Exposure of *Xenopus laevis* tadpoles to finasteride, an inhibitor of 5- α reductase activity, impairs spermatogenesis and alters hypophyseal feedback mechanisms

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Abstract

Sexual steroids have major regulatory functions in gonadal development, maturation of gametes and sexual differentiation in vertebrates. Previous studies in amphibians provided evidence that dihydrotestosterone and activity of 5- α reductases might play a significant role in androgen-mediated reproductive biology. To test the involvement of 5- α reductases in maturation of gametes in amphibians, *Xenopus laevis* was exposed to finasteride (FIN), a known inhibitor of 5- α reductase enzyme activity. In a long-term exposure from stage 46 to 66, severe disruption of spermatogenesis was observed in histological analysis of testes as detected by occurrence of empty spermatocysts, while ovaries remained unaffected. Real-time PCR analyses of male and female brain revealed an increase of LH β mRNA and a decrease of FSH β mRNA in males, suggesting a signalling on testes that could result in increased steroidogenesis and reduced Sertoli cell proliferation. Accordingly, the mRNA expression of P450 side chain cleavage enzyme and 5- α reductase type 2 was increased in testes, while no effects could be observed on steroidogenic genes in ovaries. A short-term exposure to testosterone, FIN and testosterone+FIN showed that transient effects of FIN targeted males selectively and, in particular, interfered with the hypothalamus–pituitary–gonad axis. Furthermore, a negative feedback of testosterone on LH β was observed on males and females. This study provides evidence that exposure of *X. laevis* to FIN, an inhibitor of 5- α reductases, impaired spermatogenesis and involved sex-specific hypophyseal feedback mechanisms.

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Introduction

Synthesis of sexual steroids is a conserved process in vertebrates starting with cholesterol that is converted by the actions of multiple enzymes into testosterone. Testosterone can either exert androgen action by itself or can be further converted into dihydrotestosterone (DHT) or into 17 β -oestradiol (Payne & Hales 2004). Leydig cells in testis and theca/granulosa cells in ovaries are the main producing cells of sexual steroids that are subsequently released into the bloodstream. Biological action in peripheral tissues often needs the conversion of testosterone into DHT that is accomplished by the enzyme 5- α reductase. Two isozymes exist for 5- α reductase, named type 1 (Srd5a1) and type 2 (Srd5a2), which are differentially distributed in many tissues. In rats, Srd5a2 is confined to androgen-dependent tissues, while Srd5a1 is present in various tissues (Torres *et al.* 2003). Especially, Srd5a2 activity is believed to play a key role in androgen-regulated reproductive biology.

In mammals, as in amphibians, the more potent androgen is DHT unlike in fish, where 11-ketotestosterone is the most potent androgen (Norris 1997). DHT promotes in humans and mammals the development of external genitalia or androgenic tissues like prostate, while testosterone is responsible for the development of male internal genitalia. Finasteride (FIN), a well-known inhibitor of 5- α reductase activity with higher potency to Srd5a2 than to Srd5a1 (George *et al.* 1989, Prahalada *et al.* 1997, Ellsworth *et al.* 1998), was frequently used to experimentally unravel functions of 5- α reductases being involved in male pattern hair loss (Kawashima *et al.* 2004) or benign prostate hyperplasia or prostate cancer (Luo *et al.* 2003). If administered *in utero*, external genitalia were feminized in rats (Imperato-McGinley *et al.* 1992) or developed abnormalities in rhesus monkeys (Pralhada *et al.* 1997). Humans with deficiency of Srd5a2 developed feminized genitalia (Imperato-McGinley *et al.* 1981), and polymorphisms in Srd5a2 gene were associated with differences in sperm concentration and motility (Elzanaty *et al.* 2006).

In the Srd5a2 gene promoter region, a progesterone and an androgen response element was identified in mouse, suggesting that different androgens might be able to regulate gene expression of Srd5a2 (Matsui *et al.* 2002). Testosterone regulated enzyme activity of 5- α reductase in an *in vitro* system (Andersson *et al.* 1989), mRNA expression of Srd5a2 in prostate of intact and castrated rats *in vivo* (Torres *et al.* 2003) and in rat brain *in vivo* and *in vitro* (Poletti *et al.* 1998). However, also DHT is described to induce 5- α reductase activity in rats (George *et al.* 1991). Effects of both testosterone and DHT are mediated via the androgen receptor, and it is known that some genes are differentially regulated by testosterone and DHT respectively (Avila *et al.* 1998). The mechanism for this is still unclear, but it is proposed that especially in peripheral tissues with low testosterone level, specific action of 5- α reduced androgens is needed.

In amphibians, some studies provided evidence that DHT is a decisive androgen for sexual differentiation in *Xenopus laevis*: DHT but not testosterone at a concentration of 10^{-8} M induced sex reversal in an exposure experiment (Bögi *et al.* 2002, Kloas 2002), and a sex-specific pattern of Srd5a2 mRNA expression during ontogeny suggested an important function in male gamete maturation (Urbatzka *et al.* 2007). The aim of the present study was to analyse the effect of FIN on the development of gonads in *X. laevis* and to gain insights into the physiological function of 5- α reductases in regulating reproductive biology of amphibians. Therefore, tadpoles of *X. laevis* were exposed to FIN during their sensitive phase of gonadal development and sexual differentiation with the intention to decrease the activities of 5- α reductase enzymes in *X. laevis*. The effects of FIN on gonad histology of *X. laevis* were monitored at stage 66, just after the completion of metamorphosis. Additionally, the expression of LH β and FSH β mRNA was analysed in brain samples and the expression of selected steroidogenic genes (P450 side chain cleavage enzyme (P450scc), steroidogenic acute regulatory protein (StAR), Srd5a1, Srd5a2 and aromatase (Aro)) in gonad samples. In complement to the analysis of long-term effects of FIN, a short-term exposure was conducted to analyse the transient effects of FIN. The short-term exposure included three treatment groups, testosterone, FIN and testosterone+FIN, to address the question of a potential regulation of 5- α reductase by testosterone in amphibians, a regulation that was frequently observed in mammalian model species. In this study, *X. laevis* was used for the first time as a comparative model to unravel the potential function of 5- α reductases on spermatogenesis in lower vertebrates.

Material and methods

Exposure

X. laevis was taken from the breeding stock of the Institute of Freshwater Ecology and Inland Fisheries, Berlin, Germany. Adult *X. laevis* was injected with human chorionic gonadotrophin (Sigma) into the dorsal lymph sac to induce spawning as described previously (Kloas *et al.* 1999). Fertilized eggs and tadpoles were reared in 50-l tanks containing reconstituted tap water using deionized water supplemented with 2.5 g marine salt (Tropic Marin Meersalz, Tagis, Dreieich, Germany) per 10 l. Tanks were aerated and temperature was adjusted to 22 ± 1 °C. Tadpoles were kept at a 12 h light:12 h darkness cycle and were fed daily with Sera Micron (Sera GmbH, Heinsberg, Germany). The developmental stages of the tadpoles were determined according to the Normal Table of *X. laevis* (Nieuwkoop & Faber 1994).

For a long-term exposure, tadpoles at stage 46 were transferred to a 10-l aerated glass aquaria at a density of 30 tadpoles per tank containing 7 l of reconstituted tap water. Tadpoles were exposed to FIN (APIN Chemicals Ltd, a gift of Dr John Ashby, Central Toxicology Laboratory, Cheshire, Syngenta, UK), a known 5- α reductase enzyme inhibitor. FIN was dissolved in dimethylsulphoxide (DMSO, Sigma) and the highest soluble concentration was chosen for exposure, which did not recrystallize if added to the water. The final concentration of FIN was 7×10^{-6} M (2.66 mg/l) and DMSO concentration was 0.0286%. Exposure was conducted in duplicate tanks (total $n=60$) and included a solvent control. Water and chemicals were renewed every Monday, Wednesday and Friday. Tadpoles were sampled after the completion of metamorphosis (stage 66). Sex of tadpoles was determined by gross morphology of the gonads using a stereo microscope before gonads were fixed in Bouin's solution for 24 h. Fixed gonads were transferred to 80% ethanol (EtOH) for 24 h and then stored in fresh 80% EtOH until further processing for histological analyses. Further gonad and brain samples of male and female tadpoles were dissected, immediately snap frozen in liquid nitrogen and stored at -80 °C until further analyses.

In the short-term exposure, tadpoles were reared in aerated glass aquaria until they reached stage 58. At this stage, tadpoles were transferred to the exposure tanks in a density of 18 tadpoles per tank containing 7 l of reconstituted tap water. Tadpoles were exposed to testosterone at a supraphysiological concentration of 10^{-7} M, to FIN at a concentration of 7×10^{-6} M and to a combination of both. A solvent control (DMSO 0.0867%) and a control without solvent were included in the experimental set-up. Exposure lasted

4 days and water and chemicals were changed daily. Brain and gonads of six male and female tadpoles were dissected at day 5, directly snap frozen in liquid nitrogen and stored at -80°C until further analyses.

Histology

From the long-term exposure, six males and six females of the control and the FIN treatment were analysed for histological alterations of the gonads. From paraffin blocks, 3–4 μm sections were cut using a microtome. Tissue sections were deparaffinized in xylene for 15 min, washed sequentially in 96, 90, 70, 50 and 30% EtOH (2 min each) and in aqua dest. for 5 min. Subsequently, one set of sections was stained with haematoxylin–eosin, while another set was stained by immunocytochemistry for the proliferating cell nuclear antigen (PCNA) as indication for cell proliferation. Heat-induced epitope retrieval was performed by transfer of the slides into a jar containing Sorensen's citrate buffer (pH 6) on a hot water bath ($96\text{--}99^{\circ}\text{C}$) for 30 min. After cooling down the slides for 20 min at room temperature, the slides were washed with PBS and aqua dest. Endogenous peroxidase was quenched in 3% hydrogen peroxide for 5 min with subsequent rinsing in PBS. We applied 100 μl of anti-PCNA to the slide, which was incubated at room temperature in a humidity chamber for 30 min and rinsed in PBS. The working concentration of anti-PCNA was 1:200, diluted in PBS. Next, 100 μl of peroxidase were added to the slide, followed by incubation of the slide for 30 min in a humidity chamber and rinsing with PBS. The final step comprised the application of the chromagen solution NovaRED, incubation in a humidity chamber for 15 min, washing in aqua dest. for 5 min and counter-staining of the slides with haematoxylin.

RNA isolation and reverse transcription

Extraction of total RNA of brain samples of tadpoles from the long-term exposure and brain and gonad samples of tadpoles from the short-term exposure was

performed by using RNA extraction kits of Qiagen (RNeasy Micro Kit, RNeasy Mini Kit), according to the instructions of the manufacturer and included on-column DNase ingestion (Qiagen). Transcription of RNA into cDNA was accomplished by reverse transcriptase reaction (RT) and was performed with 1 μg total RNA for the brain samples and with 200 ng total RNA for the gonad samples. For RT of brain samples, 7.5 pM poly (dT) primer (Biometra, Göttingen, Germany), 10 mM dNTP (Qbiogene, Heidelberg, Germany) and 10 U AMV reverse transcriptase (Finnzymes, Espoo, Finland) were applied in a 30 μl reaction, while for RT of gonad samples, 10 U AffinityScript reverse transcriptase (Stratagene, Amsterdam, The Netherlands) were used in a 20 μl reaction.

Real-time RT-PCR

Amplifications of cDNA specific for LH β , FSH β , P450scc, StAR, Srd5a1, Srd5a2, Aro and elongation factor 1- α as housekeeping gene of *X. laevis* were carried out by using real-time RT-PCR. Amplification was carried out in a thermal cycler (Stratagene, Mx3000P) using SYBR Green. The following thermal cycling conditions were applied: 7 min 40 s at 95°C followed by 40 cycles of 17 s at 95°C , 25 s at 62°C and 25 s at 72°C . Melting curve analyses were performed with the following setting: 40 s at 95°C , 30 s at 55°C and 30 s at 95°C . For all PCRs, cDNA was diluted 1:5 (brain samples) or 1:10 (gonad samples), and 3 μl of diluted cDNA were mixed in a 25 μl reaction with 10 mM dNTP (Qbiogene), 1:40,000 SYBR (Invitrogen), 1:200 ROX (Invitrogen), 2 mM MgCl_2 , 100–400 pM forward and reverse primer and 1 U Platinum Taq-Polymerase (Invitrogen).

Primers were designed according to sequences published in the National Centre for Biotechnology Information. All sequences were derived from *X. laevis* with the exception of Srd5a1 and Srd5a2, which were deduced from *Xenopus tropicalis*. Table 1 lists the sequences of the designed primers. To test specificity of the PCR products, the bands were run on an agarose

Table 1 Overview about primer sequences used for real-time RT-PCR of various cDNAs related to the hypothalamus–pituitary–gonad axis of *Xenopus laevis*

	Primer sequence, forward (5'–3')	Primer sequence, reverse (5'–3')
LH β , luteinizing hormone β -subunit	CACTGACGCTTCTGGGGTTCTAC	GATTGGGCAGTCGCTTTTCTCT
FSH β , follicle stimulating hormone, β -subunit	TGCTCGTTCTGTGTTGGAAGATG	CCTGTTTGATGAGTGGATGCTTTG
Srd5a1, steroid 5- α reductase, polypeptide 1	CTGAACCTCTTGGCTATG	GATGCCTAACTCGGATTG
Srd5a2, steroid 5- α reductase, polypeptide 2	CTTATCCTGCTGCTTATG	AGTCCTGTGGAAATAGTG
Aro, aromatase	CGGTTCCATATCGTTACTTCC	GCATCTTCCTCTCAATGTCTG
P450scc, P450 side chain cleavage enzyme	CAGTGTTGGCCAGGATTTTGT	GCGGAAGAGCTCATTGGTCAAG
StAR, steroidogenic acute regulatory protein	AACCCAAATGTCAAGGAAGTCAAG	ACAAAATCCCGGGCCCCCTACAATA
EF1- α , elongation factor 1- α chain	ACCGCACAGGTTATCATC	CAACAATGGCAGCATCTC

gel, extracted (QIAquick, Gel extraction kit, Qiagen) and forwarded to automated sequencing (SeqLab, Sequence Laboratories, Göttingen, Germany). Sequence comparisons by BLAST and sequence alignments (BioEdit Software, Version 7.0.9.0, Carlsbad, CA, USA) confirmed the specificity of amplified PCR products.

Data analysis and statistics

MxPro software (Stratagene) was used to analyse real-time PCR data using the $\Delta\Delta C_t$ method, including the PCR efficiencies of the genes (ranging from 91.6 to 104.5%). Values of the mRNA expressions of the target genes were normalized by the values of the house-keeping gene. After testing normal distribution, data of long-term exposure were analysed with unpaired *t*-tests. For data of short-term exposure, one-way ANOVA was used to examine significant differences between the mRNA expressions of the treatments. Either Tukey's test or Dunnett's T3-test was applied depending on the outcome of testing equality of variance by the Levene's test. Histological data were analysed by using Mann–Whitney *U* test. For all statistical analyses, differences were regarded to be significant if *P* was <0.05.

Results

Long-term exposure

Mortality of tadpoles during exposure was very low and was 2% in control tanks (one individual) and 0% in FIN treatment tanks. No differences between control and FIN treatment groups were observed regarding the time needed to complete metamorphosis and their total length at stage 66. Tadpoles developed from stage 46 to stage 66 in about 6 weeks and the mean length was 1.8 ± 0.2 cm. Exposure of *X. laevis* tadpoles during ontogeny to FIN did not affect the sex ratio of tadpoles. Sex ratio in the FIN treatment group was not significantly different from the solvent control group with 60% males and 40% females.

Histological analyses of gonads revealed that male *X. laevis* from the solvent control group had relatively immature testes containing mainly spermatogonia and spermatocytes in numerous spermatocysts (Fig. 1, panel A). A further maturation to spermatids or spermatozoa was not observed in these animals. Proliferation activity of spermatogonia and spermatocytes was high as indicated by PCNA staining (Fig. 1, panel B). FIN-exposed male *X. laevis* possessed comparable maturation stages as in the solvent control group, namely spermatogonia and spermatocytes but not spermatids or spermatozoa. In five out of six analysed FIN-treated males, empty cavities were observed in the tubules and this likely corresponded

to former spermatocysts with secondary spermatogonia (Fig. 1, panel C). Quantitative analyses of male gonadal sections revealed a statistically significant occurrence of empty spermatocysts ($P < 0.01$). While there was no significant difference, FIN-treated male animals seemed to have a lower number of tubuli, spermatogonia and spermatocytes (Table 2). Intensity of proliferation was dramatically reduced in all FIN-treated males compared with the solvent control group, indicated by the lower number of visible mitoses and reduced PCNA staining (Fig. 1, panel D).

In female *X. laevis* from the solvent control group, ovaries were in the maturation stage in that ovarian cavities were formed or expanded. In the cortical region, numerous germ cells and primary oocytes arranged in nests were observed (Fig. 1, panel E). Proliferation activity indicated by PCNA staining was moderate in germ cells and high in primary oocytes (Fig. 1, panel F). Stages of maturation and the structure of gonads of FIN-treated female *X. laevis* were similar compared with females from the solvent control (Fig. 1, panel G), and also proliferation activity was about the same (Fig. 1, panel H). The development of an ovarian cavity was not observed in one ovary.

In brain samples of male *X. laevis*, LH β mRNA expression was about ninefold increased in the FIN treatment group ($P < 0.001$), while FSH β mRNA expression was decreased in FIN-treated males ($P < 0.05$; Fig. 2). In testes, P450scc and Srd5a2 mRNA were significantly increased ($P < 0.01$), while no effects were observed for StAR, Srd5a1 and Aro mRNA expression (Fig. 2).

In brain of female *X. laevis*, the expression of both gonadotrophins was not altered by water-borne exposure to FIN (Fig. 3). In ovaries, no different expression was observed in the FIN treatment group relative to the solvent control for all analysed steroidogenic genes.

Short-term exposure

In male tadpoles from the 4 day exposure, LH β mRNA in the brain was significantly decreased by testosterone as well as by the combined exposure to testosterone and FIN. By contrast, exposure to FIN alone led to a significant increase in LH β mRNA expression and was about eightfold higher than in the control and in the solvent control (Fig. 4). FSH β mRNA expression was not changed by any treatment. In the gonads, P450scc mRNA expression was twofold elevated in response to FIN, while the other treatments had no effect. StAR, Srd5a1, Srd5a2 and Aro mRNA expression stayed at the same expression levels compared with both control groups.

In female tadpoles, LH β mRNA in the brain was significantly decreased by testosterone and the combined exposure to testosterone and FIN compared with

Figure 1 Selected pictures of histological sections of male and female gonads of *X. laevis* at stage 66 from the long-term exposure. On the left side of the figure (panels A, C, E and G), haematoxylin–eosin staining was applied; while on the right side (panels B, D, F and H), proliferating cell nuclear antigen (PCNA) staining was used. Panels A and B refer to control males, panels C and D to FIN-treated males, panels E and F to control females and panels G and H to FIN-treated females. The bar indicates a distance of 50 μ m. Full colour version of this figure available via <http://dx.doi.org/10.1677/JME-09-0058>.

the solvent control, corresponding to the results obtained in male tadpoles (Fig. 5). FIN alone did not change LH β mRNA expression in females. No significant changes of mRNA expression were observed in the other analysed genes (FSH β , P450scc, STAR, Srd5a1, Srd5a2 and Aro) relative to control and solvent control.

Discussion

Tadpoles of *X. laevis* were exposed to FIN, a known inhibitor of 5- α reductase enzyme activity (George *et al.* 1989, Ellsworth *et al.* 1998), during their sensitive phase of gonadal development and sexual

Table 2 Results of quantification of histological analyses of testis of *Xenopus laevis* at stage 66 from the long-term exposure

	tub n	sptg %	sptg n	sptc %	sptc n	sptd	sptz	fsptcst	esptcst
SoCo									
Mean	11.0	44.5	20.7	55.5	39.0	0.0	0.0	59.8	0.0
S.D.	15.9	20.2	16.4	20.2	41.3	0.0	0.0	49.2	0.0
Min	0		2		1	0	0	4	0
Max	40		40		115	0	0	132	0
FIN									
Mean	2.2	42.5	11.4	57.5	11.2	0.0	0.0	14.2	11.2
S.D.	3.5	26.5	6.8	26.5	5.8	0.0	0.0	13.1	5.8
Min	0		0		4	0	0	0	4
Max	8		18		19	0	0	25	18
Sign. diff.	–		–		–	–	–	–	*

Significant differences were analysed by Mann–Whitney *U* test (**P* < 0.01). tub, tubules; sptg, spermatogonia; sptc, spermatocytes; fsptcst, filled spermatocysts; esptcst, empty spermatocysts; SoCo, solvent control; FIN, finasteride.

differentiation. In a previous study, an important function of 5-α-reduced metabolites was suggested for spermatogenesis, observing sex-specific mRNA expression pattern of *Srd5a2* in *X. laevis* tadpoles during their ontogeny at the beginning of gamete maturation (Urbatzka *et al.* 2007). The present study demonstrated that FIN affected specifically males and not females leading to a disruption of spermatogenesis and to altered mRNA expression of gonadotrophins in the brain and of steroidogenic genes in the gonads.

Histological analyses of gonads of male *X. laevis* tadpoles exposed to FIN revealed a severe disruption of spermatogenesis. Emptied cavities were observed in the analysed gonads that corresponded to former spermatocysts with secondary spermatogonia (Fig. 1) and a reduced proliferation activity in FIN-treated males. According to these results, it was shown in male rats during puberty that a peak of 5-α reductases and 5-α-reduced metabolites coincided with the first wave of spermatogenesis, suggesting a role for 5-α reductases in the initiation of spermatogenesis (Killian *et al.* 2003). In studies during adulthood, no effects of FIN on testicular histomorphology were found in rats (George *et al.* 1989, Rhoden *et al.* 2002) and dogs (Juniewicz *et al.* 1993). By contrast, a study performed in adult rats showed that administration of FIN suppressed the testosterone-induced restoration of spermatogenesis and demonstrated clearly a role for 5-α-reduced androgens in adult spermatogenesis (O'Donnell *et al.* 1996). In contrast to the specific mode of action of FIN, the antiandrogen flutamide (FLU) inhibits the action of various androgens at the androgen receptor, and exposure experiments demonstrated the importance of androgens in spermatogenesis in different vertebrates. If rats were exposed *in utero* to FLU, the death of testicular germ cells was observed in a concentration-dependent way mediated by apoptotic gene expression (Bozec *et al.* 2004). Fish exposed to

FLU experienced spermatocyte degeneration in fat-head minnow (Jensen *et al.* 2004) or reduced numbers of spermatogenic cysts in male guppies (Kinnberg & Toft 2003).

In contrast to the results obtained in male tadpoles, histological analyses of ovaries demonstrated no alterations in the FIN treatment group compared with the control group. The single finding of one female gonad without the forming of an ovarian cavity may be an effect of FIN, but has to be verified in future experiments. In tadpoles of *Bufo bufo* and *Rana dalmatina*, the inhibition of 5-α reductase activity accelerated the ovarian differentiation in females, suggesting an inhibiting role for 5-α-reduced metabolites regarding the timing of ovarian differentiation and maybe also for oocyte maturation in females (Zaccanti *et al.* 1994, Petrini & Zaccanti 1998). However, this finding could not be confirmed in the present study.

The analysis of gonadotrophin mRNA expression in male and female *X. laevis* from the long-term exposure revealed a sex-specific hypophyseal feedback mechanism. Selectively in males, LHβ mRNA was strongly increased in the FIN treatment group, while FSHβ mRNA was decreased (Fig. 2). LH is known to act mainly on steroidogenesis in gonads, while FSH is essential for Sertoli cell proliferation (Weltzien *et al.* 2004). Therefore, this observed regulation pattern could signal an increased steroidogenesis in testes and a reduced Sertoli cell proliferation that could potentially have caused the observed impairment of spermatogonia maturation considering the central role of Sertoli cells in germ cell development. Mitotic activity of Sertoli cells in fish was highest during spermatogonial proliferation and considered as an important factor determining testis size and sperm production (Schulz *et al.* 2005). The modulatory effect of FIN on gonadotrophin mRNA expression in male *X. laevis* is shown for the first time and demonstrated an involvement of

Figure 2 Real-time RT-PCR data of male *X. laevis* at stage 66 from long-term exposure. LH β and FSH β mRNA expression were analysed in brain, while P450 side chain cleavage enzyme (P450scc), steroidogenic acute regulatory protein (StAR), 5- α reductase type 2 (Srd5a2) and type 1 (Srd5a1) and aromatase (Aro) mRNA expressions were studied in gonads. Gene expression was normalized by the housekeeping gene elongation factor 1- α . Data are represented as mean $\pm$ s.d. derived from six individuals each. Significant differences between solvent control (SoCo) and finasteride treatment group (FIN) were analysed by *t*-test, (** P <0.01; *** P <0.001; * P <0.05).

a physiological regulation of the H-P-G axis. This finding fits to a study in rats where a high dose of FIN (250 mg/kg per day) increased the plasma LH level that correlated with Leydig cell hyperplasia (Prahallada *et al.* 1994), while in another study, plasma LH level remained unchanged at a concentration of 20 mg/kg per day (Ribeiro & Pereira 2005). The contrary regulation of LH β and FSH β mRNA expression in response to sexual steroids was confirmed in further experiments from our workgroup, even if the mechanism remains unclear (Urbatzka *et al.* 2008). From studies in fish, it is known that sexual steroids can regulate gonadotrophins differently, inhibiting one gene and stimulating the other (Sohn *et al.* 2001, Mateos *et al.* 2002).

In gonads of long-term-exposed animals, steroidogenic gene expression was changed in males, but not in females (Figs 2 and 3). FIN treatment increased

P450scc and Srd5a2 mRNA expression in testes, while StAR, Srd5a1 and Aro mRNA expression was not affected. It is well known that steroidogenesis is under the control of gonadotrophins (Weltzien *et al.* 2004), and therefore it is likely that increased LH β mRNA in brain stimulated the transcription of P450scc. Interestingly, StAR mRNA expression was only slightly, but not significantly, increased. StAR is the enzyme responsible for cholesterol transport into the mitochondria where it is converted by P450scc to pregnenolone (Arukwe 2008). Studies in fish showed that StAR and P450scc mRNA expression patterns were different under some experimental conditions regarding to exposure time, compounds and their concentration (Arukwe 2005, Kortner *et al.* 2009a,b). The increase of Srd5a2 mRNA in testis in FIN-treated animals is suggested to be a counter-regulation to the inhibition of 5- α reductase enzyme activities and indicated the presence of a regulatory feedback loop. Thereby, the counter-regulation could be driven directly by the assumed lower DHT level or by the observed feedback on LH β mRNA expression. The Srd5a1 mRNA expression was only slightly increased, but not significant, and could be associated to the fact that FIN inhibits 5- α reductase type 2 enzyme with higher potency than 5- α reductase

Figure 3 Real-time RT-PCR data of female *X. laevis* at stage 66 from the long-term exposure corresponding to Fig. 2.

Figure 4 Real-time RT-PCR data of male *X. laevis* tadpoles at stage 58 exposed for 4 days to 10^{-7} M testosterone (T), 7×10^{-6} M finasteride (FIN) or a combination of both (T+FIN). The short-term exposure included a solvent control group (SoCo) and a control group without solvent (Co). LH β and FSH β mRNA expression were analysed in brain, while P450 side chain cleavage enzyme (P450scc), steroidogenic acute regulatory protein (StAR), 5- α reductase type 2 (Srd5a2) and type 1 (Srd5a1) and aromatase (Aro) mRNA expressions were studied in gonads. Gene expression was normalized by the housekeeping gene elongation factor 1- α . Data are represented as mean $\pm$ s.d. derived from six individuals each. Different letters denote significant differences between treatment groups that were analysed by ANOVA (Tukey's or Dunnett's T3 test, $P < 0.05$; n.s., not significant).

type 1 enzyme (Prahalada *et al.* 1997, Ellsworth *et al.* 1998). Aro mRNA expression stayed at the same expression level indicating no interference of FIN with oestrogen synthesis.

Male and female tadpoles at stage 58 were exposed to testosterone, FIN and a combination of both in a short-term exposure to analyse transient effects on gonadotrophin mRNA expression in the brain and steroidogenic gene expression in the gonads. Testosterone was chosen as an androgen since it has a stimulatory function on 5- α reductase mRNA expression and enzyme activity in the peripheral tissues (Andersson *et al.* 1989, Poletti *et al.* 1998, Torres *et al.* 2003). LH β mRNA expression was significantly reduced

by testosterone and testosterone+FIN in males and females, but no effects were observed on FSH β mRNA expression or steroidogenic gene expression in the gonads. The negative feedback of androgens on the H-P-G axis is known from previous studies in mature amphibians. DHT reduced the plasma LH level in male, adult *Rana pipiens* (Tsai *et al.* 2005) and methylidihydrotestosterone (MDHT) decreased the LH β mRNA expression in male, adult *X. laevis* (Urbatzka *et al.* 2006). Thus, it is suggested that decreased LH β mRNA levels in testosterone and testosterone+FIN treatment are due to negative feedback of testosterone. A regulation of Srd5a2 by testosterone like in mammals or *in vitro* (Andersson *et al.* 1989, George *et al.* 1991, Poletti *et al.* 1998, Torres *et al.* 2003) was not observed in this study for *X. laevis*. In accordance with the long-term exposure, the effect of FIN was restricted to males and included an increase in LH β mRNA and P450scc mRNA indicating an increase in steroidogenesis mediated by feedback on LH. FIN treatment in rat resulted in an increase in testosterone and androstenedione, a decrease in DHT (George 1997) and additionally increased oestradiol in humans (Castro-Magana *et al.* 1996). If a similar effect of FIN on steroids is assumed in this study, the increased LH β mRNA expression in the

Figure 5 Real-time RT-PCR data of female *X. laevis* tadpoles at stage 58 corresponding to Fig. 4.

FIN treatment group is unlikely to be induced by the increase in testosterone or oestrogens since exposure to testosterone was demonstrated in this study to inhibit LH β mRNA expression, an effect also known for exposure of amphibians to oestrogens (Urbatzka *et al.* 2006). Furthermore, androstenedione is known to have only a low affinity to the androgen receptor and is therefore an improbable candidate for steroidal feedback. In contrary, we suggest that the increase of LH β in the FIN treatment could be due to the assumed lower DHT levels, a regulation that would fit to another experiment where exposure to FLU, an androgen receptor antagonist, increased LH β mRNA expression in males (Urbatzka *et al.* 2008). A decrease of FSH β mRNA and an increase of Srd5a2 mRNA as detected in males from the long-term exposure could only slightly be observed in the short-term exposure and was not significant different from the control. Therefore, it may indicate that these effects are time dependent. Some effects may be unmasked only after a prolonged exposure time. Furthermore, transient effects could be mediated by altered enzyme levels, while long-term effects involve *de novo* synthesis of mRNA.

Interestingly, no effect of FIN was observed in females in the short- and long-term exposures either on gonadotrophin expression in the brain or on steroidogenic gene expression in ovaries. The reasons for these results remain speculative and may be owed to a differential regulation of feedback mechanisms in the brain.

The present study provided clear evidence that FIN affected the expression of gonadotrophin mRNA in long- and short-term exposures. However, the effect of FIN on gonadotrophins could be potentially mediated by the inhibition of 5- α reductase activity or by a direct effect. The present data indicated an increased Srd5a2 mRNA expression in the long-term exposure as counter-regulation to the assumed inhibition of 5- α reductase enzyme activity. This result is in line with the fact that FIN is a specific inhibitor of 5- α reductase type 2 enzyme activity in mammals (Prahallada *et al.* 1997, Ellsworth *et al.* 1998). Accordingly, specific inhibition of 5- α reductase enzyme activity was reported in sea urchins (Wasson & Watts 1998) suggesting that FIN is acting specifically in vertebrates and non-vertebrates. An assumed lower DHT level in the FIN treatment group could have affected the gonadotrophin mRNA expression in the pituitary, since it is well known that sexual steroids feedback on the hypothalamus and pituitary. In further experiments of our workgroup, it was demonstrated that LH β mRNA expression in male *X. laevis* was increased in an exposure to the androgen receptor antagonist FLU (Urbatzka *et al.* 2008) providing further indications for this hypothesis. Nevertheless, both hypotheses are suggested to be possible until a proof of decreased enzymatic levels of

5- α reductase or DHT levels is shown in response to FIN treatment in amphibians.

Summarizing, it was shown that exposure of *X. laevis* tadpoles to FIN, an inhibitor of 5- α reductase activity, during the phase of gonadal development and sexual differentiation affected only the males and impaired spermatogenesis in testes of metamorphosed frogs. The long- and short-term exposures to FIN demonstrated a potential involvement of sex-specific hypophyseal feedback mechanisms, in particular an elevation of LH β mRNA in brain and of P450scc mRNA in gonads. Only the long-term exposure revealed an additional decrease of FSH β mRNA in brain and an increase of Srd5a2 mRNA in gonads. Decreased FSH β mRNA expression may have caused a reduced Sertoli cell proliferation, important cells for germ cell development, while increased LH β mRNA expression was suggested to stimulate steroidogenesis as counter-regulation to FIN.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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