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

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Key Words
- 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.

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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-butylocarbamoyl]-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 [Sainte-Marie et al., 2008]. We hypothesize that chemically-induced 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 [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, srd5alpha, 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 (oadc), 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’-tggagcagcagagcgaa-3’; reverse: 5’-cataagccgagggagggagt-3’) were designed to have melting temperatures that were similar to the reference gene. Furthermore, primer efficiencies were calculated to ensure that the PCR efficiency for each gene was > 95%.

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Increased in finasteride-induced intersex compared to efficiencies were 90–110% with \( R^2 \) gene) using the slope of the standard curves and for all the genes efficiencies were determined by the MxPro 4.0 software (Strata- using equal parts of cDNA from each sex and treatment. Reaction genes within each sample. The standard curves were generated using 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 rp18 did not change with either finasteride or fadrozole treatment (data not shown), therefore transcript level data are normalized to rp18 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 and radioactivity was counted. Aromatase activity is expressed as fmol \(^3\)H\(_2\)O/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 of 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 srd5-alpha1, srd5alpha2, srd5alpha3, srd5beta, avt and odc, tralpha, and dio2 (table 1) and the activity of cyp19 (fig. 2B).

Table 1. Comparison between brain and hepatic gene expression changes following a chronic exposure to finasteride (25 \( \mu \)M) during \( S. \) tropicalis development

| Genes       | Brain\(^a\) | Liver\(^b\) |
|-------------|------------|------------|
|             | male | female | intersex | male | female | intersex |
| ar          | 1.3\(^d\) | –      | –        | –      | –      |
| srd5alpha1  | –     | –      | –        | –      | –      |
| srd5alpha2  | –     | –      | –        | 31.8\(^e\) | 25.7\(^e\) | 36.5\(^e\) |
| srd5alpha3  | –     | –      | –        | –      | –      |
| srd5beta    | –     | –      | –        | 3.9\(^e\) | –      |
| eralpha     | –     | –      | 1.7\(^e\) | 4.1\(^e\) | 6.1\(^e\) | –        |
| erbeta      | –     | –      | 1.6\(^d\) | –      | –      |
| cyp19       | –     | –      | 1.7\(^e\) | –      | –      |
| tralpha     | –     | –      | –        | –      | –      |
| trbeta      | 1.8\(^e\) | –      | 2.2\(^e\) | 3.1\(^e\) | –      |
| dio2        | –     | –      | –        | 4.8\(^e\) | –      | 6.1\(^e\) |
| dio3        | 2.3\(^f\) | –      | 3.8\(^f\) | 3.9\(^e\) | –      | 16.1\(^l\) |
| 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 (\( p < 0.05 \)).
**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-
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 antiandrogen 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) [Katznenellenbogen, 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-
thyroid 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 *srd5alpha2* 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., amplexic clamping 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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