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Expression Profiles of Reproduction- and Thyroid Hormone-Related Transcripts in the Brains of Chemically-Induced Intersex Frogs

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5-alpha reductase · Aromatase · 5-beta reductase · Fadrozole · Finasteride · Receptor · Thyroid hormone · *Xenopus tropicalis*

Abstract
Endocrine disrupting chemicals can induce intersex animals in amphibians and fish. Our previous study in frogs demonstrated that chemically-induced intersex animals can display different hepatic profiles of transcript levels than normal animals. In this study, we extend the observations to the developing frog brain. We investigated the effects of finasteride and fadrozole known to induce female- and male-biased sexual development on *Silurana tropicalis* brain mRNA levels. Real-time RT-PCR analysis of transcript levels of sex steroid- and thyroid hormone-related genes in the brain demonstrated that in finasteride-induced intersex animals, the mRNA levels of aromatase, estrogen receptor α, thyroid hormone receptor β and deiodinase type 3 were higher compared to both control males and females. Furthermore, finasteride-induced intersex animals expressed higher mRNA levels of both androgen receptor and estrogen receptor β compared to control females and to control males, respectively. Furthermore, fadrozole did not affect any of the genes analyzed in the brain but was effective at reducing aromatase activity. Intersex animals display different profiles of transcript levels in the brain whether the intersex condition was induced by an anti-androgen or anti-estrogen treatment. Finally, we conclude that a complex relationship exists between thyroid hormone-responsive genes and androgen status in frogs.

There is evidence that endocrine disrupting chemicals (EDCs) can upset sexual development and induce intersex (presence of testicular oocytes) phenotypes in wild amphibian populations [Hayes et al., 2003; McCoy et al., 2008]. Recent studies have shown that exposure to anti-androgen (e.g., finasteride) and anti-estrogen (e.g., fadrozole) treatments can alter amphibian gonadal differentiation and induce the intersex condition [Duarte-Guterman et al., 2009; Olmstead et al., 2009]. Synthesis of estrogens and androgens proceeds largely through similar enzymatic steps; however, the final step of the biosynthesis requires the action of aromatase (cyp19) and steroid 5α- and 5β-reductases (srd5alpha type 1, 2, 3 and srd5beta), respectively. In a previous study from our lab-

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oratory, we obtained intersex animals after blocking androgen or estrogen synthesis [Duarte-Guterman et al., 2009]. To block the androgen pathway, we used finasteride, a synthetic srd5alpha (type 1 and 2) inhibitor used to treat prostate cancer and benign prostatic hyperplasia in humans (17β-[N-tert-butylcarbamoyl]-4-aza-5α-androst-1-en-3-one; MK-906) [Stoner, 1990]; while estrogen synthesis was blocked using fadrozole (4-(5,6,7,8-tetrahydroimidazo[1,5-a]pyridin-5-yl)benzonitrile monohydrochloride; CGS 16949A), a specific inhibitor of cyp19 in frogs [Langlois et al., 2010] and other vertebrates [e.g., Steele et al., 1987; Ankley et al., 2002]. We demonstrated previously that intersex animals obtained after chronic exposure to either finasteride or fadrozole result in differential hepatic profiles of transcript levels of sex steroid- and thyroid hormone (TH)-related genes in *Silurana (Xenopus) tropicalis* when compared to normal males and females [Duarte-Guterman et al., 2009]. Therefore, the aim of this study was to investigate if the brains of *S. tropicalis* were also affected by these steroidogenic inhibitors and if the intersex animals exhibit different mRNA level patterns compared to normal males and females.

Sex steroids are involved in gonadal differentiation in frogs [Hayes, 1998]; however, much less is known about their roles in brain development and sexual differentiation. In most vertebrates, sex steroids are responsible for sexual differentiation of neural structures and reproductive behaviors [fish, reviewed in Diotel et al., 2010; birds, reviewed in Hutchison and Steimer, 1981; mammals, reviewed in Morris et al., 2004]. Furthermore, in the brain of birds, while 17β-estradiol and 5α-dihydrotestosterone are involved in sexual behaviors, the 5β-reduction of testosterone into 5β-dihydrotestosterone was suggested to be involved in the control of brain sensitivity to sex steroids [Hutchison and Steimer, 1981]. In addition, the developing vertebrate brain is a target of THs (thyroxine [T4] and triiodothyronine [T3]). Deficiency in THs can result in extreme defects in brain development such as cell migration and differentiation of both neuronal and glial cells [reviewed in Bernal et al., 2003]. There is also evidence of cross-talk between the developing reproductive and TH axes to control transcription in the frog brain [Hogan et al., 2007; Duarte-Guterman and Trudeau, 2010].

EDCs can detrimentally affect neuroendocrine control mechanisms and sexual behavior in vertebrates [Martiniuk et al., 2006; Mennigen et al., 2008; Ottinger et al., 2009; Hayes et al., 2010]. Therefore, one objective of this study was to identify genes in the brain that are susceptible to change if sex steroid levels are chemically altered in developing frogs. The target genes that were chosen included those involved in the synthesis and action of sex steroids and THs. A second objective of this project was to determine the profiles of transcript levels in the brain of *S. tropicalis* intersex animals. We used finasteride as a model anti-androgen and fadrozole as a model anti-estrogen compound to obtain intersex tadpoles at metamorphic climax. Apart from our previous liver study [Duarte-Guterman et al., 2009], the intersex pathology has not been further characterized in the literature. We hypothesize that chemically-induced intersex *S. tropicalis* will express different brain mRNA profiles compared to males and females (both control and exposed) and that these profiles will vary depending on the mode of action of the chemical.

Material and Methods

Animals and Exposures

Tadpoles of *Silurana tropicalis* were exposed to finasteride (25 μM) dissolved in ethanol (EtOH; 0.05% final concentration) and fadrozole (2 μM) dissolved in water from Nieuwkoop-Faber (NF [Nieuwkoop and Faber, 1994]) stage 12 until stage 60, along with their respective controls (EtOH and water controls) as previously described in Duarte-Guterman et al. [2009]. At the end of the exposure, brains were dissected and stored at –80°C. Homogenization and disruption of individual brain samples was achieved using an MM301 Mixer Mill (Retsch, Newton, Pa., USA) at 20 Hz for 3 min. Total RNA was obtained using the RNeasy Micro Kit (Qiagen, Mississauga, Ont., Canada). Isolated RNA was resuspended in RNase-free water and concentrations of RNA were determined using the NanoDrop-1000 spectrophotometer (NanoDrop Technologies Inc.). Total cDNA was prepared from 1 μg of total RNA and 0.2 μg random hexamer primers using Superscript II reverse transcriptase (Invitrogen). Sex of the animals (male, female or intersex) was based on the gonadal analysis previously described in Duarte-Guterman et al. [2009]. Intersex was defined as the presence of at least one oocyte in the testes. Intersex samples displayed a low number of oocytes (< 10 oocytes) which allowed the comparison within and between finasteride and fadrozole treatments. Gonadal histology demonstrated that 27% male, 53% female and 20% intersex individuals were produced after finasteride treatment, and 55% male, 30% female and 15% intersex individuals were obtained after fadrozole treatment.

Real-Time RT-PCR

Real-time RT-PCR assays were performed in a MX3005P real-time polymerase chain reaction system (Stratagene, La Jolla, Calif., USA). The transcript levels of *cyp19*, *srd5alpha1, srd5alpha2*, *srd5alpha3*, *srd5beta*, estrogen receptor α *(eralpha)*, estrogen receptor β *(erbeta)*, androgen receptor *(ar)*, TH receptor α *(tralpha)*, TH receptor β *(trbeta)*, deiodinases type 2 and 3 *(dio2, dio3)*, ornithine decarboxylase *(odc)*, arginine vasotocin *(avt)* and the reference gene ribosomal protein L8 *(rpl8)* were measured as described in Langlois et al. [2010]. Real-time PCR primers for *avt* (forward: 5′-tggacgacgagagcgaaa-3′; reverse: 5′-cataagccgcagga-

gaaagtc-3′; product size: 92 bp; primer concentration: 300 nM each; annealing temperature: 58°C) were also designed based on the complete sequence published in GenBank (accession no. XM_002936358) and primers were optimized following the protocol described in Langlois et al. [2010]. All the gene expression analyses were performed on eight individual brains per phenotype per treatment. The relative standard curve method was used to interpolate relative mRNA abundance of target and reference genes within each sample. The standard curves were generated using equal parts of cDNA from each sex and treatment. Reaction efficiencies were determined by the MxPro 4.0 software (Stratagene) using the slope of the standard curves and for all the genes efficiencies were 90–110% with $R^2 \geq 0.990$. The mRNA levels of the reference gene *rpl8* did not change with either finasteride or fadrozole treatment (data not shown), therefore transcript level data are normalized to *rpl8* and are presented as fold changes relative to EtOH-control males in the case of finasteride and to water-control males in the case of fadrozole.

Aromatase Activity

Aromatase activity was measured using a modified radiometric method optimized for amphibian tissue as described in Langlois et al. [2010]. Briefly, cyp19 activity was measured in pools of 2 to 4 brains of animals of the same sex (NF 60; n = 2–5 pools). Cofactor and ^{3}H-androstenedione were first incubated for 30 min at 37°C. After this pre-incubation, brains were sonicated in phosphate buffer and incubated for 80 min at 25°C. Aromatase activity was determined by tritiated water release from the C-1β carbon atom of 1β-^{3}H-androstenedione during its conversion to estrogen. Tritiated water was extracted with a charcoal solution and radioactivity was counted. Aromatase activity is expressed as fmol ^3H_2O/h·mg protein.

Statistical Analysis

Data for all the genes and for cyp19 activity were first tested for normality and homogeneity of variance using the Kolmogorov-Smirnov and the Levene tests, respectively. When the assumptions were not met, the data were transformed as required (e.g., $\log_{10}$, square root) and re-tested for normality and homogeneity of variance. Data were analyzed by one-way analysis of variance (ANOVA) followed by Bonferroni's post-hoc test for multiple comparisons. When data failed to meet assumptions even after being transformed, the nonparametric Kruskal-Wallis test on ranks was used. Differences were accepted as significant when $p < 0.05$.

Results

The effects of the anti-androgen finasteride on sex steroid- and TH-related mRNA levels in the brain of *S. tropicalis* metamorphs are shown in figure 1. Chronic exposure to finasteride significantly increased *cyp19* (1.7-fold; fig. 1B), *eralpha* (1.7-fold; fig. 1C), *dio3* (3.8-fold; fig. 1E) and *trbeta* (2.2-fold; fig. 1F) in intersex individuals with respect to EtOH-exposed control males and control females. Transcript levels of *ar* and *erbeta* were also increased in finasteride-induced intersex compared to EtOH-exposed control females (1.2-fold; fig. 1A) and control males, respectively (1.6-fold; fig. 1D). Finasteride did not affect the transcriptional regulation of *srd5alpha1*, *srd5alpha2*, *srd5alpha3*, *srd5beta*, *avt* and *odc*, *tralpha*, and *dio2* (table 1) and the activity of cyp19 (fig. 2B).

Exposure to the anti-estrogen fadrozole did not result in any changes in mRNA levels in the frog brain; however, cyp19 activity was almost completely inhibited (by approximately 93%) in the brain of fadrozole-treated males, females and intersex compared to water-controls (fig. 2A). Results for *eralpha*, *erbeta*, *cyp19* and *avt* are shown as examples in figure 3. The remaining gene expression data for the fadrozole exposure (i.e., *srd5alpha1*, *srd5alpha2*, *srd5alpha3*, *srd5beta*, *ar, odc*, *tralpha*, *trbeta, dio2 and dio3)* are not shown because there were no effects of treatment. No differences in mortality were observed between treated and control individuals after chronic exposure to either finasteride or fadrozole (data not shown).

Table 1. Comparison between brain and hepatic gene expression changes following a chronic exposure to finasteride (25 μM) during *Silurana tropicalis* development

Genes	Brain[a]			Liver[b]		
	male	female	intersex	male	female	intersex
ar	1.3 ↑[d]	–	1.2 ↑[d]	–	–	–
srd5alpha1	–	–	–	–	–	1.9 ↓[e]
srd5alpha2	–	–	–	31.8 ↓[c]	25.7 ↓[c]	36.5 ↓[c]
srd5alpha3	–	–	–	–	–	–
srd5beta	–	–	–	–	3.9 ↓[c]	3.3 ↓[c]
eralpha	–	–	1.7 ↑[c]	4.1 ↑[c]	6.1 ↑[c]	–
erbeta	–	–	1.6 ↑[d]	–	–	–
cyp19	–	–	1.7 ↑[c]	nd	nd	nd
tralpha	–	–	–	–	–	–
trbeta	1.8 ↑[c]	–	2.2 ↑[c]	3.1 ↑[c]	–	–
dio2	–	–	–	4.8 ↑[c]	–	6.1 ↑[c]
dio3	2.3 ↑[c]	–	3.8 ↑[c]	3.9 ↓[c]	–	16.1 ↓[c]
avt	–	–	–	na	na	na
odc	–	–	–	na	na	na

nd = Not detectable; na = not available; – = no changes.

Fadrozole did not affect any of the transcripts measured in the frog brain. Statistically significant fold changes (with respect to EtOH-control males) are reported along with arrows indicating increase or decrease of mRNA levels.

[a] Gene expression results reported in this study.

[b] Duarte-Guterman et al. [2009].

[c] Different from both control males and females ($p < 0.05$).

[d] Different from control males ($p < 0.05$).

[e] Different from control females ($p < 0.05$).

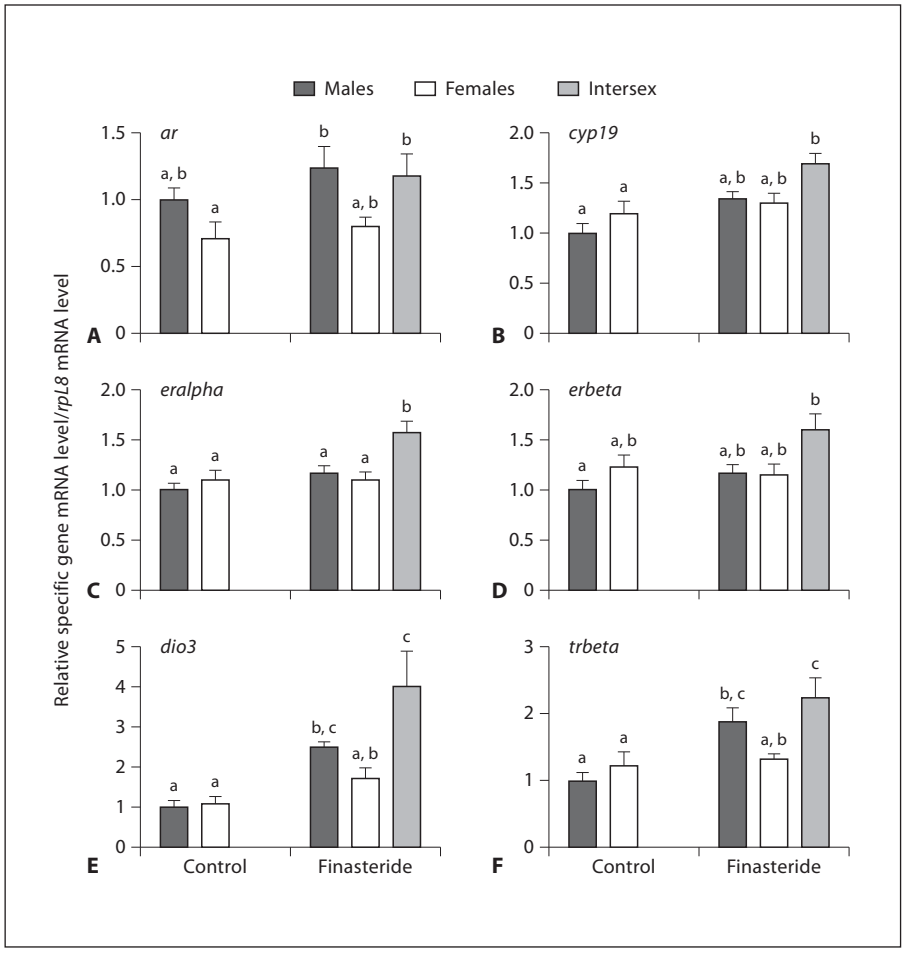

Fig. 1. Effects of finasteride on *ar* (**A**), *cyp19* (**B**), *eralpha* (**C**), *erbeta* (**D**), *dio3* (**E**) and *trbeta* (**F**) mRNA levels in NF60 *Silurana tropicalis* brains. Chronic exposures of *S. tropicalis* from NF12 to NF60 to either 0.05% EtOH (control) or 25 μM finasteride (delivered in 0.05% EtOH) were performed. The mRNA levels are expressed relative to the male control group and are normalized to the ribosomal protein L8 *(rpl8)* mRNA levels. Bars represent the mean mRNA level + SEM. Different letters indicate statistically significant differences between sex treatments (one-way ANOVA; $n = 8$, $p < 0.05$). The scales of the y-axis vary between graphs. ar: androgen receptor, cyp19: aromatase, dio3: deiodinase type 3, eralpha: estrogen receptor α, erbeta: estrogen receptor β and trbeta: thyroid hormone receptor β.

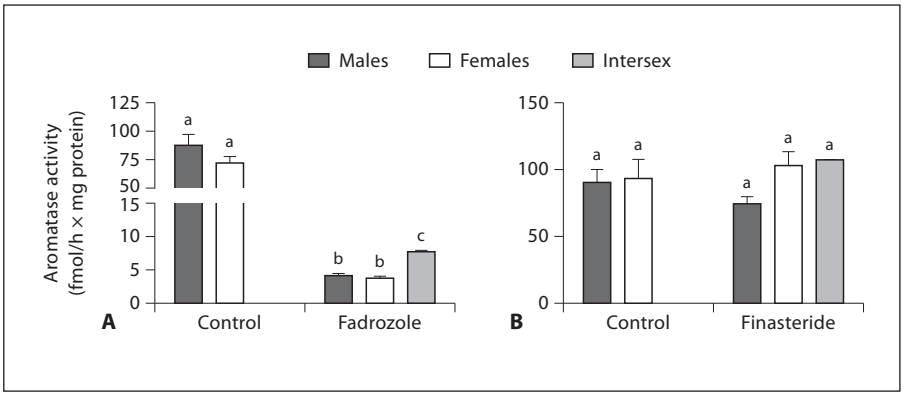

Fig. 2. Effects of fadrozole (**A**) and finasteride (**B**) on aromatase activity in the brain of NF60 *S. tropicalis*. Chronic exposures of *S. tropicalis* from NF12 to NF60 to 25 μM finasteride (delivered in 0.05% EtOH) or 2 μM fadrozole (delivered in water) were performed. Enzyme activity is expressed in fmol/h normalized to total protein content (mg). Bars represent the mean + SEM. Data were analyzed using one-way ANOVA (2–4 brains per sample; $n = 2–5$; $p < 0.01$). Different letters indicate statistically significant differences between sex treatments.

Discussion

Our previous work showed that chronic exposures to anti-androgen or anti-estrogen chemicals altered sex ratios in *S. tropicalis* [Duarte-Guterman et al., 2009]. Gonadal histology demonstrated that 27% male, 53% female and 20% intersex individuals were produced after finasteride treatment, and 55% male, 30% female and 15% intersex individuals were obtained after fadrozole treatment. The water and EtOH controls exhibited 53% male, 47% female individuals, and 54% male, 46% female individuals, respectively. We used hepatic profiles of tran-

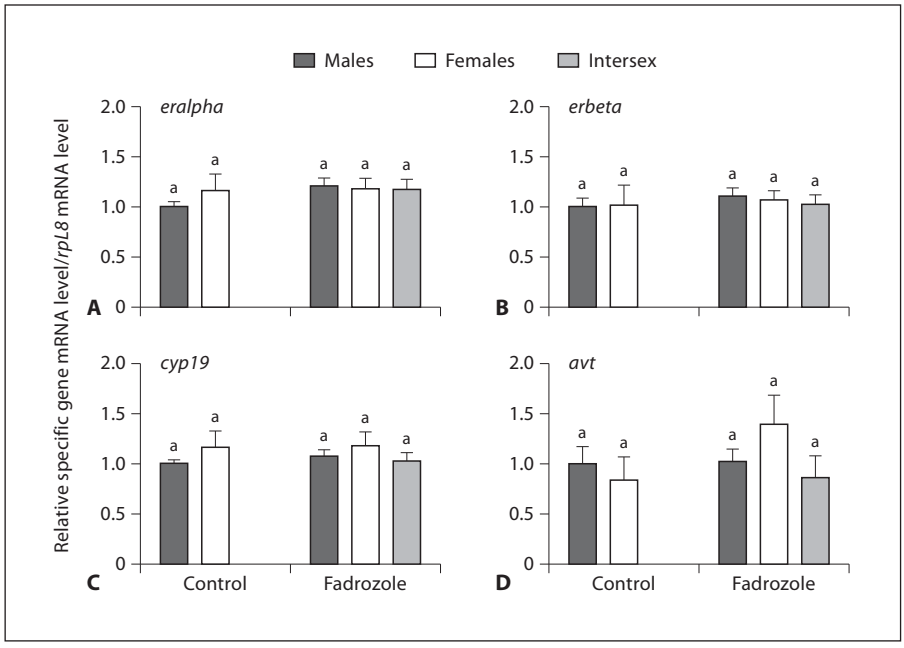

Fig. 3. Effects of fadrozole on *eralpha* (**A**), *erbeta* (**B**), *cyp19* (**C**), and *avt* (**D**) mRNA levels in NF60 *Silurana tropicalis* brains. Chronic exposures of *S. tropicalis* from NF12 to NF60 to either water (control) or 2 μM fadrozole were performed. The mRNA levels are expressed relative to the male control group and are normalized to the ribosomal protein L8 *(rpl8)* mRNA levels. Bars represent the mean mRNA level + SEM. Different letters indicate statistically significant differences between sex treatments (one-way ANOVA; n = 8, $p < 0.05$). avt: arginine vasotocin, cyp19: aromatase, eralpha: estrogen receptor α, erbeta: estrogen receptor β.

script levels to show that the physiological status of normal and intersex tadpoles is different [Duarte-Guterman et al., 2009]. Here we extend these observations to the developing brain.

In the frog brain, waterborne exposure to the anti-androgen finasteride altered the mRNA levels of sex steroid- and TH-related genes while the anti-estrogen fadrozole did not induce any changes in transcriptional regulation for any of the analyzed genes. Finasteride-induced intersex tadpoles exhibited increases in the levels of the three sex steroid receptor *(ar, eralpha* and *erbeta)* mRNAs and the estrogen synthesis enzyme *cyp19* mRNA in the brain. There is very limited information regarding the regulation of our target genes after finasteride treatment in vertebrates. However, it has been demonstrated in humans that one consequence of finasteride treatment is an increase in plasma testosterone [Habib et al., 1997; Roehrborn et al., 2003]. This increase would provide potential substrate for cyp19 to produce 17β-estradiol which in turn could autoregulate the transcription of its own steroidogenic enzyme and its receptors (due to the presence of an estrogen-responsive element in the promoter region of these genes in many species including frogs) [Katzenellenbogen, 1996; Akatsuka et al., 2005]. We propose that the increase in mRNA levels of the estrogen-related genes in the frog brain measured in our study could be explained by a putative increase in testosterone level in the brain. Finasteride did not affect *srd5alpha* (type 1, 2, 3) and *srd5beta* mRNAs in the brain which contrasts with the effects in livers of the same animals. Finasteride significantly decreased hepatic *srd5alpha2* and *srd5beta* mRNA levels in metamorphic *S. tropicalis* tadpoles (NF 60) [Duarte-Guterman et al., 2009] and in whole larvae (NF 46) [Langlois et al., 2010]. Interestingly, the transcriptional regulation of *srd5alpha3* mRNA was not affected by finasteride treatment in *S. tropicalis* in any tissue or developmental stage studied [NF 60 brain, present study; NF 60 liver Duarte-Guterman et al., 2009; NF 46 whole larvae Langlois et al., 2010]. Srd5alpha3 is a newly discovered enzyme and its regulation and function have not yet been fully explored [Tamura et al., 2007; Uemura et al., 2008], especially not in frogs. Therefore, there is a possibility that *srd5alpha3* may be differentially affected by finasteride or differentially regulated compared to *srd5alpha1* and *srd5alpha2.* To our knowledge, this is the first study assessing the effects of finasteride on *srd5alpha3* mRNA levels in the amphibian brain.

Finasteride treatment also increased brain *trbeta* and *dio3* mRNA levels in treated males and intersex animals. During metamorphosis, as TH levels rise, and after treatment with T3, *trbeta* and *dio3* mRNA increase in the brain [Morvan Dubois et al., 2006; Hogan et al., 2007; Wang et al., 2008]. Our results suggest that an inhibition of srd5alpha (type 1, 2, 3) and srd5beta would favor an increase in TH levels in the brain. Based on these data, we can speculate that there is an interaction between the androgen and TH axes, and this is supported by three other studies. Hy-

pothyroid rats exhibit decreased hepatic *srd5alpha1* transcript level and activity; while T4 addition restores both *srd5alpha1* mRNA level and activity [Ram and Waxman, 1990]. Furthermore, *S. tropicalis* larvae (NF 52–54) treated with T3 exhibit increased brain *srd5alpha1* and *srd5-alpha2* mRNA levels [Duarte-Guterman and Trudeau, 2010]. Finally, chronic exposure to finasteride in *S. tropicalis* results in a hepatic increase in the transcript level of the enzyme involved in the activation of TH *(dio2)* but in a reduction of the mRNA level of the enzyme responsible for TH inactivation (*dio3*; NF 60) [Duarte-Guterman et al., 2009]. However, the precise relationship and physiological consequences of cross-talk between the androgen and TH axes remains to be determined.

When comparing the brain and hepatic profiles of transcript levels in finasteride-induced intersex individuals in *S. tropicalis* (table 1), our data support the evidence that intersex animals express a different endocrine physiology when compared to non-exposed males and females. Furthermore, there is a clear difference in tissue sensitivity in response to finasteride between brain and liver frog tissues which adds to the complexity of the intersex pathophysiology (table 1).

In contrast to finasteride, fadrozole did not affect the transcriptional regulation of any of the genes analyzed. This is in marked contrast with previous studies on *S. tropicalis* liver [Duarte-Guterman et al., 2009] and fish brain [Villeneuve et al., 2009; Zhang et al., 2009] that have shown that fadrozole affects mRNA levels of many genes. Interestingly, we found that cyp19 activity was almost completely inhibited in the brain of fadrozole-treated animals compared to controls; while none of the estrogen-responsive genes were affected *(cyp19, eralpha, erbeta* and *avt).* Research from our laboratory has shown that exposure to the synthetic estrogen, ethinylestradiol increases the transcript levels of estrogen-responsive genes, i.e., *cyp19* and *eralpha,* in the frog brain [Duarte et al., 2006]. However, the fadrozole-induced decline in estrogen levels does not appear to have the opposite effect of an estrogen exposure since no transcriptional changes were observed in our study. Future studies should investigate other endpoints (e.g., brain morphology, sex steroid levels, and other transcripts) in more specific areas of the brain to understand the consequences of a lack of estrogen during amphibian development. Indeed, recent studies have demonstrated that estrogens are critical for neuronal development [Diotel et al., 2010]. Furthermore, we observed a 2-fold difference in cyp19 activity levels in the brain between the fadrozole induced-intersex and the treated males and females. This difference in response between intersex and differentiated males and females (both fadrozole treated and control) was also observed in the hepatic profiles of mRNA levels of the same animals [Duarte-Guterman et al., 2009] supporting the idea that intersex animals are different from both normal, untreated animals, and males and females from the treatment groups.

Studies in many vertebrate species have shown that avt is an important neuropeptide regulating social behaviors such as vocalization, parental and sexual behaviors [reviewed in Goodson and Bass, 2001]. In amphibians, avt regulates reproductive behaviors (e.g., amplectic clasping of females and release calls) [Moore and Miller, 1983]. In this study, we used *avt* in the brain as an endpoint to assess whether this neuropeptide could be affected in intersex and sex-reversed individuals after exposure to fadrozole or finasteride. Expression of *avt* was not affected after exposure to either chemical. In adult amphibians, concentrations of avt are higher in certain brain regions in males relative to females [Boyd and Moore, 1992; Boyd et al., 1992]. However, in the brains of our control groups, *avt* did not show dimorphic expression which suggests that sex-specific regulation by avt may not be fully in place at the end of metamorphosis. Future research should investigate specific brain regions and the long-term consequences of sex steroid synthesis inhibition on the *avt* system and other related endpoints in the tadpole brain.

In conclusion, exposures to finasteride and fadrozole resulted in very distinct profiles of gene expression in the brains of *S. tropicalis*. We showed that finasteride-induced intersex animals were distinguishable from normal males and females in the EtOH-control groups. On the other hand, mRNA levels in the brain of fadrozole-induced intersex individuals were similar to normal males and females. Although gonads of finasteride- and fadrozole-induced intersex animals are morphologically similar, 'intersex' is a heterogeneous condition which according to the chemical mode of action leads to different endocrine pathophysiologies. Whether chemically-induced differences in gene expression in the brain lead to developmental and reproductive abnormalities remains to be further elucidated. Finally, this study supports evidence of a crosstalk between the androgen and TH systems in amphibians.

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