDNAs encoding the FSH and LH/choriogonadotropin receptors, Fshra and Lhcgrba following the nomenclature proposed by Chauvigné et al. [6] (GenBank accession nos. GQ472139 and GQ472140, respectively), were cloned into the *Eco*RI/*Xba*I sites of the pcDNA3 vector with the addition of a epitope FLAG tag (DYKDDDDK) inserted by PCR in the C-terminus of the encoded protein before the stop codon.

Production of Senegalese Sole Recombinant Gonadotropins

Chinese hamster ovary (CHO) cells were cultured in Dulbecco modified Eagle medium (DMEM) supplemented with penicillin/streptomycin and 5% fetal bovine serum, at 37°C with 5% CO_2. Cells were transfected with pcDNA3-rFsh or pcDNA3-rLh expression plasmids (each bearing the β or α subunit as indicated above) in 24-well plates using the Lipofectamine Reagent (Life Technologies). To obtain the stable clones, 48 h after transfection cells were replated in 96-well plates and selected using Geneticin antibiotic (G418; Sigma-Aldrich) at 500 μg/ml. Stable clones were isolated and expanded, and their efficiency of production was evaluated by the luciferase reporter assay on HEK293T cells stably expressing the European sea bass gonadotropin receptors [18]. For large-scale production, selected clones were grown at 37°C with 5% CO_2 in 225-cm^3 flasks containing 35 ml of complete DMEM. When the cells reached confluence, they were incubated at 28°C and 5% CO_2 in DMEM without serum during 8 to 10 days. After this production period, the medium was centrifuged at 15 000 × *g* for 15 min, and the supernatant containing recombinant hormones was purified by immobilized metal affinity chromatography using His GraviTrap columns (GE Healthcare). The final elution step was carried out with 500 mM imidazole, and the eluate was concentrated 250 times using Amicon Ultra-15 3K centrifugal filter devices (Millipore). The concentration of imidazole in the concentrated solution was reduced to approximately 0.1 mM by successive washes in PBS (pH 7.5). A sample of the medium was recovered at every step of the purification procedure, as well as after concentration, mixed with Laemmli sample buffer, boiled, and examined with SDS-PAGE using 12% acrylamide minigels. Proteins were visualized by silver staining, and the recombinant gonadotropins were detected by Western blotting (see below). Protein concentration was measured using the Bio-Rad Protein Assay kit (Bio-Rad Laboratories), and the purity of the recombinant hormones was estimated using densitometry of SDS-PAGE analysis with the Quantity-One software (Bio-Rad Laboratories).

Production of Polyclonal Antisera for Senegalese Sole Gonadotropin Receptors

Antisera were raised in rabbits against synthetic peptides corresponding to amino acid residues 657–671 or 641–657 for Senegalese sole Fshra and Lhcgrba, respectively [6], with the predicted initiation codon (methionine, ATG) designated as residue 1 (AntibodyBcn). The antisera were affinity purified against the synthetic peptides (AntibodyBcn), and their specificity was confirmed by ELISA and immunofluorescence microscopy on HEK293T cells (see below).

Functional Analysis of Senegalese Sole Recombinant Gonadotropins

Senegalese sole gonadotropin receptor cDNA constructs were stably or transiently expressed in HEK293T cells. The cells were grown in DMEM supplemented with penicillin/streptomycin, 2 mM glutamine, and 10% fetal bovine serum (Life Technologies), and were incubated at 37°C in 5% CO_2. Receptor activation was measured using a reporter gene assay following the protocol described by Andersson et al. [13]. At approximately 70% confluence, cells were transfected in 24-well plates with 300 ng of total DNA per well using the calcium phosphate method. Cells were transfected for 18 h with 75 or 150 ng of each receptor or empty pcDNA3, 75 ng of cAMP-responsive reporter gene plasmid (pCRE-luc; Agilent Technologies), and 75 ng of β-galactosidase (β-Gal) plasmid (Promega Corp.). After 36 h, the medium was replaced with serum-free DMEM medium for at least 3 h, and cells were incubated for 6 h with different concentrations of forskolin as positive control, hCG, or Senegalese sole rFsh or rLh. All treatments were performed in triplicate. The cells were washed in PBS and lysed with 100 μl of Reporter Lysis Buffer (Promega Corp.), and luciferase and β-Gal activities were measured by

luminescence or colorimetric detection, respectively, using reconstituted Luciferin (Biothema) or *o*-nitrophenyl βD-galactopyranoside (Sigma-Aldrich) substrates. The data on luciferase activity were normalized to those of β-Gal.

Immunoblotting

Protein samples containing rFsh or rLh were denatured at 95°C, subjected to 12% SDS-PAGE, and blotted into nitrocellulose membranes (Sigma-Aldrich). Membranes were blocked with 5% nonfat dry milk in TBST (20 mM Tris, 140 mM NaCl, 0.1% Tween [pH 7.6]) for 1 h at room temperature, and incubated with anti-sea bass Fshβ or Lhβ antibodies (1:1000) [45, 46] overnight at 4°C. Bound antibodies were detected with horseradish peroxidase (HRP)-coupled anti-rabbit immunoglobulin G (IgG) secondary antibodies and Chemiluminescent Picomax Sensitive HRP Substrate (Rockland Immunochemicals Inc.). Alternatively, membranes were incubated with 6xHis monoclonal antibody-HRP conjugate (Clontech Laboratories Inc.) and revealed with Immobilon Western (Millipore). An aliquot of purified recombinant hormones was deglycosylated by incubation with 500 units of *N*-Glycosidase F (PNGase F; New England Biolabs Inc.) for 3 h at 37°C prior to SDS-PAGE.

For Western blotting of gonadotropin receptors, a piece of testis (20 mg) was homogenized in 150 mM NaCl, 50 mM Tris (pH 7.4), 1% Triton X-100, 5 mM ethylene diamine tetraacetic acid, 5 mM ethylene glycol tetraacetic acid, 0.5% sodium deoxycholate, 0.1% SDS, 1 mM PMSF, and a cocktail of protease inhibitors (Roche Applied Science, Mannheim, Germany). The homogenate was incubated on ice for 15–20 min before sonication (3× 1 min on ice), and centrifuged at 14 000 × *g* at 4°C during 10 min. Untreated protein extracts and extracts treated with PNGase F were electrophoresed in 8% SDS-PAGE gels, transferred to nitrocellulose membranes, and blocked as above. Membranes were incubated overnight at 4°C with anti-Senegalese sole Fshra or Lhcgrba antisera (1:500). Bound antibodies were detected as described above.

Immunohistochemistry and Immunofluorescence Microscopy

For immunohistochemistry, subsamples of testes were fixed in 4% paraformaldehyde (PFA) in PBS for 6 h, washed, dehydrated, and embedded in Paraplast (Sigma-Aldrich). Sections of 7 μm were then rehydrated in decreasing ethanol solutions, permeabilized with 0.2% Triton X-100 in PBS, and blocked in 5% goat serum, 0.1% bovine serum albumin (BSA) in PBST (0.1% Tween in PBS). Incubation with the Senegalese sole Fshra or Lhcgrba antisera (1:400) was performed overnight at 4°C in 1% goat serum, 0.1% BSA in PBST. After washing, sections were incubated with a secondary anti-rabbit IgG HRP-coupled antibody (Rockland Immunochemicals Inc.) for 2 h, and specific staining was revealed by 3,3′-diaminobenzidine (Sigma-Aldrich). Sections were counterstained with hematoxylin, dehydrated, and mounted in Fluoromount Aqueous Mounting Medium (Sigma-Aldrich).

Immunofluorescence on cultured HEK293T cells was carried out on cells stably transfected with FLAG-tagged Senegalese sole Fshra or Lhcgrba and grown on coverslips close to confluence. Cells were washed in PBS containing 1 mM $MgCl_2$, fixed in methanol for 6 min at −20°C, and permeabilized in acetone for 30 sec at −20°C. The Fshra and Lhcgrba antibodies (1:500) or anti-FLAG M2 monoclonal antibody (1:400; Sigma-Aldrich) were applied overnight at 4°C and revealed by anti-rabbit or anti-mouse fluorescein isothiocyanate (FITC)-coupled secondary antibodies (1:1000; Sigma-Aldrich).

For immunofluorescence microscopy on testis sections, the protocol was the same as that used for immunoperoxidase, but the anti-rabbit IgG HRP-coupled secondary antibody was replaced with that coupled to FITC or Cy3. In colocalization experiments, sections were first incubated with anti-Fshra antibodies followed by the anti-rabbit IgG Cy3-coupled secondary antibody. After postfixation with 4% PFA for 15 min, sections were incubated with anti-Lhcgrba antibodies labeled with FITC using the Lightning-Link Fluorescein Conjugation kit (Innova Biosciences) overnight at 4°C. The sections were washed in PBS and the cell nucleus counterstained with 4′,6-diamidino-2-phenylindole dihydrochloride (DAPI; 1:3000; Sigma-Aldrich) for 3 min, and finally mounted with Fluoromount.

Combined In Situ Hybridization and Immunofluorescence Microscopy

For colocalization of mRNA and protein, in situ hybridization was performed first, followed by immunofluorescence. Samples of testis were fixed in 4% PFA for 16 h at 4°C and were subsequently dehydrated and embedded as described above. In situ hybridization was performed on 7-μm sections using digoxigenin-incorporated cRNA probes synthesized with SP6 and T7 RNA polymerases using the DIG RNA Labeling Kit (Roche Applied Science). Probes were synthesized for Senegalese sole *lhcgrba* (nucleotides 763-1555) and for steroidogenic acute regulatory protein (*star*; GenBank accession no. HQ392856; nucleotides 483-1051). Hybridization was performed as previously described [35] with some modifications to allow subsequent immunofluorescence. The proteinase K step was avoided and was replaced with the incubation of the sections in TBS containing 0.1% Triton X-100 for 10 min to permeabilize the tissue. The acetylation step (0.25% acetic anhydride in 0.1 M triethanolamine [pH 8.0]) was reduced to 5 min, and the posthybridization washing was 50% formamide in 2× saline-sodium citrate (SSC) at 50°C, followed by two washes in 2× SSC for 10 min at 50°C and a final wash in 0.5× SSC at 42°C. After 30 min, sections were blocked with 0.5% BSA, and hybridized riboprobes were detected with mouse anti-digoxigenin antibody (1:250; Roche Applied Science) overnight at 4°C. The green fluorescence was obtained by using Alexa 488-conjugated goat anti-mouse IgG secondary antibodies (1:500; Molecular probes; Life Technologies) in TBST for 2 h at room temperature. The sections were subsequently refixed with 4% PFA for 15 min and incubated with the Senegalese sole Fshra or Lhcgrba antisera (1:250 in PBST with 0.1% BSA) overnight at 4°C. The red fluorescence was obtained by using Cy3-coupled sheep anti-rabbit secondary antibody (1:400; Sigma-Aldrich) in PBST for 2 h at room temperature. Sections were finally washed in PBS, counterstained with DAPI, and mounted as above.

In Vitro Culture of Testicular Explants

Pieces of the testes collected from males with a GSI of 0.13 ± 0.03 were washed in PBS containing 1 mM $MgCl_2$ and 1 mM $CaCl_2$, transferred to a Petri dish with Leibovitz L-15 culture medium without phenol red (Life Technologies), and cut into smaller fragments of approximately 5–15 mg. The pieces were placed into 48-well plates with 350 μl of L15 medium and incubated in triplicate with rFsh or rLh during 24 h at 18°C in a temperature-controlled incubator. The culture medium was collected in three 100-μl aliquots, and the testis explants were weighted, deep-frozen in liquid nitrogen, and stored at −80°C. In some experiments, explants were preincubated with different doses (1 or 5 μM) of the adenylyl cyclase inhibitor (MDL-12330A; Sigma-Aldrich), the cAMP-dependent protein kinase A (PKA) inhibitor H-89 (Cayman Chemical Co.), or the 3β-hydroxysteroid dehydrogenase (3β-Hsd) inhibitor trilostane (TRIL; Selleck Chemicals), 1 h before gonadotropin addition.

In Vivo Action of Gonadotropins on Androgen Production

Senegalese sole F1 males (1227 ± 92 g; n = 13) were collected in March and injected intramuscularly with 40 μg/kg body weight rFsh or rLh in ~200 μl of vehicle (PBS with 0.1 mM imidazole [pH 7.5]). Control fish were injected with vehicle alone. Before hormonal treatments were administered, and at 24 and 48 h after injection, animals were sedated with 500 ppm phenoxyethanol, and approximately 0.5 ml of blood was extracted from the caudal vein with a heparinized syringe, centrifuged, and the plasma stored at −80°C until analysis.

Steroid Determination

Levels of testosterone (T) and 11-ketotestosterone (11-KT) in plasma and culture medium were determined by commercial enzyme immunosorbent assay (EIA; Cayman Chemical) as previously described [42, 47]. Free steroids were extracted from plasma (3.5 μl) in methanol, whereas culture medium was diluted in EIA buffer (1:20 and 1:100 for T and 11-KT determinations, respectively). All samples were analyzed in duplicate, and for each EIA plate a separate standard curve was run. The lower limits of detection for T and 11-KT were 5.69 pg/ml and 1.20 pg/ml, respectively. The intraassay coefficients of variance were less than 10% and 13% for T and 11-KT, respectively, whereas the interassay coefficients of variance were less than 5% and 10% for T and 11-KT, respectively. Steroid levels in the culture medium were normalized with respect to the weight of the testis explants.

Statistics

Data analyses were carried out by one- or two-way ANOVA, after log-transformation of the data when needed, followed by the Duncan multiple range test. A value of $P < 0.05$ was considered to be significant.

RESULTS

Production and Purification of Senegalese Sole Single-Chain rFsh and rLh

Recombinant Fsh and Lh were efficiently produced and secreted in the culture medium using mammalian CHO cells.

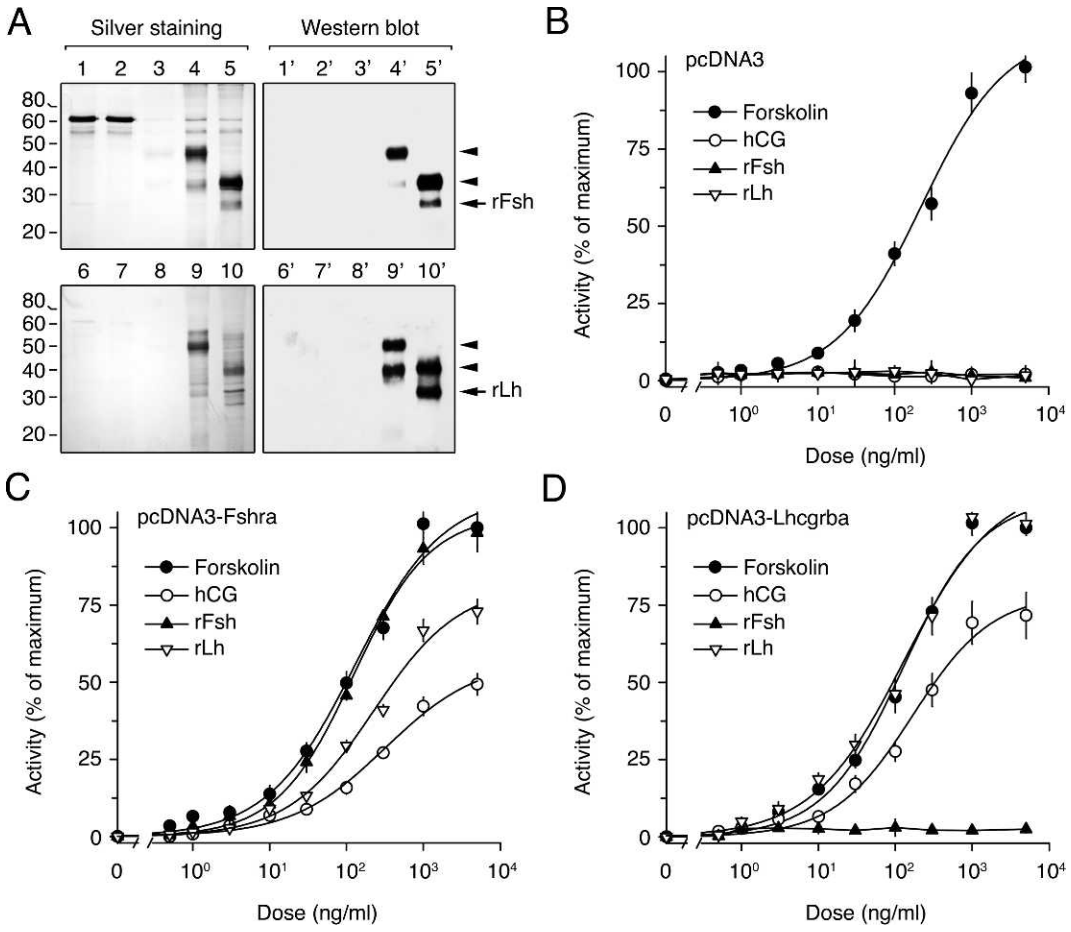

FIG. 1. Production of Senegalese sole rFsh and rLh and response of cognate receptors. **A**) SDS-PAGE (left) and Western blot (right) analyses of culture medium from CHO cells producing rFsh (upper) and rLh (lower). SDS-PAGE was conducted under reducing conditions, and Western blot analysis was carried out using anti-sea bass Fsh and Lh antibodies. Lanes 1 and 6, culture medium; lanes 2 and 7, flow-through fraction of Ni-NTA column; lanes 3 and 8, fraction eluted with 500 mM imidazole; lanes 4 and 9, eluate 100× concentrated; lanes 5 and 10, concentrated eluate treated with PNGase F. The arrows indicate single-chain deglycosylated peptides, whereas the arrowheads indicate glycosylated forms. Molecular mass markers (kDa) are on the left. **B**–**D**) Effect of sole rFsh and rLh, hCG, or forskolin on HEK293T cells transiently transfected with reporter gene constructs alone (pcDNA3; **B**) or in combination with either Senegalese sole Fshra (pcDNA3-Fshra; **C**) or Lhcgrba (pcDNA3-Lhcgrba; **D**) expression plasmids. Hormone-induced cAMP production was indirectly quantified by measuring the luciferase activity from the reporter vector. The responses were normalized to the maximal activity induced by forskolin, which was set at 100%. Data (mean ± SEM) are derived from three to five independent transfections.

Culture media from CHO selected clones were concentrated after His GraviTrap column purification and were analyzed by SDS-PAGE and Western blotting (Fig. 1A). Thick positive bands reacting with the sea bass Fsh and Lh antisera, as well as with the anti-His monoclonal antibody (data not shown), with molecular masses of approximately 45 and 52 kDa, were considered to be rFsh and rLh, respectively (Fig. 1A, lanes 4 and 9). When eluates were treated with PNGase F, minor bands of approximately 27 and 30 kDa were visible, indicating that rFsh and rLh were *N*-glycosylated (Fig. 1A, lanes 5 and 10). The purities of rFsh and rLh in the first trial of purification were estimated to be 89% and 91%, respectively. The amounts of rFsh and rLh recovered from 350 ml of medium were 689 and 594 μg, respectively. In the second trial, the purity of rFsh and rLh was estimated to be 91% for both, and the amounts recovered were 383 and 200 μg, respectively.

Functional Analysis of Recombinant Gonadotropins and Receptor Specificity

The biological activity and specificity of rFsh and rLh were tested by ligand-induced cAMP production in HEK293T cells transiently transfected with Senegalese sole *fshra* and *lhcgrba*. The recombinant gonadotropins, as well as hCG and forskolin, were assayed at doses ranging from 0 to 5 μg/ml. In cells transfected with empty pcDNA3 expression plasmid and the reporter gene plasmids pCRE-Luc and β-Gal, forskolin-induced cAMP levels resulted in a concentration-dependent increase in luciferase activity (Fig. 1B). Incubation with rFsh, rLh, or hCG did not induce measurable increases in luciferase activity, showing that HEK293T cells do not endogenously respond to Senegalese sole gonadotropins. When cells were transfected with a combination of pCRE-Luc/β-Gal and pcDNA3-Fshra expression plasmid, incubation with Senegalese sole rFsh induced concentration-dependent activation equivalent to forskolin (Fig. 1C). Interestingly, Senegalese sole rLh and hCG also activated reporter gene expression in cells expressing the Fshra, although the half-maximal effective concentrations (EC_{50}) of rLh and hCG were 4- and 44-fold higher, respectively, compared with that of rFsh (Fig. 1C). Cells expressing sole Lhcgrba showed a clear response to rLh and hCG (Fig. 1D). The response to hCG was 3.5-fold lower than to rLh. No response was noted for rFsh up to a dose of 5

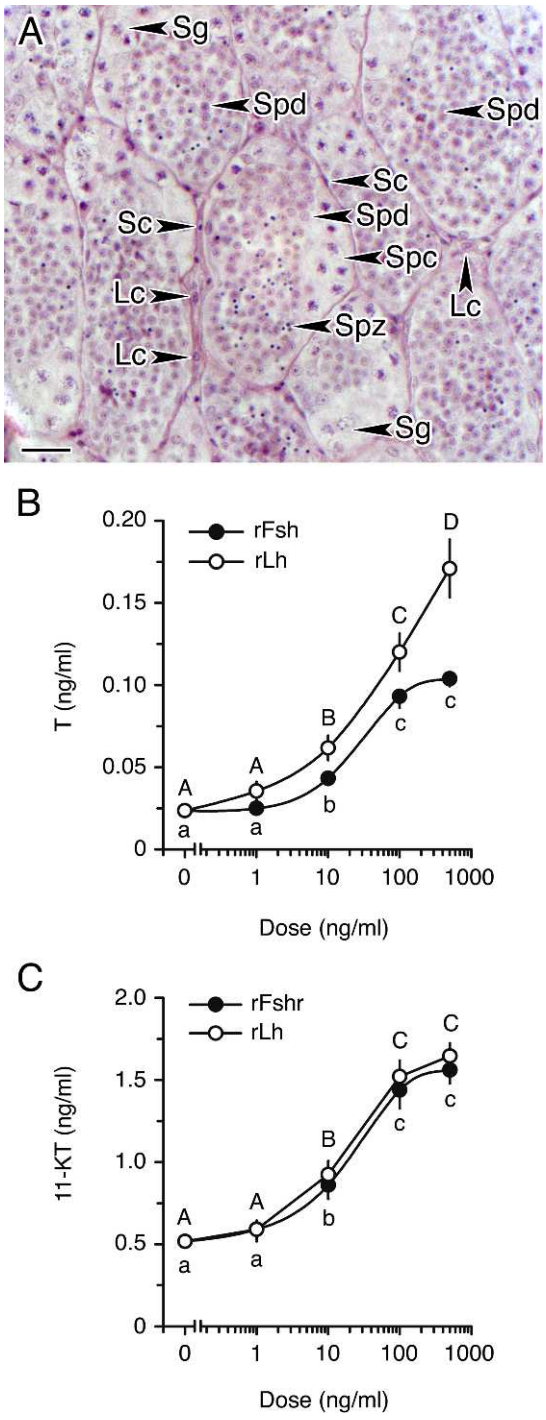

FIG. 2. Gonadotropin-stimulated androgen release by Senegalese sole testicular explants. **A**) Photomicrograph of a representative histological section of the testis explants used for the experiments. Lc, Leydig cell; Sc, Sertoli cell; Sg, spermatogonia; Spc, spermatocyte; Spd, spermatid; Spz, spermatozoa. Bar = 20 μm. **B** and **C**) Amounts of T (**B**) and 11-KT (**C**) measured in incubation media after 24 h of exposure to increasing concentrations of rFsh and rLh. Values (mean ± SEM) represent compiled data from three experiments on three different males, each with three replicates per ligand concentration. Different letters denote significant differences for each hormone ($P < 0.05$).

μg/ml. The receptors did not display signs of constitutive activity because their expression in cells did not increase basal (i.e., nonstimulated) luciferase activity or potentiate the response to forskolin (data not shown).

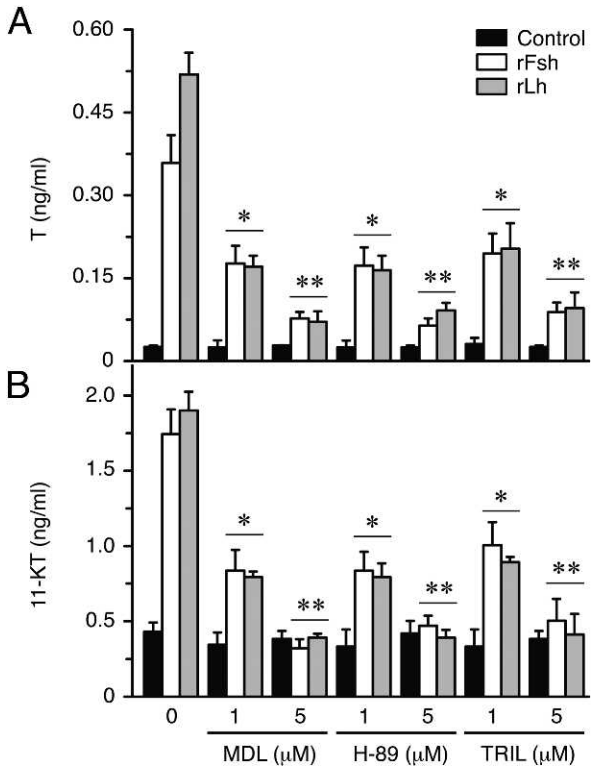

FIG. 3. Effects of inhibitors of adenylyl cyclase (MDL-12330A; MDL), PKA (H-89), or 3β-Hsd (TRIL) on gonadotropin-stimulated androgen release by Senegalese sole testicular explants. Amounts of T (**A**) and 11-KT (**B**) measured in incubation media after 24 h of exposure to 250 ng/ml rFsh or rLh, and in combination with 1 or 5 μM MDL-12330A, H-89, or TRIL. Values (mean ± SEM) represent compiled data from two experiments on two different males, each with three replicates per condition. Asterisks denote significantly different values (*$P < 0.05$; **$P < 0.01$) with respect to the groups stimulated with gonadotropins alone.

Gonadotropin Stimulation of Androgen Production In Vitro

To investigate whether rFsh and rLh were able to stimulate testicular androgen release in Senegalese sole, explants of testes were incubated in the presence of increasing doses of rFsh or rLh, ranging from 0 to 500 ng/ml (Fig. 2). The histological analysis of the testes used for these experiments showed that most of the seminiferous tubules were filled with spermatids and few spermatozoa (Fig. 2A). The basal levels of 11-KT released by the testicular explants were ~26-fold higher than those of T (0.52 and 0.02 ng/ml, respectively; Fig. 2, B and C). When the explants were incubated with rFsh, androgen secretion increased gradually with the gonadotropin concentration. A dose of 10 ng/ml rFsh elicited the first significant elevation of both T (Fig. 2B) and 11-KT (Fig. 2C) release (~1.8- and ~1.6-fold above basal levels, respectively). Maximum androgen release (~4-fold for T and ~2.8-fold for 11-KT) was reached at 100 ng/ml rFsh. No further significant increase in T or 11-KT release was observed at 500 ng/ml rFsh.

For rLh, a dose of 10 ng/ml also induced the first significant increase in both T and 11-KT secretion (~2.6- and ~1.7-fold above basal levels, respectively; Fig. 2, B and C). However, in this case maximum T production was observed at 500 ng/ml rLh (~7.2-fold increase), whereas 11-KT secretion was similar at 100 and 500 ng/ml rLh (~3-fold above basal levels). Thus, rLh appeared to be more potent than rFsh at inducing T release, whereas both gonadotropins equally stimulated 11-KT secretion.

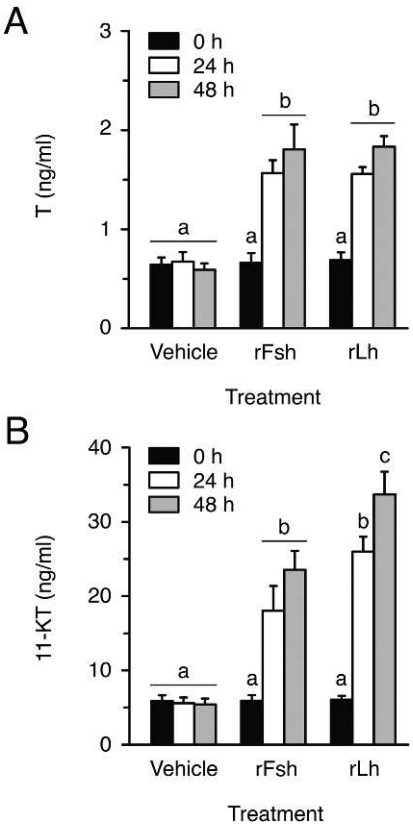

FIG. 4. In vivo modulation of plasma levels of T (**A**) and 11-KT (**B**) by recombinant gonadotropins in Senegalese sole males. Males were injected intramuscularly with ~40 μg/kg body weight of rFsh or rLh, or with the hormone vehicle, and steroids were determined before injection (0 h) and after 24 and 48 h. Data (mean ± SEM, n = 4) with different superscripts are significantly different ($P < 0.05$).

Involvement of the cAMP/PKA Pathway and 3β-Hsd in Gonadotropin-Mediated Androgen Release

To investigate whether Senegalese sole gonadotropins stimulate steroid release via activation of the cAMP/PKA pathway and steroidogenesis, testicular explants were cultured with 250 ng/ml rFsh or rLh, in the presence or absence of 1 or 5 μM adenylyl cyclase inhibitor MDL-12330A, PKA inhibitor H-89, or trilostane, which inhibits 3β-Hsd (the enzyme indispensable for the production of T and 11-KT; Fig. 3). As reported above, the production of T (Fig. 3A) and 11-KT (Fig. 3B) was enhanced after stimulation with 250 ng/ml rFsh (~16- and ~4-fold above basal levels, respectively) or rLh (~18- and ~5-fold, respectively). However, in the presence of MDL-12330A, H-89, or trilostane, rFsh- and rLh-mediated stimulation of T and 11-KT secretion was inhibited in a dose-response manner (Fig. 3, A and B). Therefore, for both Senegalese sole recombinant gonadotropins, the cAMP-PKA pathway and the activation of 3β-Hsd seem to be the major mediators of the steroidogenic response.

Androgen Production by Gonadotropins In Vivo

To test the in vivo bioactivity of the Senegalese sole recombinant gonadotropins, F1 males were injected intramuscularly with either rFsh or rLh, or with the hormone vehicle as control, and plasma levels of T and 11-KT were measured at 24 or 48 h by EIA (Fig. 4). Both gonadotropins were equally potent at increasing the plasma concentrations of T (~2.6-fold increase above controls) within 24 h after injection. At 48 h, levels of T were slightly higher (Fig. 4A). Treatment with rFsh also resulted in an increase of circulating 11-KT (~3.5-fold above controls) at 24 and 48 h, whereas rLh induced a first rise in 11-KT plasma levels at 24 h (~4.7-fold above controls) and a further increase (~6.0-fold above controls) at 48 h (Fig. 4B). At 48 h, plasma levels of 11-KT were higher in rLh-treated males than in those injected with rFsh (23.54 ± 2.56 and 33.70 ± 3.06 ng/ml, respectively), suggesting that rLh elicited a more potent in vivo response compared with rFsh.

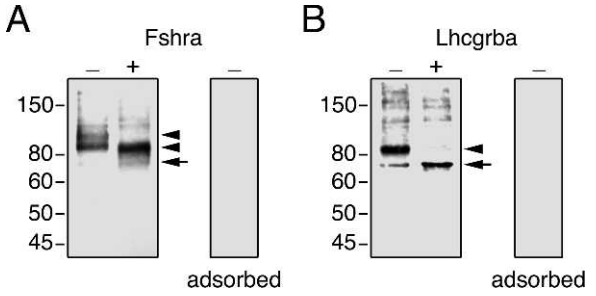

FIG. 5. Representative Fshra (**A**) and Lhcgrba (**B**) immunoblots of testicular protein extracts (30 μg). Duplicated membranes were incubated with the Fshra and Lhcgrba antisera (left) or with the antibodies preabsorbed with the synthetic peptide used for immunization (right). Plus or minus indicates preincubation of protein extracts with PNGase F prior to SDS-PAGE and Western blot. The arrow indicates Fshra or Lhcgrba monomers, whereas the arrowheads point to PNGase F-sensitive and insensitive posttranslational modifications of the proteins. Molecular mass markers (kDa) are on the left.

Immunolocalization of Gonadotropin Receptors in the Senegalese Sole Testis

The ability of rFsh to stimulate androgen production in vitro and in vivo led us to examine the cellular sites of Fshra and Lhcgrba expression in the Senegalese sole testis by using polyclonal antibodies generated against synthetic peptides corresponding to amino acid sequences of the C-terminus of Fshra and Lhcgrba. The specificity and potential cross-reactivity of these affinity-purified antisera were determined on HEK293T cells stably transfected with Senegalese sole FLAG-tagged Fshra or Lhcgrba (Supplemental Fig. S1, available online at www.biolreprod.org). Immunofluorescence using the anti-FLAG monoclonal antibody revealed a strong expression of both receptors in each cell clone, whereas cells transfected with empty pcDNA3 gave no signal (Supplemental Fig. S1, C, F, and I). The anti-Fshra antibody stained exclusively the cells expressing Fshra (Supplemental Fig. S1, D and G), whereas the Lhcgrba antiserum only reacted with cells expressing Lhcgrba (Supplemental Fig. S1, E and H). Neither of the antisera reacted with pcDNA3 empty-transfected cells (Supplemental Fig. S1, B and C). These data indicated that the antibodies against Senegalese sole gonadotropin receptors were specific because no cross-reaction was observed.

The presence of Fshra and Lhcgrba polypeptides in the Senegalese sole testis was confirmed by Western blot analysis. The antibodies generated against Fshra identified a single smeared band of ~85 kDa in testicular protein extracts, which is near the predicted molecular mass of the Senegalese sole Fshra (77.2 kDa; Fig. 5A). When protein extracts were treated with PNGase F, other polypeptides of lower molecular mass, of ~80 and 78 kDa, became visible. With the Lhcgrba antibody, one major reactive band of ~85 kDa and additional weaker

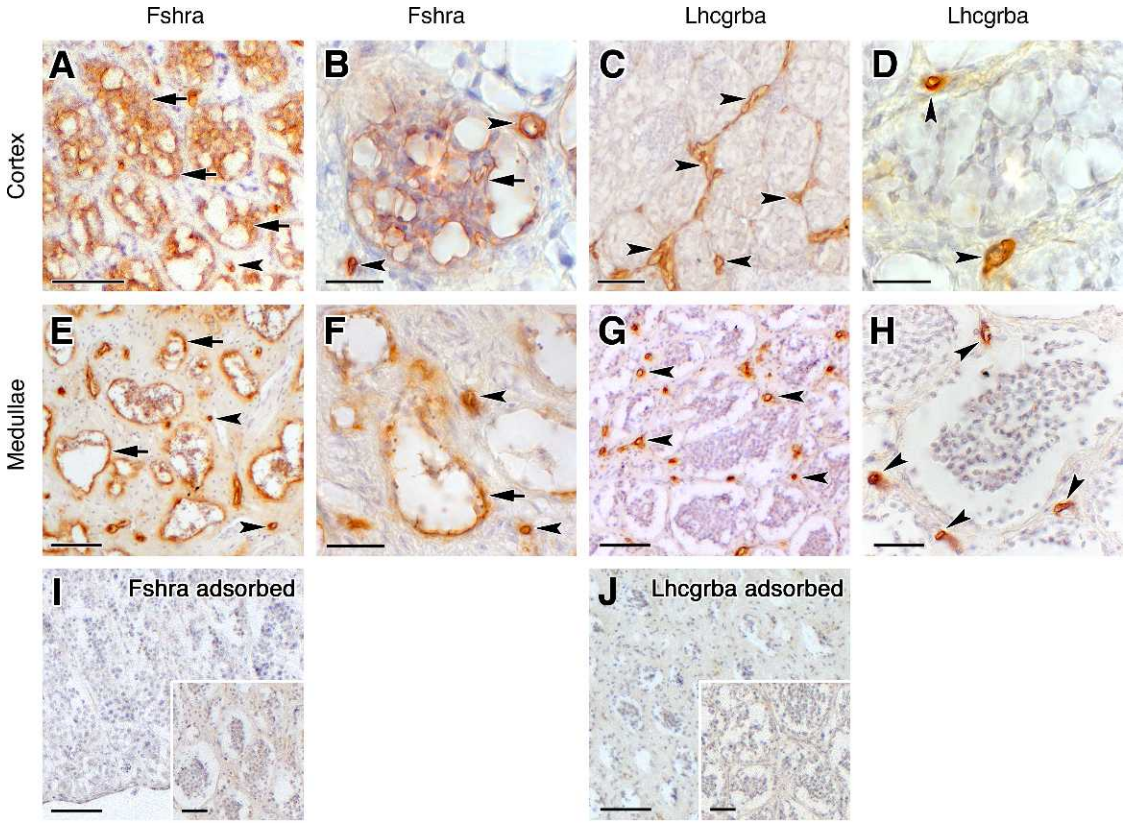

FIG. 6. Characterization of Fshra and Lhcgrba proteins in the testis of Senegalese sole using specific affinity-purified polyclonal antibodies. **A**–**J**) Immunoperoxidase staining of Fshra and Lhcgrba in the cortex (**A**–**D**) and medullae (**E**–**H**) of the testis. The arrows indicate Sertoli cells, and the arrowheads indicate Leydig cells. Note Fshra immunostaining in Leydig cells (**A**, **B**, **E**, and **F**). Negative controls (**I** and **J**) were incubated with preabsorbed Fshra or Lhcgrba antisera. Sections were counterstained with hematoxylin. Bars = 50 μm (**A**, **E**, **C**, **G**, **I**, and **J**), 40 μm (**B**, **F**, **D**, and **H**), and 25 μm (**I** inset and **J** inset).

bands of ~150, 130, and 78 kDa, were detected in the absence of PNGase F (Fig. 5B). After deglycosylation with PNGase F, a single major band of ~78 kDa was detected, which coincided with the predicted molecular mass of the Senegalese sole Lhcgrba (78.1 kDa). All of the Fshra and Lhcgrba reactive bands were no longer detected after preincubation of the antisera with large amounts of the corresponding immunizing peptides (Fig. 5, right panels). These data indicate that both antisera were specific, and that particularly Fshra, but also Lhcgrba, seems to be posttranslationally modified via *N*-glycosylation.

Immunohistochemistry was subsequently carried out using the antibodies described above in order to determine the cell types expressing the Fshra and Lhcgrba in the Senegalese sole testis. In the cortical region of the testis, strong Fshra signals were detected in the membrane of cytoplasmic extensions of Sertoli cells surrounding spermatogonia and spermatocytes (Fig. 6, A and B). In the medullar region, Fshra was also found in Sertoli cells lining the lumen of the seminiferous tubules in which spermatids and spermatozoa are released (Fig. 6, E and F). In both cortical and medullar regions of the testis, Lhcgrba was exclusively detected in Leydig cells distributed in the interstitial compartment as single cells or in small groups of a few cells (Fig. 6, C, D, G, and H; see also Fig. 2A). A faint staining was also noted in the spermatids within some tubules (data not shown). Interestingly, intense Fshra staining was observed in Leydig cells within the interstitial compartment in both medullar and cortical testicular regions (Fig. 6, A, B, E, and F; arrowheads). Control sections incubated with the Fshra and Lhcgrba antibodies preadsorbed with the peptides used for immunization were negative (Fig. 6, I and J).

To further corroborate the presence of both Fshra and Lhcgrba in Leydig cells of the Senegalese sole testis, we carried out in situ hybridization using molecular markers specific for Leydig cells, such as *star* and *lhcgrba* [6], followed by immunofluorescence using anti-Fshra antibodies, as well as double immunofluorescence for Fshra and Lhcgrba (Fig. 7). These experiments confirmed that *star* and *lhcgrba* were expressed exclusively in Leydig cells (Fig. 7, B and F), and showed consistently that these transcripts colocalized with Fshra polypeptides (Fig. 7, D and H). In addition, Leydig cells stained with the Lhcgrba antiserum also reacted with the Fshra antiserum, whereas Sertoli cells only reacted with the Fshra antiserum (Fig. 7, J–L). These observations thus demonstrate that in the Senegalese sole testis, Leydig cells synthesize the Lhcgrba as well as Fshra, whereas Sertoli cells only express the Fshra.

DISCUSSION

The mechanisms of gonadotropin signaling in the teleost testis using homologous gonadotropin preparations, together with the establishment of the cellular sites of Fshra and Lhcgrba expression, have so far only been reported in species that belong to basal lineages, such as eels, catfish, cyprinids, and salmonids [4, 5]. From these studies, a putative model has emerged in which Leydig cells expressing Fshra mediate the strong steroidogenic activity of piscine FSH-type gonadotropins [14, 22, 27]. However, it has remained unclear whether

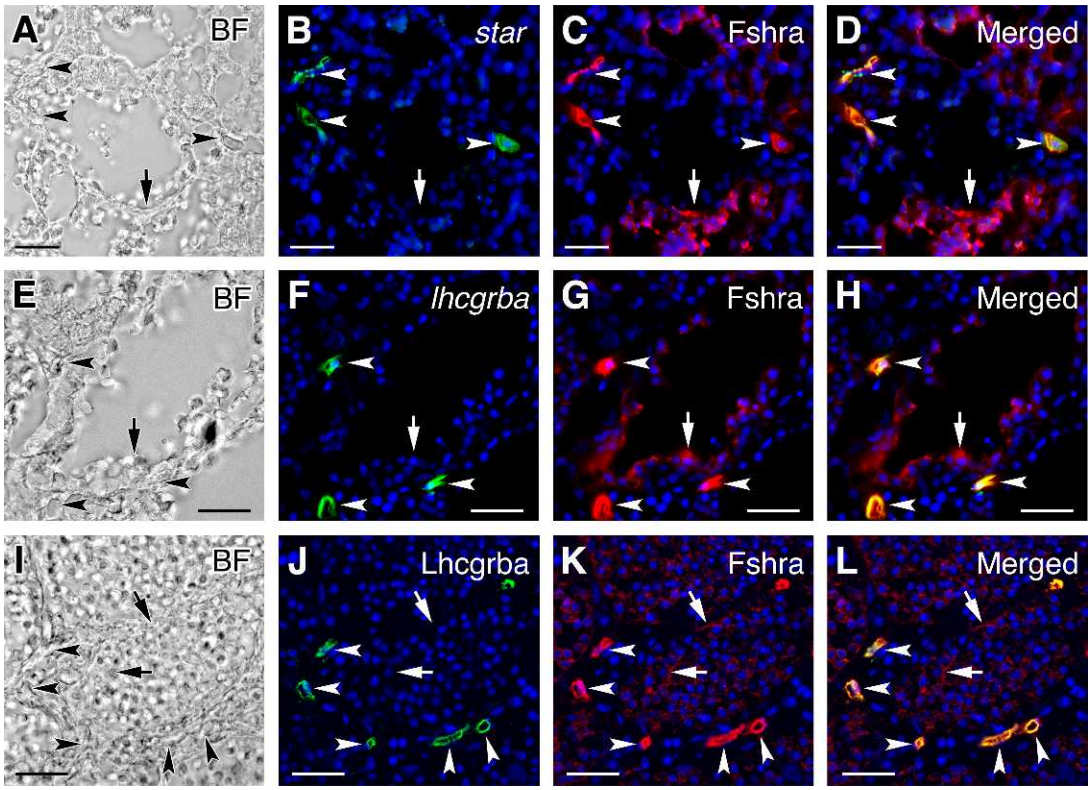

FIG. 7. Localization of Fshra in Leydig cells in the Senegalese sole testis. Representative bright-field (BF; **A**, **E**, and **I**) and epifluorescence (**B**–**D**, **F**–**H**, and **J**–**L**) photomicrographs showing multiple labeling combining in situ hybridization (**B** and **F**) and immunofluorescence (**C**, **G**, **J**, and **K**). The arrows indicate Sertoli cells, and the arrowheads indicate Leydig cells. The Leydig cell-specific markers *star* (**B**) and *lhcgrba* (**F**) were detected by in situ hybridization and anti-DIG FITC-labeled secondary antibodies (green). The Lhcgrba (**J**) and Fshra (**C**, **G**, and **K**) polypeptides were detected with the corresponding specific polyclonal antibodies labeled with FITC or Cy3 (red), respectively. In all sections, cell nuclei were stained with DAPI (blue). The fluorescence of different channels and the merged images (**D**, **H**, and **L**) shown were derived from the same section. Bars = 20 μm.

this mechanism exists in other more evolved major taxonomic groups of teleosts. By using homologous recombinant gonadotropins and specific antibodies against gonadotropin receptors, we show in the present study that the dual expression of Fshra and Lhcgrba in Leydig cells, as well as the androgenic function of Fsh, are conserved features in an evolutionary advanced teleost, the pleuronectiform Senegalese sole.

To establish this insight, we successfully produced relatively large amounts of Senegalese sole rFsh and rLh in mammalian CHO cells using constructs designed on the previously cloned sequence of the sole gonadotropin subunits [35]. Both recombinants were produced and secreted as single-chain polypeptides consisting of the specific β chain and the common α chain linked by the carboxyl-terminal peptide of hCG. Single-chain gonadotropins have been previously produced in different teleosts, including the Nile tilapia (*Oreochromis niloticus*) [48, 49], Japanese eel [50], Manchurian trout [16], zebrafish [27, 51], and European sea bass [18], using either the methylotrophic yeast *Pichia pastoris*, insect or mammalian cell lines, or baculovirus-silkworm systems, and are suggested to have a longer half-life compared with the production of separate subunits [52, 53]. By adding a His tag to the protein, which does not seem to affect the bioactivity of the hormones [27, 50], sole recombinant gonadotropins could be efficiently purified from the culture medium (~90%) using affinity chromatography. Western blot analysis using sea bass anti-Fsh and anti-Lh polyclonal antibodies, or anti-His monoclonal antibody, and PNGase F treatment revealed that both sole recombinant gonadotropins were extensively glycosylated. These findings are consistent with the in silico detection of at least one *N*-glycosylation site in each of the Fshβ and Lhβ subunits [35]. The glycosylation of gonadotropin subunits is known to be crucial for biosynthesis, subunit assembly, stability of the heterodimer, and protection against clearance by hepatic asialoglycoprotein receptors [52, 54–56], as well as for the proper binding to the corresponding receptors [9, 12].

The functional activity and specificity of Senegalese sole rFsh and rLh were assessed by transactivation assays on HEK293T cells transiently transfected with the previously cloned and characterized sole *fshra* and *lhcgrba* [6], together with a cAMP-responsive luciferase reporter. Recombinant gonadotropins induced a cAMP response after activation of their cognate receptors equivalent to that observed with forskolin, suggesting that rFsh and rLh maximally activated Fshra and Lhcgrba, respectively. However, Senegalese sole rFsh was unable to activate the Lhcgrba up to a dose of 5 μg/ml, whereas rLh activated both the Lhcgrba and Fshra, albeit the latter with a potency 4-fold less than that of rFsh. These results confirm previous data obtained using *Xenopus laevis* oocytes expressing Senegalese sole Fshra and Lhcgrba and European sea bass recombinant gonadotropins [6]. The promiscuous activation of the Fshra by Lh has also been reported in some salmonids [7], catfish [8–10], and cyprinids [11, 12], but in other teleosts, such as the rainbow trout [15], Manchurian trout [16], and European sea bass [17, 18], each gonadotropin seems to exclusively activate specific cognate receptors. Our observations suggest that the cross-activation of the Fshra by Lh is a conserved mechanism in acanthomorph teleosts such as the Senegalese sole. However, because of the

absence of specific immunoassays, the physiological significance of this mechanism remains intriguing because the endogenous levels of circulating gonadotropins in male Senegalese sole during spermatogenesis are unknown.

A general feature observed in most teleosts studied to date is that both Fsh and Lh stimulate testicular androgen production both in vivo and in vitro. Because Fsh can elicit a significant response at lower concentrations than those required to cross-activate the Lhcgrba, it has been suggested that Fsh-stimulated steroid release occurs via a Fshra-dependent mechanism [14]. In the present study, in vitro incubation of Senegalese sole testicular explants with subthreshold doses of rFsh that do not activate Lhcgrba, as well as with rLh, promoted the secretion of T and 11-KT. Similarly, injection of males with rFsh or rLh in vivo increased plasma androgen concentrations. No differences in gonadotropin response were observed between wild-caught and F1 males reared in captivity with a similar GSI (data not shown). These data therefore confirm the steroidogenic function of Fsh during spermatogenesis in Senegalese sole.

In mammals, androgen production by Leydig cells is mainly regulated directly by LH, in an endocrine or trophic manner, and indirectly by FSH-mediated paracrine effects of Sertoli cell-derived factors [57]. This mechanism is supported by the mutually exclusive expression of FSHR in Sertoli cells and LHCGR in Leydig cells, which trigger cAMP/PKA-independent and cAMP/PKA-dependent signaling pathways, respectively [3, 57, 58]. In teleosts, such as the African catfish (*Clarias gariepinus*) and zebrafish, coexpression of *fshra* and *lhcgrba* or *lhcgrbb* in Leydig cells has been reported [14, 27], whereas the presence of the Fshra protein product in Leydig cells has only been shown in the Japanese eel [22]. Based on these observations, it has been proposed that in teleosts Fsh directly stimulates testicular androgen release via its trophic action on Fshra-expressing Leydig cells [14]. In the Senegalese sole, an initial study using in situ hybridization indicated *fshra* transcripts exclusively in the cytoplasm of Sertoli cells surrounding type A and early type B spermatogonia, whereas *lhcgrba* and *star* were found only in Leydig cells [6]. Based on these observations, we initially suggested that the specific expression pattern of gonadotropin receptors in the Senegalese sole testis appeared to fit with the mammalian model [6].

The steroidogenic action of rFsh observed in the present work prompted us to reexamine the cellular distribution of the Fshra and Lhcgrba in the Senegalese sole testis by immunohistochemistry using specific antibodies. These experiments confirmed that the Lhcgrba was exclusively expressed in Leydig cells, whereas the Fshra was observed in both Sertoli and Leydig cells. This was unambiguously demonstrated in the present study by double immunofluorescence with the Fshra and Lhcgrba antibodies, as well as by fluorescent in situ hybridization using Leydig cell-specific molecular markers (*lhcgrba* and *star*) coupled with Fshra immunofluorescence. The expression level of *fshra* in Leydig cells may be below the detection limits of colorimetric in situ hybridization, which would explain why this was not revealed in earlier studies [6]. Nevertheless, the present findings suggest that the Fsh-mediated androgenic action through the activation of cognate receptors in Leydig cells is likely a conserved feature in more advanced teleosts such as Pleuronectiformes. The observation that the inhibition of adenylyl cyclase, PKA, or 3β-Hsd was effective at blocking both rLh- and rFsh-induced androgen secretion in vitro in Senegalese sole reinforces this notion.

Although both Senegalese sole rFsh and rLh were steroidogenic, we noted that rFsh was as potent as rLh in the stimulation of 11-KT production in vitro, but it was less efficient than rLh in inducing T secretion. However, when the recombinant gonadotropins were injected in vivo, the opposite effect was observed, rLh being as potent as rFsh in increasing T plasma levels but being slightly more effective than rFsh in increasing the plasma levels of 11-KT at 48 h. The in vitro data may agree with observations reported in Atlantic halibut where both native Fsh and Lh promoted 11-KT secretion in vitro with equivalent potency, whereas Fsh seemed to be less efficient in stimulating T release [20]. By contrast, zebrafish rFsh is at least 20-fold more potent in stimulating androgen production compared with rLh [27]. The causes for the differences between in vitro and in vivo experiments in Senegalese sole and with other species are unknown, but they might be related to the possible different developmental stages of the testis of the animals used in the in vitro and in vivo studies, the expression of steroidogenic enzymes in germ cells that can potentially synthesize androgens under rLh regulation, and/or the differential regulation of the expression of steroidogenic enzymes by Fsh and Lh. In zebrafish, rFsh up-regulates the testicular mRNA levels of a number of steroidogenesis-related genes both in vitro and in vivo, whereas rLh and hCG do not [27], and in European sea bass expression of 11β-hydroxylase, involved in the conversion of T into 11-KT, has been found in germ cells [59]. Although similar studies are not yet available for Senegalese sole, a recent report has shown that in vivo treatment of sole males with hCG increases the testicular expression of several steroidogenic enzymes, such as *star*, *3bhsd*, and *17bhsd* [40]. However, because we found here that hCG can activate both the Fshra and Lhcgrba (although with less potency for Fshra), the specific contribution of Fsh or Lh in the transcriptional regulation of steroidogenic enzymes remains unknown. Therefore, additional studies will be necessary to elucidate the physiological function of Fsh- and Lh-mediated steroid production in the regulation of spermatogenesis in Senegalese sole.

In summary, the present study shows that the Senegalese sole Fshra is activated by rFsh and rLh, whereas the Lhcgrba is specific for the rLh ligand. Our data also demonstrate that Leydig cells express the Fshra, together with *star* and the Lhcgrba, which most likely explains the cAMP/PKA pathway-mediated androgenic action of rFsh. The promiscuity of the Fshra in relation to its ligand, and the expression of the Fshra in Leydig cells, have previously only been described in species belonging to more ancestral orders of teleosts, such as Anguilliformes, Siluriformes, and Cypriniformes. The present data reveal that these features are conserved in an advanced pleuronectiform teleost. In the semicystic testis of the Senegalese sole, a potential role of Fsh may be to support long-term spermatogenesis and to assure the prevalence of spermatids within the testis throughout the year, whereas Lh may play a more acute function triggering the differentiation of spermatids into spermatozoa and subsequent spermiation. Further studies are needed to test these hypotheses.

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