Studies in Zebrafish Reveal Unusual Cellular Expression Patterns of Gonadotropin Receptor Messenger Ribonucleic Acids in the Testis and Unexpected Functional Differentiation of the Gonadotropins

Ángel García-López, Hugo de Jonge, Rafael H. Nóbrega, Paul P. de Waal, Wytske van Dijk, Wieger Hemrika, Geir L. Taranger, Jan Bogerd, and Rüdiger W. Schulz

Division of Endocrinology and Metabolism (Á.G.-L., H.d.J., R.H.N., P.P.d.W., W.v.D., J.B., R.W.S.), Department of Biology, Faculty of Science, Utrecht University, and U-Protein Express B.V. (W.H.), 3584 CH Utrecht, The Netherlands; and Department of Physiology of Growth and Reproduction in Fish (G.L.T., R.W.S.), Institute of Marine Research, 5817 Bergen, Norway

This study aimed to improve, using the zebrafish model, our understanding of the distinct roles of pituitary gonadotropins FSH and LH in regulating testis functions in teleost fish. We report, for the first time in a vertebrate species, that zebrafish Leydig cells as well as Sertoli cells express the mRNAs for both gonadotropin receptors (*fshr* and *lhcgr*). Although Leydig cell *fshr* expression has been reported in other piscine species and may be a common feature of teleost fish, Sertoli cell *lhcgr* expression has not been reported previously and might be related to the undifferentiated gonochoristic mode of gonadal sex differentiation in zebrafish. Both recombinant zebrafish (rzf) gonadotropins (*i.e.* rzfLH and rzfFSH) stimulated androgen release *in vitro* and *in vivo*, with rzfFSH being significantly more potent than rzfLH. Forskolin-induced adenylate cyclase activation mimicked, whereas the protein kinase A inhibitor H-89 significantly reduced, the gonadotropin-stimulated androgen release. Therefore, we conclude that both FSH receptor and LH/choriogonadotropin receptor signaling are predominantly mediated through the cAMP/protein kinase A pathway to promote steroid production. Despite this similarity, other downstream mechanisms seem to differ. For example, rzfFSH up-regulated the testicular mRNA levels of a number of steroidogenesis-related genes both *in vitro* and *in vivo*, whereas rzfLH or human chorionic gonadotropin did not. Although not fully understood at present, these differences could explain the capacity of FSH to support both steroidogenesis and spermatogenesis on a long-term basis, whereas LH-stimulated steroidogenesis might be a more acute process, possibly restricted to periods during which peak steroid levels are required. **(*Endocrinology* 151: 2349–2360, 2010)**

The pituitary gonadotropins LH and FSH play critical roles in regulating male reproduction across vertebrates (1–3). In mammals, the specific activities of both gonadotropins are clearly defined, given the highly specific interactions between each hormone and its respective receptor, LH/choriogonadotropin receptor (Lhcgr) and FSH receptor (Fshr). In addition, Lhcgr and Fshr expression in mammalian testis is restricted to Leydig and Sertoli cells, respectively (1, 2, 4). Hence, LH predominantly controls Leydig cell steroidogenesis, whereas FSH regulates Sertoli cell functions (2, 4–6).

In teleost fish, conversely, the biological activities of FSH and LH seem to be broader because both are strong steroidogenic hormones (7–16). Our present concept on

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Abbreviations: Cq, Threshold cycle; FACS, fluorescence-activated cell sorting; Fshr, FSH receptor; hCG, human chorionic gonadotropin; 3β-Hsd, 3β-hydroxysteroid dehydrogenase; 11-KT, 11-ketotestosterone; Lhcgr, LH/choriogonadotropin receptor; OHA, 11β-hydroxyandrostenedione; PKA, protein kinase A; qPCR, quantitative PCR; rzf, recombinant zebrafish.

how gonadotropins develop their bioactivity in fish testis is mainly based on a groundbreaking study in coho salmon, *Oncorhynchus kisutch*, showing LH binding to Leydig cells (indicating the presence of Lhcgr) and FSH binding to Sertoli cells (indicating the presence of Fshr), although FSH binding to Leydig cells could not be unequivocally demonstrated or clearly excluded (17). Recently, Fshr protein (Japanese eel, *Anguilla japonica*) (18) and mRNA (African catfish, *Clarias gariepinus*) (7) have been demonstrated in teleost Leydig cells, findings compatible with a direct trophic effect of FSH on Leydig cell functioning, including the regulation of steroid release (7). Thus, a revision of the concept of gonadotropin mode of action in fish testis is required. For instance, in the presence of Fshr in both Leydig and Sertoli cells, FSH alone may regulate the activities of both cell types during early-mid spermatogenesis, when plasma LH levels are very low or undetectable in seasonally reproducing species (19–21).

Another distinctive feature of teleost gonadotropin bioactivity is the limited hormone-binding selectivity that the gonadotropin receptors exhibit (7, 14, 16, 17, 22–26). This may lead to receptor cross-activation, most probably LH interaction with Fshr (see references above), especially during the spawning season when LH reaches peak plasma levels (19–21, 27). The physiological relevance of this phenomenon (if any) is unknown at present.

To understand how gonadotropins modulate gonadal functions, knowledge about the identity and the relevant characteristics of the gonadotropin target cells is imperative. This information is still missing in zebrafish, *Danio rerio*, an important vertebrate model species. Thus, the first objective of the present report was to identify the cell types expressing either the *fshr* or the *lhcgr* mRNA in zebrafish testis. We then set out to produce recombinant zebrafish (rzf) gonadotropins for studies on their biological activities regarding testicular androgen release and expression of selected testicular genes.

Materials and Methods

Animals

Sexually mature zebrafish from the Tübingen AB strain, either wild type or transgenic [expressing enhanced green fluorescent protein (EGFP) under the control of the germ cell-specific *vas* promoter; *vas::EGFP* (28)], and outbred fish were used. Animal housing (29) and experimentation were consistent with Dutch national regulations and were approved by the Utrecht University Animal Use and Care Committee.

Cellular localization of gonadotropin receptor gene expression in zebrafish testis

The localization of *lhcgr* and *fshr* mRNA expression in zebrafish testis was investigated by *in situ* hybridization, laser microdissection of testis sections, and fluorescence-activated sorting of testicular cell suspensions.

In situ hybridization for *lhcgr*, *fshr*, and *insulin-like peptide 3* (*insl3*), a Leydig cell-specific transcript (30), was performed on 10-μm-thick cryosections from wild-type Tübingen AB zebrafish testis using digoxigenin-labeled cRNA probes (7). Gene-specific primers used to generate DNA templates for probe synthesis are shown in Supplemental Table 1 (published on The Endocrine Society's Journals Online web site at http://endo.endojournals.org).

Laser microdissection of zebrafish testis sections was carried out using a PALM MicroBeam Instrument (PALM Microlaser Technologies, Bernried, Germany). Two microdissected fractions were analyzed from two independent biological samples for *lhcgr* and *fshr* mRNA abundance: interstitial tissue, identified by 3β-hydroxysteroid dehydrogenase (3β-Hsd) staining of Leydig cells, and intratubular tissue, containing spermatogenic cysts (germ/Sertoli cells units). See Supplemental Materials and Methods and Supplemental Fig. 1 for further details.

Fluorescence-activated cell sorting (FACS) was used to isolate a germ-cell-enriched population from *vas::EGFP* zebrafish testis. Both EGFP intensity and cell size decrease as spermatogenesis progresses (31), whereas somatic cells are EGFP negative and have variable sizes. This allowed obtaining cell populations enriched in spermatogonia and primary spermatocytes by selecting for cells showing strong EGFP intensity and large size (Supplemental Fig. 1, D and E). Dissociated testicular cells were prepared from two independent batches of 10–12 fish each (32), resuspended in 1 ml D-PBS+ (Invitrogen, Carlsbad, CA), and then immediately subjected to FACS using an inFlux cell sorter (Becton Dickinson Biosciences, Franklin Lakes, NJ). The obtained cell suspension was centrifuged at 50 $\times$ *g* for 10 min followed by total RNA extraction using the RNAqueous-Micro kit (Ambion, Austin, TX). Synthesis of cDNA from total RNA samples was performed as described (26).

Primers to detect zebrafish *fshr* mRNA, *lhcgr* mRNA, *piwi-like 1* (*piwil1*) mRNA (predominantly expressed in spermatogonia) (33), *synaptonemal complex protein 3 like* (*sycp3l*) mRNA (expressed by primary spermatocytes) (34, 35), *outer dense fiber 3 like* (*odf3l*) mRNA (expressed by spermatids) (35), *gonadal somatic cell-derived factor* (*gsdf*) mRNA (expressed by Sertoli cells) (36), *insl3* mRNA (expressed by Leydig cells) (30), and the reference endogenous control gene *β-actin1* (Supplemental Table 2) were designed and validated for specificity and amplification efficiency on serial dilutions of testis cDNA (26). All real-time quantitative PCRs (qPCRs) and calculations were performed as described previously (7, 26, 37).

Gonadotropins

The rzfFSH and rzfLH proteins used for these experiments were produced as detailed in the Supplemental Materials and Methods and Supplemental Fig. 2. Human chorionic gonadotropin (hCG) was obtained from Organon (Oss, The Netherlands).

In vitro androgen release response to increasing gonadotropins and forskolin concentrations

Testicular tissue was challenged in concentration-response bioassays with either rzfFSH (from 12.5–1000 ng protein/ml), rzfLH (from 100-2000 ng protein/ml), or the adenylate cyclase

activator forskolin (from 0.1–25 μM; Sigma-Aldrich, St. Louis, MO). Testis tissue was collected from 12 outbred zebrafish per condition tested, and the two testes from each fish were incubated in parallel, one of them (randomly chosen left or right) serving as control for the contralateral one. Incubations lasted 18 h in a humidified air atmosphere at 25 C in 96-well flat-bottom plates (Corning Inc., Corning, NY) using a final volume of 200 μl culture medium (38). After incubation, tissue explants were weighed and discarded, while the medium was processed for the quantification of 11-ketotestosterone (11-KT) and 11β-hydroxyandrostenedione (OHA) levels by RIA (39).

Because of the experimental design used (one testis assigned to basal condition and the contralateral one to experimental condition), we obtained data for basal steroid release for all concentrations of the compounds assayed. Homogeneity of basal steroid release among the different replicates was tested by one-way ANOVA. Because no statistically significant differences ($P > 0.05$) were identified, basal steroid release data were compiled into one single basal steroid release condition for each compound tested. Thereafter, significant differences among the different concentrations of each substance were identified by one-way ANOVA followed by the Student-Newman-Keuls test ($P < 0.05$).

Role of the cAMP/protein kinase A (PKA) pathway on the gonadotropin-mediated stimulation of androgen release *in vitro*

Testis tissue explants were incubated with rzfFSH (250 ng/ml) or rzfLH (1000 ng/ml) in the absence or presence of 100 μM of the PKA inhibitor H-89 (Sigma-Aldrich) (7). Fish origin, batch and age, number of replicates per condition tested, tissue preparation, culture conditions, and analyses performed were the same as described above. For each fish, one testis was incubated with recombinant gonadotropin, whereas the contralateral one was incubated with gonadotropin plus H-89. Significant differences were identified by the Student's paired *t* test ($P < 0.05$).

In vitro short-term actions of gonadotropins on testis functions

The capacities of rzfFSH (100 ng/ml) and rzfLH (500 ng/ml) to modulate the mRNA levels of a number of testicular genes were investigated over a 2-h incubation period. Origin of the fish (n = 8 per condition), tissue preparation, culture conditions, and analyses performed were the same as described above, except that testis explants were saved for gene expression studies.

Total RNA was extracted from testis explants using the RNAqueous-Micro kit (Ambion). Further processing to determine the threshold cycle (Cq) values of the reference endogenous control gene *β-actin1* as well as of *fshr*; *lhcgr*; the Leydig cell genes *insl3*, *steroidogenic acute regulatory protein* (*star*), and *cytochrome P450*, *family 17*, *subfamily A*, *polypeptide 1* (*cyp17a1*); and the Sertoli cell genes *androgen receptor* (*ar*; expression not detectable in zebrafish Leydig cells by *in situ* hybridization) (37), *anti-Müllerian hormone* (*amh*), and *gsdf* (primer sequences are listed in Supplemental Table 2) by qPCR analysis was performed as reported (26, 37). No significant differences ($P > 0.05$) were found among the mean *β-actin1* Cq values in the different treatment groups (Supplemental Fig. 3A), thus validating *β-actin1* as a suitable reference for the current experiments.

Because the two testes from each fish were incubated in parallel, the amounts of androgens released into the incubation media and the relative mRNA expression levels were compared between treated and respective control groups by the Student's paired *t* test ($P < 0.05$). Thereafter, values were expressed as percentage of respective basal levels, and differences between each gonadotropin treatment were identified by the Student's unpaired *t* test ($P < 0.05$).

In vitro medium-term actions of gonadotropins on testis functions

The capacities of rzfFSH (100 ng/ml) and rzfLH (500 ng/ml) to modulate the mRNA levels of a number of testicular genes were investigated in a medium-term organ culture system (38). In addition, the relative contribution of steroid production to gonadotropin-induced changes in gene expression was assessed by including 25 μg/ml of the 3β-Hsd inhibitor trilostane (Chemos, Regenstauf, Germany) in the media. After 2 d incubation in a humidified air atmosphere at 25 C, explants (n = 8 per condition) were processed as above for gene expression analysis. No significant differences ($P > 0.05$) were found among the mean *β-actin1* Cq values in the different treatment groups (Supplemental Fig. 3B).

Incubation media were recovered after culture and stored at −25 C until 11-KT and/or OHA quantification (39). Pilot studies determined that the 11-KT antibody cross-reacted with trilostane at the concentration used, and therefore, trilostane-containing incubation media were assayed for OHA only. Recovery studies using tritiated androgens showed that 47 ± 1% of total steroids added to culture wells was present in the incubation medium after an overnight equilibration period (n = 8), whereas the remaining steroid was trapped in the agar cylinder. The results obtained were corrected accordingly.

Data were compared between treated and respective control explants by the Student's paired *t* test ($P < 0.05$). Thereafter, values were expressed as percentage of respective basal levels, and differences between treatments (*i.e.* gonadotropin *vs.* gonadotropin plus trilostane) were identified by the Student's unpaired *t* test ($P < 0.05$).

In vivo short-term actions of gonadotropins on testis functions

In this experiment, outbred zebrafish received an ip injection of 100 ng/g body weight rzfFSH or rzfLH or 10 IU/g body weight hCG in a total volume of approximately 5 μl (n = 8 fish per condition). Control fish received a 5-μl PBS injection. Two hours after the injections, fish were euthanized in ice water, the caudal peduncle cut, and a sample of blood collected using heparinized syringes. Samples were then transferred to heparinized tubes and the 11-KT plasma levels quantified (39). Testes were used for gene expression analysis as reported above. No significant differences ($P > 0.05$) were found among the mean *β-actin1* Cq values in the different treatment groups (Supplemental Fig. 3C).

Significant differences among the different treatments were identified by one-way ANOVA followed by the Student-Newman-Keuls test ($P < 0.05$).

Results

Zebrafish Leydig and Sertoli cells express both *fshr* and *lhcgr* mRNA

A clear *in situ* hybridization signal on interstitial Leydig cells was obtained with *fshr* antisense probe (Fig. 1A). The

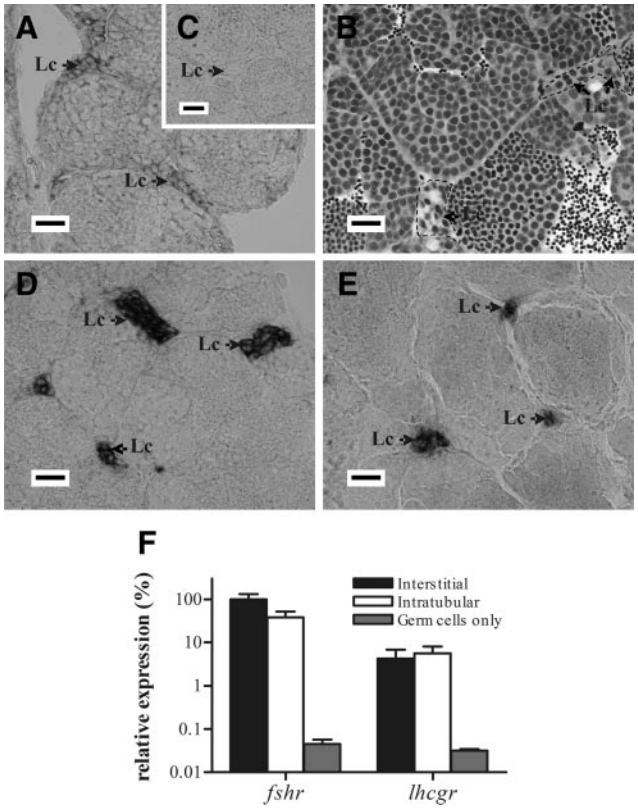

FIG. 1. Cellular localization of *fshr* and *lhcgr* mRNA expression in zebrafish testis. A and C, *In situ* hybridization with either an antisense (A) or a sense (C) riboprobe for zebrafish *fshr*; note the positive staining in Leydig cells (Lc) in the interstitial tissue in A and the absence of signal in C. In both cases, germ cells were devoid of staining. B, Toluidine blue-stained 2-μm-thick plastic section showing groups of Leydig cells (Lc) within the interstitial compartment (*enclosed by broken lines*). D and E, Leydig cells are also identified by their positive staining with an *insl3* antisense cRNA probe (D) and with the 3β-Hsd enzymatic reaction (E). *Scale bars*, 20 μm. F, Relative *fshr* and *lhcgr* mRNA expression levels in two microdissected testis tissue fractions (interstitial and intratubular) and a germ-cell-enriched population obtained from *vas::EGFP* transgenic zebrafish by FACS (germ cells only). Data correspond to values from two experiments, each with duplicate measurements (mean ± SEM), normalized to *β-actin1* mRNA levels, and expressed as percentage of *fshr* transcript amounts in the interstitial fraction. Note the logarithmic scale.

arrangement of cells stained was characteristic of Leydig cells as observed on histological sections (Fig. 1B) and resembled those obtained with *insl3* antisense probe (Fig. 1D) and with the 3β-Hsd enzyme-histochemical reaction (Fig. 1E). Sertoli and germ cells were negative for *fshr* mRNA by *in situ* hybridization. No signal was obtained for *lhcgr* mRNA. The *fshr* mRNA-positive Leydig cells present were stained with an approximately similar intensity, and no apparent spatial distribution pattern of Leydig cells (*e.g.* rostrocaudal, ventrodorsal, or central *vs.* peripheral in the testis) was observed. Hybridization with sense cRNA probes for *fshr* (Fig. 1C), *lhcgr*, or *insl3* (not shown) did not yield any staining.

Confirmation of *fshr* mRNA expression by interstitial Leydig cells was obtained by qPCR analysis of laser microdissected testis fractions (Fig. 1F). This analysis also revealed expression of *fshr* mRNA in the intratubular compartment [~3-fold lower than in the interstitial (Leydig cell) fraction]. Moreover, *lhcgr* mRNA expression was found in the interstitial and, remarkably, also in the intratubular samples (~24- and ~18-fold lower than *fshr* mRNA levels measured in the interstitial compartment, respectively). The purity of the intratubular fraction was confirmed by measuring mRNA levels of the Leydig cell-specific gene *insl3*. Its expression level was approximately 265-fold lower in the intratubular than in the interstitial fraction (Supplemental Fig. 1C). Contamination of the interstitial testis tissue fraction with intratubular Sertoli cells was assessed by measuring the mRNA levels of the Sertoli cell-specific gene *gsdf*. Its expression level in the interstitial fraction was approximately 11-fold lower than in the intratubular fraction (Supplemental Fig. 1C). These data indicated a small degree of Sertoli cell contamination in the interstitial fraction but negligible contamination of the intratubular fraction with interstitial elements. Still, *lhcgr* mRNA levels were even 1.3-fold higher in the intratubular than in the interstitial fraction.

Identification of the intratubular cell type expressing the gonadotropin receptor mRNAs was accomplished by qPCR analysis of a germ-cell-enriched population obtained by FACS of testis cell suspensions prepared from *vas::EGFP* transgenic zebrafish (Fig. 1F). This cell population exhibited abundant *piwi1l* and *sycp3l* mRNA expression in addition to intermediate *odf3l* mRNA levels (*i.e.* the cell population contained mainly spermatogonia and spermatocytes but also spermatids), whereas the levels of the somatic transcripts *gsdf* and *insl3* were at least 60-fold lower than *piwi1l* (Supplemental Fig. 1F), indicating the high purity of the samples obtained. Because both *fshr* and *lhcgr* mRNA levels were very low in the germ cells compared with the whole intratubular compartment (Fig. 1F), we concluded that the intratubular expression of both *fshr* and *lhcgr* genes resided in the only non-germ-cell type in the intratubular compartment, the Sertoli cell.

Altogether, our results indicate that in zebrafish testis, both Leydig and Sertoli cells express both *fshr* and *lhcgr* mRNA, whereas germ cells are devoid of such transcripts.

Recombinant, single-chain zebrafish gonadotropins and forskolin stimulate androgen production in testicular explants

When zebrafish testes were incubated with rzfFSH, androgen secretion increased gradually with the gonadotropin concentrations (Fig. 2A). The lowest rzfFSH concentration tested (12.5 ng/ml) elicited significant elevations of both 11-KT and OHA release (~4.7- and ~3.5-fold above basal levels, respectively). Maximal androgen release

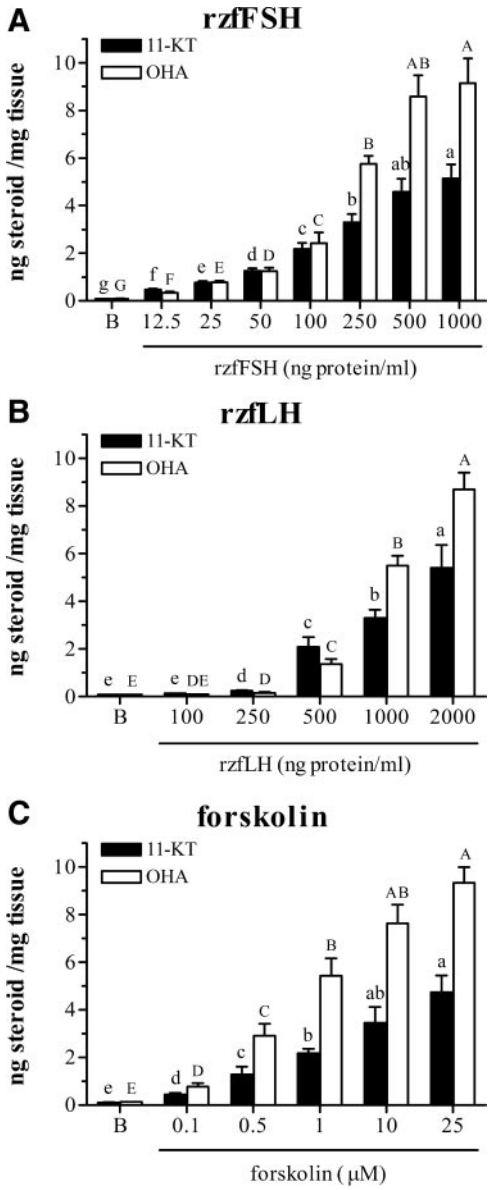

FIG. 2. Stimulation of androgen release by zebrafish testicular explants. Amounts of 11-KT and OHA (mean ± SEM) measured in incubation media after overnight (18 h) exposure to increasing concentrations of zfFSH (panel A), rzfLH (panel B), or the adenylate cyclase activator forskolin (panel C). B, Basal release. Values represent compiled data from two experiments, each with six replicates per ligand concentration. *Different letters* denote significant differences among groups ($P < 0.05$).

(~47-fold for 11-KT and ~84-fold for OHA) was reached at 500 ng/ml rzfFSH. No further increase was observed by doubling the concentration.

For rzfLH (Fig. 2B), 250 ng/ml induced the first significant increase in both 11-KT and OHA secretion (~2.5-and ~1.8-fold above basal levels, respectively). From 250-2000 ng/ml, androgen secretion kept increasing significantly. Higher rzfLH concentrations could not be tested. However, the androgen release induced by 2000 ng/ml rzfLH was similar to those observed in response to maximally effective rzfFSH or forskolin concentrations, suggesting that maximal androgen release had been reached.

Incubation with 0.1 μM forskolin, the lowest concentration tested, already induced significant increases in both 11-KT and OHA production by zebrafish testis (~4.0- and ~5.9-fold above basal levels, respectively) (Fig. 2C). The maximal steroidogenic response was obtained with forskolin concentrations between 10 and 25 μM.

The cAMP/PKA pathway is involved in both the FSH- and LH-stimulated androgen production

When testis tissue was incubated with 250 ng/ml rzfFSH or 1000 ng/ml rzfLH in the presence of 100 μM of the PKA inhibitor H-89, androgen production was strongly reduced (22- to 16-fold) compared with the levels measured in the absence of the inhibitor (Fig. 3). Therefore, for both rzf gonadotropins, the cAMP-PKA pathway is the major mediator of the steroidogenic response. This is further supported by comparing the residual 11-KT release observed in the presence of gonadotropin and H-89 with basal 11-KT release from 49–82 individual testis incubations of fish of the same origin and age (basal release from the dose-response experiments described above; see Fig. 2): all 11-KT release levels were within the basal release range, whereas the values of the precursor OHA showed a somewhat higher dispersion with part of the data being above the basal range (Supplemental Fig. 4).

The rzf gonadotropins show differential effects on steroidogenesis and testicular gene expression *in vitro* and *in vivo*

Zebrafish testis explants incubated for 2 d with either 100 ng/ml rzfFSH or 500 ng/ml rzfLH displayed a signif-

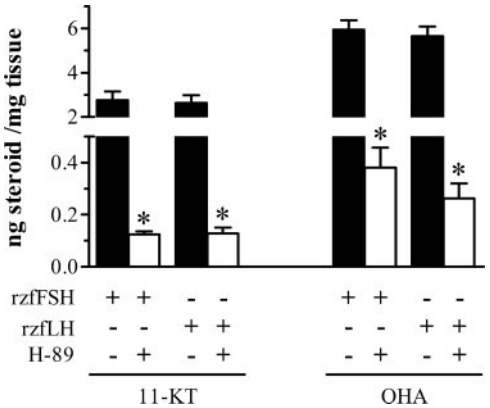

FIG. 3. Effects of the PKA inhibitor H-89 on the gonadotropin-stimulated androgen release by zebrafish testicular explants. Amounts of 11-KT and OHA (mean ± SEM) measured in incubation media after overnight (18 h) exposure to 250 ng/ml rzfFSH or 1000 ng/ml rzfLH alone and in combination with 100 μM H-89. Values represent compiled data from two experiments, each with six to seven replicates per condition. *, Values are significantly different ($P < 0.05$) from the respective gonadotropin-only condition.

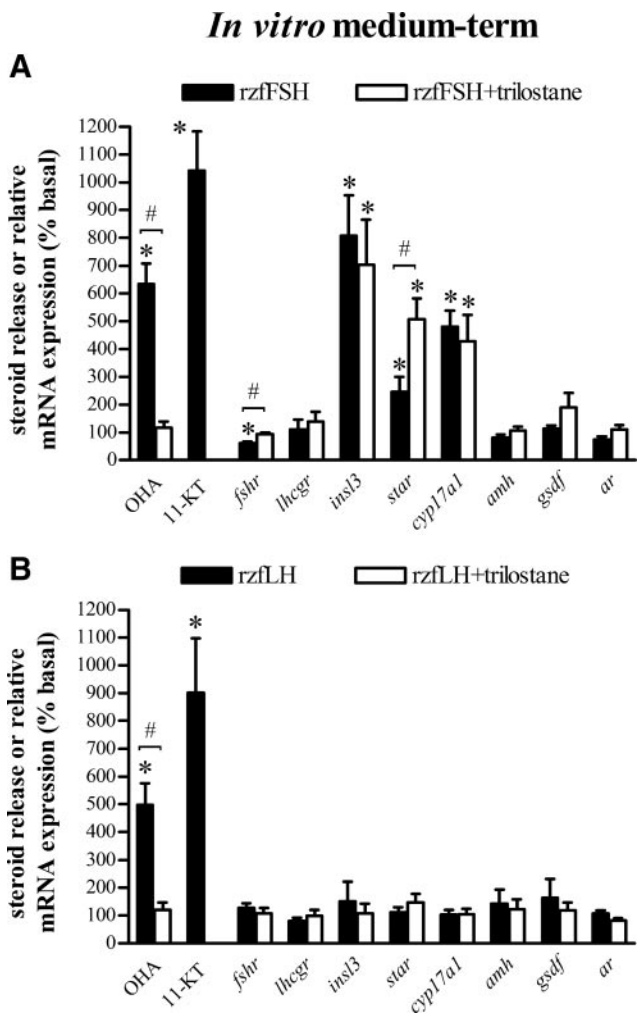

FIG. 4. *In vitro* medium-term (2 d) modulation of androgen release and testicular gene expression by rzf gonadotropins. Amounts of the androgens OHA and 11-KT measured in incubation media and relative mRNA expression levels of several testicular genes (*fshr*, *lhcgr*, *insl3*, *star*, *cyp17a1*, *amh*, *gsdf*, and *ar*) after 2 d exposure to 100 ng/ml rzfFSH (A) or 500 ng/ml rzfLH (B) alone (*black bars*) and in combination with 25 μg/ml of the 3β-Hsd inhibitor trilostane (*white bars*). Data (mean ± SEM) come from an experiment with eight replicates per condition and are expressed as percentage of basal levels, which were set to 100% for each parameter analyzed. Basal androgen release was 237 ± 18 pg OHA/mg tissue and 147 ± 17 pg 11-KT/mg tissue. Gene expression levels were normalized to *β-actin1* mRNA levels. *, Values are significantly different ($P < 0.05$) from the respective basal condition in the absence of recombinant gonadotropin; #, significant difference ($P < 0.05$) between the absence and the presence of trilostane.

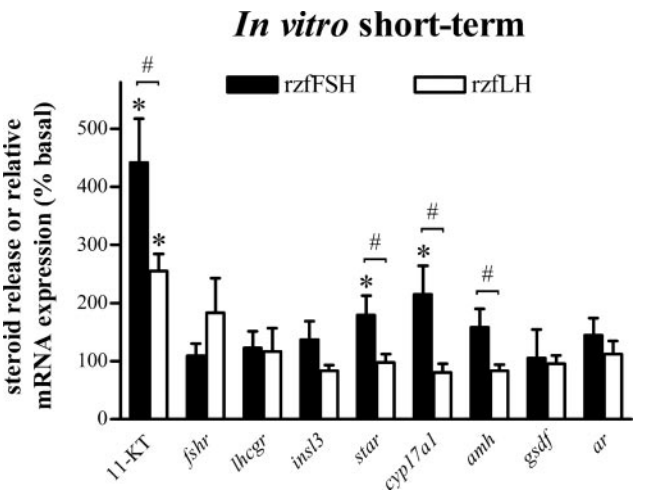

FIG. 5. *In vitro* short-term (2 h) modulation of androgen release and testicular gene expression by rzf gonadotropins. Amounts of androgen (11-KT) measured in incubation media and relative mRNA expression levels of several testicular genes (*fshr*, *lhcgr*, *insl3*, *star*, *cyp17a1*, *amh*, *gsdf*, and *ar*) after 2 h exposure to 100 ng/ml rzfFSH (*black bars*) or 500 ng/ml rzfLH (*white bars*). Data (mean ± SEM) come from an experiment with eight replicates per condition and are expressed as percentage of basal levels, which were set to 100% for each parameter analyzed. Basal androgen release was 40.0 ± 4.9 pg 11-KT/mg tissue. Gene expression levels were normalized to *β-actin1* mRNA levels. *, Values are significantly different ($P < 0.05$) from the respective basal condition in the absence of recombinant gonadotropin; #, significant difference ($P < 0.05$) between treatment with rzfFSH and rzfLH.

icant up-regulation (5- to 10-fold) of androgen release (Fig. 4). However, we observed significant changes in gene expression levels only in response to rzfFSH. The steady-state mRNA levels of a number of Leydig cell genes (*i.e. insl3*, *star*, and *cyp17a1*) increased (2.5- to 8-fold), whereas *fshr* mRNA levels were reduced to 60% of control values (Fig. 4A). No significant changes were observed for the Sertoli cell genes *amh*, *gsdf*, or *ar* mRNA levels (Fig. 4A). In the presence of the 3β-Hsd inhibitor trilostane, both rzfFSH- and rzfLH-stimulated androgen release was completely abolished (Fig. 4). Although trilostane did not modify the rzfFSH-stimulated *insl3* and *cyp17a1* mRNA overexpression, it prevented rzfFSH-induced *fshr* mRNA down-regulation and further increased *star* mRNA levels (Fig. 4A). The presence of trilostane in combination with rzfLH did not modify the mRNA levels of any of the transcripts analyzed (Fig. 4B).

The short-term culture approach (Fig. 5) provided a picture similar to that obtained in the medium-term study; both rzfFSH and rzfLH stimulated testicular 11-KT release ($P < 0.05$), whereas significant changes in steroidogenesis-related transcripts (*star* and *cyp17a1*) were detected only after rzfFSH treatment. Incubation with rzfLH elicited a 1.8-fold increase in *fshr* mRNA levels, but statistical significance was not reached. Moreover, no significant changes were observed for any of the Sertoli cell genes assayed as compared with basal samples, although *amh* mRNA expression differed statistically between rzfFSH- and rzfLH-treated explants.

Both recombinant gonadotropins exhibited bioactivity also *in vivo*, as reflected in significantly elevated plasma 11-KT levels and the change in the mRNA levels of several genes expressed in zebrafish testis (Fig. 6). At the dose (100 ng/g body weight) and time after administration (2 h) tested, rzfFSH showed significantly higher steroidogenic potency than rzfLH, although the difference between the

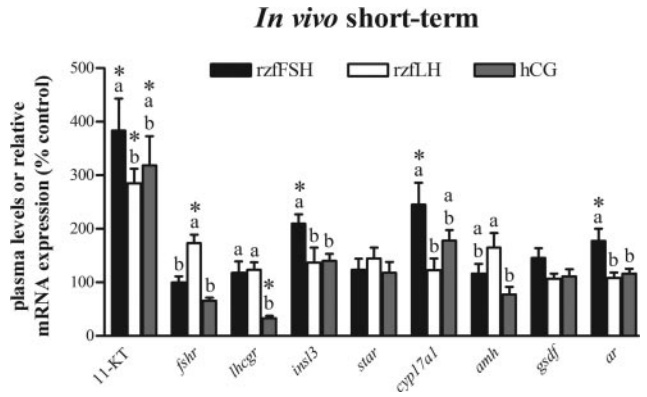

FIG. 6. *In vivo* short-term (2 h) modulation of plasma androgen levels and testicular gene expression by rzf gonadotropins and hCG. Circulating 11-KT concentrations and relative mRNA expression levels of several testicular genes (*fshr*, *lhcgr*, *insl3*, *star*, *cyp17a1*, *amh*, *gsdf*, and *ar*) 2 h after injection with 100 ng/g body weight rzfFSH (*black bars*), rzfLH (*white bars*) or 10 IU/g body weight hCG (*gray bars*). Data (mean ± SEM) come from an experiment with eight fish per condition and are expressed as percentage of control levels, which were set to 100% for each parameter analyzed. Control androgen levels were 3.6 ± 0.9 ng 11-KT/ml plasma. Gene expression levels were normalized to *β-actin1* mRNA levels. *, Values are significantly different ($P < 0.05$) from the basal control condition. *Different letters* denote significant differences among groups ($P < 0.05$).

two hormones was less pronounced than that observed *in vitro*. Considering changes in gene expression, the rzfFSH treatment up-regulated *insl3*, *cyp17a1*, and *ar* mRNA levels (1.8- to 2.5-fold). *In vivo* administration of rzfLH, conversely, had only minor effects on testicular gene expression. An interesting exception was the significant 1.7-fold up-regulation of *fshr* mRNA levels. Injection of hCG also elicited a significant increase in plasma 11-KT levels that was intermediate between those observed after the treatments with rzfFSH and rzfLH (Fig. 6). Similar to rzfLH, hCG injection did not change the mRNA levels of the steroidogenesis-related genes (*e.g. star* and *cyp17a1*) or the Leydig cell-specific gene *insl3*, the only exception being the previously unreported down-regulation of *lhcgr* transcription.

Discussion

The pituitary gonadotropins LH and FSH, acting via their receptors, Lhcgr and Fshr, are the main factors controlling testis functions across vertebrates, including teleost fish (1–3, 40). Knowledge about the identity and characteristics of testicular cell types responding to gonadotropic signals, *i.e.* expressing the gonadotropin receptors, is crucial for understanding how gonadotropins regulate testis functions. This information is, however, lacking in most fish species. The available data are restricted to coho salmon, Japanese eel, and African catfish (7, 17, 18), although a complete data set has been reported only for the latter; African catfish Leydig cells express both *lhcgr* and *fshr* mRNA, whereas Sertoli cells solely express *fshr* mRNA (7). In view of the importance of zebrafish as an experimental model and the essential roles that pituitary gonadotropins play in the regulation of testis physiology, we considered it imperative to identify gonadotropin receptor-expressing cells in the zebrafish testis.

In situ hybridization and qPCR analysis of laser microdissected testicular interstitial tissue showed that, also in zebrafish, the steroid-producing Leydig cells express both *fshr* and *lhcgr* mRNAs. These results support the view that *fshr*/Fshr expression by Leydig cells is a common feature among teleost fish, which is further supported by the strong steroidogenic activity displayed by several piscine FSH proteins (7–16). Therefore, we propose to explain the steroidogenic activity of FSH-like piscine gonadotropins by a trophic, direct effect on Leydig cells steroid release (7).

qPCR-based analyses of samples obtained by laser microdissection and FACS demonstrated *fshr* expression in zebrafish Sertoli cells, as typically reported in other vertebrates (1, 2, 4) including fish (7, 17, 18). Surprisingly, we also found, for the first time in any vertebrate, *lhcgr* mRNA expression in zebrafish Sertoli cells. This striking testicular expression pattern of *lhcgr* in zebrafish may be related to its undifferentiated gonochoristic mode of gonadal sex differentiation, in which the indifferent gonad initially develops as an ovary-like structure in all individuals, but in future males, developing oocytes soon degenerate and the gonads transform into testes (41–43). Accordingly, in undifferentiated gonochoristic fish, the default differentiation pathway of the germ-cell-supporting somatic cell precursor (common for both granulosa and Sertoli cells) (44, 45) may be toward granulosa-like cells, which may retain the potential to transdifferentiate to Sertoli cells (46–49) and/or may regress, whereas another population of undifferentiated somatic cells differentiates into Sertoli cells (50). Because mammalian as well as salmon granulosa cells (51–53) express both Fshr and Lhcgr protein and the respective mRNAs, it seems possible that the coexpression of both *fshr* and *lhcgr* mRNA in zebrafish Sertoli cells is related to the transitory female developmental stage observed in this species.

In the present report, we show that rzfFSH was at least 20-fold more potent in stimulating androgen production than rzfLH, whereas both hormones reached similar maximal stimulation levels. This difference in the steroidogenic potency may be related to the higher mRNA expression levels shown by *fshr* in comparison with *lhcgr*, which may lead to a lower abundance of Lhcgr protein on the Leydig cell membrane. This may also represent an adaptive mechanism to balance the constitutive activity of the zebrafish Lhcgr (54) in favor of a tight regulation of Leydig

cell steroid release. Functional characterization of zebrafish gonadotropin receptors has shown that the Lhcgr was exclusively activated by LH, whereas both FSH and LH were able to activate the Fshr, which showed a slightly higher preference for FSH (24). Therefore, we conclude that the stimulation of steroid release elicited by rzfFSH was exclusively mediated via Fshr-dependent mechanisms.

Incubation of zebrafish testicular explants with increasing concentrations of the adenylate cyclase activator forskolin resulted in a concentration-dependent stimulation of androgen release, as previously shown in other fish (8, 55–57). In the current study, forskolin and both recombinant gonadotropins reached similar maximal induction levels, whereas the PKA inhibitor H-89 strongly inhibited gonadotropin-stimulated androgen release. Together, these data suggest a prominent role for the cAMP/PKA pathway in both Fshr- and Lhcgr-mediated regulation of testicular steroid production (7, 8, 55–57), although other signaling pathways may have (a quantitatively minor) role in the process, as proposed for other fish (8, 55, 58, 59) and mammalian species (5, 60, 61).

Although both FSH and LH make use of the cAMP/PKA pathway in zebrafish Leydig cells, other downstream mechanisms that the gonadotropins use seem to differ, as demonstrated by analyzing expression levels of steroidogenesis-relevant transcripts; only rzfFSH up-regulated the testicular mRNA levels of *star* and *cyp17a1*. Our results are similar to those recently obtained for Japanese eel where the two gonadotropins showed similar potencies in stimulating testicular androgen release, but only recombinant FSH induced major changes in steroidogenic gene expression (16). Because up-regulation of gene expression is essential for the long-term maintenance of steroid production (60, 62), piscine FSH seems to be the main hormone sustaining Leydig cell steroidogenesis.

The finding that zebrafish (this study) and Japanese eel (16) recombinant LH proteins have limited capacities to support expression of steroidogenesis-related genes may indicate that their bioactivity regarding steroid production relies predominantly on nongenomic mechanisms. Although the present results do not allow identifying such mechanisms, possible explanations could include direct stimulation of the catalytic activity of steroidogenic enzymes, recruitment of mRNAs encoding for steroidogenic enzymes and/or steroidogenic acute regulatory protein from previously produced and stabilized pools, and/or activation (*e.g.* by phosphorylation) of already synthesized but inactive protein pools (60, 62). However, all these assumptions would postulate the use by FSH of yet unidentified downstream pathways, which may or may not involve the participation of cAMP and/or PKA, to induce the observed up-regulation of steroidogenesis genes expression. As mentioned previously, the latter is essential for the sustained production of steroids, suggesting that the effects exerted by piscine LH on steroidogenesis have a limited duration. This may constitute an additional mechanism in zebrafish Leydig cells to counterbalance the constitutive activity of the Lhcgr (54). Hence, we propose that the LH bioactivity on Leydig cells may be restricted to a transient response of the testicular steroidogenic system, such as described in spawning goldfish, a close relative of the zebrafish, with processes of short duration requiring a quick response in males once they perceive the stimuli from ovulatory females (27, 63). Such events are characterized by the presence of high plasma sex steroid levels, and the two gonadotropins may cooperate to produce the high steroid output required for semen hydration, for stimulating courtship/spawning behavior, and/or for the release of pheromones into the water (7, 27, 63, 64).

Although rzfLH did not change the mRNA levels of steroidogenesis-related genes analyzed, it did increase *fshr* mRNA levels in our short-term experiments (2 h). Interestingly, rzfFSH induced a partial down-regulation of *fshr* mRNA levels after 2 d culture, which, together with the short-term LH-induced up-regulation, may represent a regulatory loop to achieve a balanced testicular gonadotropin receptor expression.

The ability of hCG to stimulate steroidogenesis in fish is well known (65) and is explained by its capacity to activate the Lhcgr but not the Fshr in different species, including the zebrafish (23, 54). In our *in vivo* study, hCG elicited downstream effects on zebrafish testis similar to rzfLH (*i.e.* stimulation of androgen release without up-regulating gene expression), although the effects at the receptor transcription level were different: *lhcgr* mRNA down-regulation occurred after hCG but not after rzfLH treatment. This could be associated with the long half-life of hCG (65) or the comparatively high concentration used (10 IU to ~715 ng); also, the high specificity of hCG for the Lhcgr of fish is remarkable because piscine (purified or recombinant) LH proteins, although preferring the Lhcgr, also activate the Fshr (7, 22, 24, 52).

The experimental setup involving the 3β-Hsd inhibitor trilostane revealed inhibitory effects of androgens on rzfFSH-induced changes in both *fshr* and *star* mRNA levels. Both genes are situated far upstream in the steroidogenic process (60), and thus, androgen-mediated inhibition may be part of a negative feedback loop to prevent overstimulation of steroidogenesis. This is supported by ongoing studies in our laboratory showing that testicular *star* mRNA levels are down-regulated by exposure to 11-KT (unpublished) as well as by the information available for mammalian models (66, 67).

Further confirmation of the differential roles of FSH and LH on zebrafish Leydig cell functions was obtained in the current study by measuring mRNA levels of the Leydig cell-specific gene *insl3*. Its expression remained unchanged after exposure to rzfLH or hCG but was strongly up-regulated by rzfFSH in a steroid-independent manner. The latter observation agrees with own *in vitro* assays in zebrafish testis showing no effect of increasing concentrations of 11-KT on *insl3* mRNA transcription (unpublished), although it contrasts with previous studies reporting stimulatory effects of androgens on testicular *insl3* expression in amphibians (68). In mammals, although one study has reported that *Insl3* expression was regulated by androgens both in primary cultures of rat Leydig cells and in the mouse MA-10 tumor Leydig cell line (69), another study using the same cell line concluded that *Insl3* was expressed in a constitutive manner (70). To our knowledge, the present report is the first demonstrating in any teleost species a stimulatory effect of FSH on *insl3* expression, a Leydig cell-specific factor with antiapoptotic effects on meiotic germ cells in mammals (71) but with yet unknown functions in fish.

Although there is substantial evidence supporting the critical nursing functions of Sertoli cells during germ cell proliferation and differentiation in fish spermatogenesis (40), very little is known about the mechanisms by which Sertoli cells relay the gonadotropic signals toward the developing germ cells. Thus, in an attempt to characterize gonadotropin effects on zebrafish Sertoli cells, we measured the mRNA levels of a number of Sertoli cell-specific genes in several bioassays. However, among the genes examined, we found little response to both gonadotropins, the exceptions being the transient increases in *fshr* or *ar* mRNA levels after 2 h *in vivo* rzfLH and rzfFSH treatment, respectively. In general, our candidate gene approach regarding gonadotropin-sensitive Sertoli cell transcripts was of limited success; future studies should therefore include unbiased approaches.

Whether the effects elicited by recombinant hormones (in this and other studies, *e.g.* Refs. 11, 13, 16, and 72–76) reflect the biological activities of the pituitary hormones could be answered only by comparing highly purified gonadotropins with homologous recombinant proteins, a setting not feasible in zebrafish due to its small size. Nevertheless, different recombinant gonadotropins, including single-chain and/or N-terminal His-tagged molecules (like the hormones used in this study), have been shown to elicit biological effects comparable to heterodimeric purified native hormones, which has been explained by the receptors' capacity to specifically recognize their ligands even if those are presented in different conformations (77–82). Moreover, based on structural analysis of human FSH in complex with its receptor (80), the His tag used for purification is pointing away from the major receptor interaction sites, and therefore, it is unlikely to interfere with receptor activation. Whether this applies to fish gonadotropins and their receptors as well remains to be explored with species large enough to allow purification of pituitary hormones for comparative studies. However, our His-tagged, single-chain gonadotropins were able to fully exploit the steroidogenic potency of zebrafish testis tissue (as measured by forskolin incubations), demonstrating their suitability as *bona fide* ligands to entirely activate their receptors.

In summary, the zebrafish testis shows a unique, previously unreported among vertebrates, cellular pattern of *lhcgr* and *fshr* expression, because both Leydig and Sertoli cells express the mRNAs for both receptors. Particularly remarkable is the *lhcgr* expression by Sertoli cells, which may be related to the undifferentiated gonochoristic mode of sex differentiation in zebrafish. In a series of functional bioassays, rzfFSH showed a higher potency in stimulating the testicular steroidogenic system and a higher capacity to alter testicular gene expression profiles than rzfLH. These differences highlight the distinct functional domains of FSH and LH in regulating testis physiology in adult zebrafish, with a role for FSH as the constitutive driving force for both steroidogenesis and spermatogenesis, whereas the function of LH might be seen in context with an acute, additional steroid demand. Moreover, although pharmacological data show that the Fshr can be cross-activated by LH, this does not appear to happen *in vivo* or under culture conditions, because only FSH induced clear changes in the expression of selected testicular genes.

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Address all correspondence and requests for reprints to: Dr. Rüdiger W. Schulz, Utrecht University, Faculty of Science, Department of Biology, Division of Endocrinology and Metabolism, Kruyt Building Room W-604, Padualaan 8, 3584 CH Utrecht, The Netherlands. E-mail: r.w.schulz@uu.nl.

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Current address for Á.G.-L.: Institute of Marine Sciences of Andalucía (High Spanish Council for Scientific Research), Avenida República Saharaui 2, 11510 Puerto Real, Cádiz, Spain.

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