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Effect of low dose exposure to the herbicide atrazine and its metabolite on cytochrome P450 aromatase and steroidogenic factor-1 mRNA levels in the brain of premetamorphic bullfrog tadpoles (*Rana catesbeiana*)

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Abstract

The transcriptional regulator steroidogenic factor 1 (SF-1) and the enzyme cytochrome P450 aromatase (CYP19) play a central role in modulation of a broad range of tissue-specific developmental processes associated with hormone homeostasis that includes differentiation of the central nervous system. SF-1 and CYP19 expression may be targeted by a variety of endocrine disruptive agents prevalent within the environment. In the present study, we cloned and characterized partial sequences for bullfrog (*Rana catesbeiana*) SF-1 and CYP19 and examined the effects of a 48 h exposure to 1 and 100 µg/L of the herbicide atrazine (ATZ) and its major metabolite desethylatrazine (DEA), as well as 5 ng/L of the estrogenic chemical, 17α-ethynylestradiol (EE₂), and 673 ng/L of the thyroid hormone, 3,5, 3'-triiodothyronine (T₃), on SF-1 and CYP19 mRNA abundance in the brains of premetamorphic bullfrog tadpoles. Quantitative RT-PCR analysis showed an increase in CYP19 mRNA following a 48 h exposure to EE₂ but not T₃ while no significant changes in SF-1 transcript levels occurred. We observed a strong positive correlation between CYP19 and SF-1 transcript abundance in the ATZ-exposed animals which was not evident with DEA- or hormone-exposed tadpoles. Our results are intriguing in light of reported behavioral changes in ATZ-exposed frogs and suggest that further research is warranted to examine the relationship and role of CYP19 and SF-1 in amphibian brain development.

Keywords

atrazine; desethylatrazine; aromatase; steroidogenic factor 1; endocrine disruption; amphibian development; pesticides; gene expression; estrogen; thyroid hormone; low dose

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Introduction

The brain functions as an endocrine organ producing hormones that act directly on the central nervous system (CNS) as well as on peripheral tissues (Altman, 2004). Sex steroids along with other signaling molecules such as thyroid hormones (THs) play significant roles in differentiation of the CNS and perturbation of these processes can lead to permanent changes in brain function. Mechanistically, estrogens and THs influence each other leading to complex regulatory possibilities (Vasudevan et al., 2002). A pivotal enzyme required in estrogen production is cytochrome P450 aromatase (CYP19) which catalyzes conversion of testosterone to estradiol thereby contributing to a variety of hormone-dependent processes that include sexual differentiation, behavior, neural development, neuroprotection, and regulation of neurotransmitter receptor function (Hutchison et al., 1997; Li and Shen, 2005; Piferrer and Blazquez, 2005).

The nuclear transcriptional regulatory factor steroidogenic factor-1 (SF-1), also known as adrenal 4 binding protein (Ad4BP), regulates *CYP19* expression, and both are expressed in amphibian brain tissue (Mayer et al., 2002; Kuntz et al., 2004; Kloas et al., 2009). Furthermore, potential SF-1 binding sites have been identified in the promoter regions of a number of genes encoding steroidogenic enzymes in a range of taxa which suggests a broader importance of SF-1 in regulating endocrine processes (Morohashi et al., 1992; Honda et al., 1993; Lynch et al., 1993; Morohashi et al., 1993; Tanaka et al., 1995; Morohashi and Omura, 1996; Sugawara et al., 1997; Stocco et al., 2001). For example, SF-1 contributes to the development of the ventromedial hypothalamic nucleus structure (VMH) in rodents and studies in which SF-1 expression was disrupted report impaired gonadotrophs, abnormalities or absence of the VMH, and abnormal expression of VMH markers which include NKX2-1 and BDNF neurotrophin (Ikeda et al., 1995; Dellovade et al., 2000; Stallings et al., 2002; Tran et al., 2003; Davis et al., 2004). The VMH region of the brain is highly conserved across species, plays a critical role in regulating sexual behavior, feeding, energy balance, and cardiovascular function, and contains regions with a high level of estrogen receptor expression (McClellan et al., 2006).

Thus, the developing brain represents a potential target for endocrine disruption by environmental contaminants that interfere with the production or actions of estrogen. One chemical of concern is the herbicide atrazine (ATZ). Registered for commercial use in the United States in 1957, ATZ is extensively used in North America, the majority being applied to corn crops in the mid-western United States and southern Ontario, Canada. ATZ and its metabolites can be measured in rivers and streams at concentrations that rarely exceed 20 µg/L, and in reservoirs at concentrations up to 12 µg/L although higher concentrations have been reported in some instances (Solomon et al., 1996). Mounting evidence of the disruptive effects of ATZ on amphibian development can be found for such diverse processes as gonadal differentiation and laryngeal muscle development as well as maintenance of plasma steroid hormone concentrations (Hayes et al., 2002, 2003). A common mechanistic theme for ATZ appears to be potential alteration of CYP19 expression and function.

Several studies have identified ATZ as an endocrine disruptive agent in frogs with the ability to increase estrogen-regulated CYP19 mRNA expression in gonads, although contradictory results have been reported in the literature (Crain et al., 1997; Sanderson et al., 2000; Sanderson et al., 2001; Kazeto et al., 2004; Keller and McClellan-Green, 2004; Coady et al., 2005; Hecker et al., 2005b; Murphy et al., 2006a; Oka et al., 2008; Kloas et al., 2009). The disparity in ATZ effects could be due to different species or strains examined, the developmental timing of exposure, biological variability of control or reference animals, and differences in the chemical concentrations examined. Interestingly, recent studies have

shown that the induction of CYP19 by ATZ is dependent upon SF-1 related regulatory pathways (Fan et al., 2007a, 2007b; Suzawa and Ingraham, 2008).

The present study describes the cloning of partial SF-1 and CYP19 cDNA sequences from the North American bullfrog, *Rana catesbeiana*, and investigates whether short-term exposure to ATZ, or its prominent environmental metabolite, desethylatrazine (DEA) (Solomon et al., 1996), alters the steady-state levels of these transcripts in the brain of premetamorphic bullfrog tadpoles. The present study sets a critical foundation for understanding the transcript responses of two important regulators in the brain and suggests that alterations to this organ should be taken into account when considering ATZ exposure effects.

Materials and Methods

Experimental animals and chemical exposures

The care and treatment of the animals used in this study were in accordance with the guidelines of the Canadian Council on Animal Care. Wild-caught Taylor and Kollros stage VI-VIII bullfrog tadpoles (*Rana catesbeiana*) (Taylor and Kollros, 1946) were obtained from Ward's Natural Science (St. Catharines, ON, Canada) and housed at the Environment Canada Pacific Environmental Science Centre (PESC; North Vancouver, BC, Canada). Tadpoles were maintained in outdoor tanks containing well water under a natural light cycle at approximately 9°C and were acclimatized for a minimum of 48 h in the environmental control rooms at 22°C with a light:dark cycle of 16:8 h prior to chemical exposure. ATZ, DEA, EE₂ and T₃ were purchased from Sigma-Aldrich Canada Ltd. (Oakville, ON). ATZ, DEA, and EE₂ stock solutions were all prepared in 100% isopropyl alcohol (IPA; Fisherbrand, Ottawa, ON, Purity 99.9%), to limit the number of solvents used in the experiments, and chemical analyses were performed to confirm accurate nominal stock concentrations. Specifically, the nominal stock concentrations were: ATZ: 10,000 mg/L and 100 mg/L; DEA: 10,000 mg/L and 100 mg/L; and EE₂: 5 mg/L. The confirmed stock concentrations were ATZ: 10,137 mg/L and 101 mg/L; DEA: 6,400 mg/L and 64 mg/L; and EE₂: 3.29 mg/L and we will refer to the nominal concentrations in the manuscript. T₃ was prepared as previously described (Veldhoen et al., 2006) and the biological activity of this chemical was confirmed through measurement of TR β transcript level induction as was previously described (Helbing et al., 2010). All exposures had the same volume of stock solution applied and contained 0.001% (vol/vol) IPA vehicle control (a 100,000X dilution). Thus the final nominal treatment concentrations were 673 ng/L T₃, 50 ng/L EE₂, 1 μ g/L and 100 μ g/L DEA, and 1 μ g/L and 100 μ g/L ATZ.

Two separate exposure experiments were performed. The first experiment was done in December 2005 and included IPA, T₃, and the two concentrations of DEA. The second experiment was done in March 2006 and included IPA, T₃, EE₂, and the two concentrations of ATZ. Each treatment group contained 10 animals that were equally divided between two replicate 20 gallon glass aquarium tanks containing 50 L of aerated well water. Dissolved oxygen, temperature, and pH were recorded at the outset of the experiment and at termination. The tadpoles were not fed 24 h prior to treatment as well as throughout the 48 h exposure period. The average tadpole weight was 5.09 ± 0.24 g for experiment 1 and 7.35 ± 0.28 g for experiment 2. Tadpoles lost weight over the 2 day exposure period ($4.21 \pm 0.98\%$ and $5.46 \pm 0.60\%$, experiment 1 and 2, respectively), but this was not significant between treatments ($p=0.924$, $n=40$ experiment 1; $p=0.151$, $n=50$ experiment 2, ANOVA). The average tadpole loading density was 0.63 ± 0.03 g/L. No mortalities were observed in any treatment.

Following the 48 h exposure period, animals were euthanized by immersion in 0.1 % w/v tricaine methylsulfonate (MS-222) (Syndel Laboratories Ltd, Vancouver, BC) buffered with 25 mM NaHCO₃ and tissue samples were collected. Whole brains were isolated and placed in 1 ml RNAlater (Applied Biosystems Inc., Foster City, CA). Samples were kept at 4°C for one week and then stored at -20°C until RNA was isolated from the tissue according to standard protocols recommended by the manufacturer.

Total RNA isolation and cDNA synthesis

Total RNA was isolated from whole brain tissue using TRIzol Reagent as outlined by the manufacturer (Invitrogen Canada Inc., Burlington, ON). Each tissue sample was individually homogenized in a 1.5 ml Eppendorf Safe-Lock microcentrifuge tube (Fisher Scientific Ltd., Ottawa, ON) containing a 3 mm tungsten-carbide bead and 300 µl TRIzol. Tissue homogenization was achieved using a Retsch MM301 Mixermill (Fisher Scientific Ltd., Ottawa, ON) with 2 × 3 min shaking cycles at 20 Hz. The racks were rotated 180° between each shaking cycle. Total RNA was resuspended in 10 µl of diethyl pyrocarbonate (DEPC)-treated (Sigma-Aldrich Canada) dH₂O. RNA concentration was measured using a ND-1000 Nanodrop (Nanodrop Technologies Inc., Wilmington, DE). Each 20 µl cDNA synthesis reaction contained 500 ng random hexamer oligonucleotide (GE Healthcare, Baie D'Urfé, PQ), 1 µg total RNA, Superscript II RNase H⁻ reverse transcriptase, and additional required reagents as described by the manufacturer (Invitrogen Canada). cDNA samples were diluted 20-fold in PCR-grade dH₂O and stored at -20°C prior to QPCR analysis.

Development of species-specific QPCR primers

Isolation of *Rana catesbeiana* CYP19 and SF-1 cDNA sequences was carried out using either non degenerate PCR primers designed against gene-specific cDNA information from a closely related species or degenerate PCR primers developed using ClustalW (<http://www.ebi.ac.uk/clustalw/index.html>) aligned cDNA sequences available from other amphibian species deposited in the National Center for Biotechnology Information Entrez Nucleotide Database (<http://www.ncbi.nlm.nih.gov/sites/entrez?db=Nucleotide>) (Table 1). All primers were designed using Primer Premier Version 5.0 software (Premier Biosoft International, Palo Alto, CA). Gene-specific information used in SF-1 cloning primer design utilized *Rana rugosa* (GenBank Accession # AB017352) sequence while CYP19 degenerate cloning primers were generated from aligned cDNA sequence from *Xenopus laevis* (GenBank Accession # AB031278), *Rana rugosa* (GenBank Accession # AB178482), *Pleurodeles waltl* (GenBank Accession # AY135485), and *Cynops pyrrhogaster* (GenBank Accession # AB164064).

All primers were supplied by Integrated DNA Technologies (Coralville, IA, USA). DNA amplification reactions were performed on a MyCycler System (Bio-Rad Laboratories Ltd., Mississauga, ON) using 2 U PfuTurbo Hotstart DNA polymerase (Agilent Technologies, La Jolla, CA) as per the manufacturer's recommended protocol. Each 20 µl reaction prepared on ice also contained 2 µl of 20-fold diluted template cDNA and 20 pmol of each primer. The thermocycle program consisted of an initial denaturation step of 95°C (2 min) followed by 46 cycles of 95°C (15 s), 52°C (30 s), and 72°C (45 sec), and a final elongation step of 72°C (10 min). Amplified DNA products were evaluated (5 µl) by electrophoresis on a 1.5% agarose gel, visualized with ethidium bromide staining, and DNA amplicons of the correct predicted size were identified. Each positive PCR reaction (15 µl) was treated with 10 U T4 polynucleotide kinase in a 30 µl reaction for one hour at 37°C as per the manufacturer's protocol (New England Biolabs Ltd., Pickering, ON). The DNA products were separated by electrophoresis on a 1.5% agarose gel, visualized with ethidium bromide, and DNA amplicons of the correct predicted size were excised from the gel and placed in an Ultrafree-

DA extraction column (Millipore Corp., Bedford, MA). DNA was isolated from the gel matrix through centrifugation at 13,000 rpm for 10 min in a microcentrifuge at 4°C.

Purified DNA amplicons were ligated into pUC18 plasmid vector pre-cut with SmaI restriction endonuclease and dephosphorylated with calf intestinal phosphatase using the manufacturer's recommended protocols (New England Biolabs). Ligation reactions (15 µl) which included 1× ligase buffer, 50 ng of linearized pUC18 plasmid DNA, 4 µl of purified DNA amplicon, and 400 U of T4 DNA ligase (New England Biolabs) were incubated overnight at 16°C prior to transformation with 80 µl chemically competent *Escherichia coli* DH5α cells. Competent cells mixed with ligated DNA were incubated 1 h on ice followed by 2 min at 42°C and 90 sec on ice. SOC media (250 µl) was added and the cells were allowed to recover for 1 h at 37°C with shaking. *E. coli* DH5α cells transformed with ligated plasmid DNA were selected for by plating 200 µl of each transformation solution onto LB agar plates containing 100 µg/ml ampicillin, 20 mg/ml X-gal, and 0.5 mM isopropyl-beta-d-thiogalactopyranoside (IPTG). Plates were incubated overnight at 37°C.

Individual recombinant colonies were transferred onto a master plate and screened for positive clones by colony PCR in a 15 µl DNA amplification reaction containing 1 U Maxima Hot Start Taq DNA polymerase (Fermentas) and 1.5 pmol each of M13 forward and reverse universal sequencing primers. The thermocycle program consisted of an initial denaturation step of 94°C (5 min) followed by 30 cycles of 94°C (30 sec), 52°C (30 sec), and 72°C (1 min), and a final elongation step of 72°C (10 min). Amplified DNA products were separated by electrophoresis on a 1.5% agarose gel and visualized with ethidium bromide. Positive clones were selected and used to inoculate 5 ml of LB broth containing 100 µg/ml ampicillin. Cultures were grown at 37°C overnight with shaking at 250 rpm. Plasmid DNA was recovered from the cell cultures using a QiaPrep Spin Miniprep kit (QIAGEN) according to the manufacturer's instructions. DNA sequencing was performed at the Centre for Biomedical Research DNA Sequencing Facility (University of Victoria). *Rana catesbeiana* SF-1 (Genbank Accession # GU062744) and CYP19 (Genbank Accession # GU062745) cDNA was annotated by BLASTn (<http://www.ncbi.nlm.nih.gov/BLAST/>) DNA alignment analyses and used for further species-specific QPCR primer design (Table 1).

QPCR Assay

QPCR was performed using a Stratagene MX3005P thermocycler (Stratagene, LaJolla, CA) and 96 well PCR plates and optic flat caps (Ultident Scientific, St. Laurent, PQ) to measure the steady-state levels of select mRNA transcripts. Each 15 µl reaction contained 5 pmol of gene-specific primers (Table 1) and additional reagents as described previously (Crump et al., 2002). The thermocycle protocol included 9 min denaturation at 95°C followed by 40 cycles of 15 sec at 95°C, 30 sec annealing, and 45 sec elongation at 72°C. For the different mRNA targets, annealing temperatures included 58°C (SF-1), 62°C (CYP19), and 55°C (rpL8). Samples were run in quadruplicate and controls included a no cDNA and an inter-run cDNA standard for each QPCR run. Inter-run QPCR variation was $3.5 \pm 1.6\%$. QPCR data was analyzed using the comparative C_t method ($\Delta\Delta C_t$) (Applied Biosystems, 1997) with rpL8 as an invariant input normalizer ($C_t = 18.94 \pm 0.068$). Expression profiles of the rpL8 transcript normalizer were used to aid in the identification of poor quality samples which resulted in the removal of 2 out of the total of 90 brain samples from the analysis. In addition, the amplified DNA signals for all QPCR reactions were evaluated for specificity based upon their thermodenaturation profiles. Data that failed to produce a profile indicative of gene target-specific detection were removed ($n=3$ and 4 for CYP19 and SF-1, respectively). The remaining normalized data were then expressed as mean fold difference from the IPA control.

Statistical analyses

All statistical analyses were conducted using PASW18 (SPSS, Chicago, IL, USA). Percent changes in weights over the 2 day period were analysed using ANOVA followed by the Tukey posthoc test. Mean fold difference mRNA abundance data for CYP19 and SF-1 violated the assumptions of parametric analysis using the Shapiro-Wilk test for normality and the Levine's test for homogeneity of variance. Therefore, Kruskal-Wallis (KW) test was performed and pair-wise comparisons were made using a Mann-Whitney U (MWU) statistic. The two exposure experiments are shown and analyzed separately. There was no difference in CYP19 or SF-1 transcript levels between IPA controls in the two experiments ($p=0.210$ and 0.634 , respectively MWU). Nor was there a difference in these transcripts between the T_3 -treated groups in the two experiments ($p=0.077$ and 0.617 , respectively, MWU). For the correlation analysis, data from both experiments were combined and the correlations between CYP19 and SF-1 transcript levels were determined using Spearman's rho.

Results

The isolated partial bullfrog CYP19 cDNA sequence exhibited greater than 90% putative amino acid sequence similarity across the anuran species included in the alignment (*Rana pipiens*, *Rana rugosa*, *Xenopus laevis*, and *Xenopus tropicalis*), approximately 89% similarity with two salamanders (caudates) (*Pleurodeles waltl* and *Hynobius retardus*), and approximately 81% similarity with two fish species that were included in the alignment (*Oryzias latipes* and *Rutilus rutilus*) (Figure 1). At both DNA sequence and protein encoding levels, bullfrog CYP19 is highly conserved compared with the species sampled for the analysis (Table 2).

Brain and gonadal specific isoforms of CYP19 have been identified in fish, salamander, and birds and it is plausible that multiple isoforms exist in bullfrog (Tchoudakova and Callard, 1998; Ramachandran et al., 1999; Kishida and Callard, 2001; Kuntz et al., 2004). Tissue-specific isoforms from two fish species, *Oryzias latipes* (Medaka) and *Rutilus rutilus* (Roach), were included in the alignment analysis to examine whether the sequence we identified in bullfrog displayed isoform-specific characteristics (Figure 1). Based on the deduced 221 amino acid bullfrog sequence, a few notable differences exist when compared to the fish brain and gonadal isoforms of CYP19 (refer to amino acids 5, 66, and 198 in *Rana catesbeiana* in Figure 1 indicated by the asterisks), but there is not enough information to predict whether we have isolated a brain or gonadal specific isoform or whether multiple isoforms exist in bullfrogs. Brain and gonad specific isoforms have been described in the salamander *Pleurodeles waltl*, although the mRNA sequences for the two isoforms differ only in the length of the 5'-untranslated region (Kuntz et al., 2004) for which we have no sequence information in bullfrog for comparison.

The partial bullfrog SF-1 sequence presented in this study exhibited 95.9% amino acid sequence similarity with *Rana rugosa* (wrinkled frog), ~91% similarity with *Pseudacris regilla* (Pacific tree frog) and *Xenopus laevis*, and 79.4% similarity with the salamander *Pleurodeles waltl* (Spanish ribbed newt) (Figure 2). At the protein level, bullfrog SF-1 is more closely related to frog (*Rana rugosa*), chicken (*Gallus gallus*), and turtle (*Trachemys scripta*) (>80%) than to mammalian species *Homo sapiens* (62.4%) and *Mus musculus* (63.5%) or to fish (62.2% *Danio rerio*) (Table 2).

Animals were treated with T_3 or EE_2 for 48 h in this study so that we could also examine whether these hormones act as regulators of SF-1 and CYP19 in the brain of premetamorphic *Rana catesbeiana* tadpoles. Treatment with 673 ng/L (1 nM) T_3 , a quantity known to affect several transcripts in the brain (Helbing et al., 2007), had no effect on either

transcript in both experiments (experiment 1: $p=0.191$ MWU, CYP19; $p=1.000$ MWU, SF-1; experiment 2: $p=0.566$ MWU, CYP19; $p=1.000$ MWU, SF-1) (Figure 3). EE₂ treatment (50 ng/L) increased CYP19 expression 2-fold ($p = 0.007$ MWU) but had no effect on SF-1 expression ($p = 0.847$ MWU) (Figure 3).

Figure 4 summarizes the CYP19 and SF-1 data from the brains of both the DEA- and ATZ-exposed animals. Evaluation of tank effects on these results was not possible given the two-tank experimental design, however there was a trend towards a decrease in CYP19 transcript levels in DEA-exposed tadpoles ($p=0.055$ KW) and a trend towards an increase in the ATZ-exposed tadpoles ($p=0.041$ KW; Figure 4). No effect on SF-1 transcripts was observed with either treatment (DEA: $p=0.972$ KW; ATZ: $p=0.112$ KW; Figure 4).

Correlation analysis was conducted to determine whether a relationship was evident between CYP19 and SF-1 transcript levels. We found a significant positive correlation between the two transcripts in the ATZ treatment (Correlation coefficient 0.526, $p = 0.017$ Spearman's rho) whereas no relationship was observed in the IPA, EE₂, T₃, and DEA treated tadpoles (Table 3).

Discussion

A recent study in *Xenopus laevis* demonstrated that 2.5 µg/L ATZ induced feminization of genetic males which included a change in behavior (mating choice) and fertility (Hayes et al., 2009). Our data are suggestive that ATZ alters the expression relationship between two important transcripts, CYP19 and SF-1, in the brain (Table 3) and that this relationship may be linked to a trend toward an increase in CYP19 transcript levels (Figure 4). Therefore our results lend support the hypothesis that ATZ induces feminization in the brain by increasing CYP19 mRNA levels. It is interesting to note that the boxplots for the CYP19 transcript levels for both ATZ concentrations in Figure 4 shows greater variation towards an increase in mRNA levels compared to the IPA control. A more extensive study would be able to distinguish whether or not this is indicative of an ATZ exposure effect. It also remains to be determined whether aromatase protein levels and activity are similarly altered. Moreover, our results suggest that the parent compound, ATZ, has different effects compared to its metabolite, DEA. The exposure dates for the two experiments may be a potential confounding factor contributing to the difference in response of the ATZ- and DEA-exposed animals; the ATZ exposures took place in the spring (when ATZ field application occurs) whereas the DEA exposures took place in the winter. It is therefore possible that seasonality might influence the tadpoles' responses to these chemicals. However, we did not observe differences in the distribution or levels of CYP19 or SF-1 mRNAs in the IPA or T₃-exposed animals between experiments (Figure 3) lending credence to the idea that the animals were comparable between experiments.

Support for ATZ-induced feminization through the SF-1 transcript was not evident. We found a positive correlation between the two transcripts only in the ATZ exposures and not in the EE₂ exposures. We cannot discount the possibility that EE₂ exposure has a similar effect since the number of animals in this treatment condition was lower than that in the ATZ exposure used for the correlation analyses. Nevertheless, it is clear that the other treatment conditions did not show a positive correlation between steady-state CYP19 and SF-1 transcript levels (Table 3).

CYP19 transcript levels have been demonstrated to increase in the brains of *Rana pipiens* during natural metamorphosis and decrease in response to high levels (50 nM) of T₃ (Hogan et al., 2007). At lower concentrations, such as the concentration used in the present study,

Hogan et al (2007) did not observe a change in CYP19 transcript abundance which is consistent with our findings in *Rana catesbeiana*.

Estrogens regulate CYP19 expression in teleost fish (brain isoform but not gonadal isoform) (Kishida and Callard, 2001; Kazeto and Trant, 2005; Menuet et al., 2005; Pellegrini et al., 2005; Sawyer et al., 2006), mammals (Luo and Wiltbank, 2006), and the salamander *Pleurodeles waltl* gonad at specific developmental stages (Kuntz et al., 2006), and the induction of CYP19 is used as a neural marker for estrogenic effects in fish (Sawyer et al., 2006). The estrogen-mediated response of CYP19 varies within different tissues with little change in testicular activity (Hecker et al., 2005a) and decreased activity in the ovary of *Xenopus laevis* exposed to estradiol (Coady et al., 2005). An estrogen-insensitive form of CYP19 (P450AromA) has also been identified in zebrafish (Kishida and Callard, 2001; Sawyer et al., 2006). Furthermore, estrogen has been demonstrated to regulate SF-1 in salamanders and turtles (Fleming and Crews, 2001; Kuntz et al., 2006). While we found that EE₂ exposure increased CYP19 transcript levels in the *Rana catesbeiana* tadpole brain within 48 h, we found no evidence to suggest that it modulates the expression of the SF-1 sequence identified in the present study.

One potential pathway by which ATZ could increase CYP19 (and SF-1 - although not observed in this study) transcript levels in brain tissues is through alterations in the cyclic AMP mediated pathway. Gonadotropins (8-br-cAMP + FSH), forskolin, and 8-br-cAMP alone increase SF-1 mRNA *in vitro* in mouse adrenal tumor cells and rat granulosa cells (Shapiro et al., 1996; Lehmann et al., 2005) and cAMP is believed to stimulate the transcription of human aromatase in the ovary by increasing SF-1 expression and enhancing its binding to the aromatase promoter (Michael et al., 1995). ATZ inhibits phosphodiesterase activity (Roberge et al., 2004) in swine heart, brain, and lung but not in the liver or kidney (Roberge et al., 2006) and in rat liver (Messner et al., 1979) with some specificity which can lead to elevated cAMP levels. cAMP and aromatase levels increased in human H295R adrenocortical carcinoma cells exposed to isobutyl methylxanthine (IBMX), forskolin, and ATZ with the concentration response curves of IBMX and ATZ being similar, which also suggests that ATZ increases cAMP and aromatase through the inhibition of phosphodiesterases (Mehats et al., 2002; Sanderson et al., 2001, 2002). Whether this mechanism, following ATZ exposure, is important in the frog tadpole brain remains to be determined.

Conclusions

The functional consequence of an increase in CYP19 mRNA transcripts in the brains of premetamorphic bullfrog tadpoles is currently unknown. SF-1 and CYP19 expression have both been measured in brain tissues, often in the context of sex differentiation, and they potentially play a role in the extragonadal production of steroid hormones by the brain as well as the sexual differentiation of CNS and reproductive tissues (Willingham et al., 2000; Crews et al., 2001; Mayer et al., 2002; Kuntz et al., 2003; Kuntz et al., 2006). Little is known about the brain's role in frog sex differentiation. It is a plausible hypothesis that ATZ promotes the production of sex steroids at early stages of development prior to gonadal differentiation, although there is an ongoing debate about these biological outcomes based on other field and microcosm studies (Jooste et al., 2005; Murphy et al., 2006b). Sex steroids play a role in gonadal sex differentiation in amphibians (Hayes, 1998; Miyata et al., 1999; Miyata and Kubo, 2000) and further work is needed to determine the role of CYP19 in the central nervous system of amphibians and to determine whether ATZ-induced changes in aromatase transcript levels lead to functional effects related to sex differentiation in amphibians.

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[illegible]

Figure 1.

Putative protein sequence alignment of CYP19 from *R. catesbeiana* brain with other amphibian species. Clustal alignment were performed using ClustalW2 (<http://www.ebi.ac.uk/Tools/clustalw2/>) with amino acid positions identical to *R. catesbeiana* shown by a dash. Gap alignment positions are denoted by an asterisk. Amino acid positions relative to the start of the known putative sequence and sequence similarity scores generated by MatGAT 2.02 (<http://bitincka.com/ledion/matgat/>) compared to *R. catesbeiana* CYP19 are shown on the right. Tissue-specific mRNA forms observed in some species are denoted by (B) brain and (G) gonad. The asterisk indicates notable differences between the bullfrog sequence and the fish brain and gonadal isoforms.

Figure 2.

Putative protein sequence alignment of SF-1 from *R. catesbeiana* brain with other amphibian species. Clustal alignment were performed using ClustalW2 (<http://www.ebi.ac.uk/Tools/clustalw2/>) with amino acid positions identical to *R. catesbeiana* shown by a dash. Gap alignment positions are denoted by an asterisk. Amino acid positions relative to the start of the known putative sequence and sequence similarity scores generated by MatGAT 2.02 (<http://bitincka.com/ledion/matgat/>) compared to *R. catesbeiana* SF-1 are shown on the right.

Figure 3.

Relative fold difference in CYP19 and SF-1 mRNA abundance in whole brain tissue of premetamorphic *Rana catesbeiana* tadpoles exposed for 48 h to 673 ng/L T₃, 50 ng/L EE₂, or isopropyl alcohol (IPA) vehicle alone. The results from two separate experiments are shown. The medians are shown as solid black lines within the box, and the box indicates the first and third quartiles. The whiskers indicate minimum and maximum values. Outlier (cases between 1.5 and 3.0 box lengths from the upper or lower edge of the box) and extreme values (cases >3.0 box lengths from the upper or lower edge of the box) are indicated by an open circle and asterisk, respectively. Significance is indicated by an 'a' ($p=0.007$) relative to the IPA control.

Figure 4.

Relative fold difference in CYP19 and SF-1 mRNA abundance in whole brain tissue of premetamorphic *Rana catesbeiana* tadpoles exposed for 48 h to isopropyl alcohol (IPA) vehicle alone or 1 $\mu\text{g/L}$ and 100 $\mu\text{g/L}$ of ATZ or DEA. A bevel indicates increasing concentrations of ATZ or DEA. The medians are shown as solid black lines within the box, and the box indicates the first and third quartiles. The whiskers indicate minimum and maximum values. Outlier (cases between 1.5 and 3.0 box lengths from the upper or lower edge of the box) and extreme values (cases >3.0 box lengths from the upper or lower edge of the box) are indicated by an open circle and asterisk, respectively.

Table 1

Primer sequences use for targeted cDNA isolation and QPCR.

Gene Transcript	GenBank Accession #	Experimental Method	Primer Name	Primer Sequence	Amplicon Length (bp)
tpL8	AY452063	Cloning	L8-2 UP	5'-CAGGGGACAGAGAAAAGGTG-3'	712
			L8-2 DN	5'-ACGACGACGACGCAATAAGAC-3'	
		QPCR	L8-2 UP ^a	5'-CAGGGGACAGAGAAAAGGTG-3'	270
			UL8 DN	5'-TGAGCTTCTTGCCACAG-3'	
SF-1	GU062744	Cloning	424 UP	5'-AAAGCAGCAGAGAAAAGC-3'	549
			424 DN	5'-CGAGCCCACTCTACTATG-3'	
		QPCR	USF1 UP	5'-GATCCGTGCGAATGGTAT-3'	527
			424 DN ^a	5'-CGAGCCCACTCTACTATG-3'	
CYP19	GU062745	Cloning	1331 UP	5'-TGCAGATCCAGAAATACTTT-3'	703
			1331 DN	5'-RCTTTCATCATYACCATGGC-3'	
		QPCR	Q1331 UP	5'-CCGCTGAGAAATGTGAACC-3'	249
			Q1331 DN	5'-GCATGACCAAGCCTACCA-3'	

^aIdentical DNA primer was used for gene sequence isolation and QPCR analysis.

Table 2
Cross-species comparison of cDNA and putative protein sequences for SF-1 and CYP19^a.

Species	SF-1		CYP19	
	Accession #	cDNA	Protein	Accession #
<i>R. rugosa</i>	AB017352	89.2	95.9	AB178482
<i>T. scripta</i>	AF033833	70.2	85.3	AF178949
<i>G. gallus</i>	AB002404	67.5	80.0	J04047
<i>H. sapiens</i>	U76388	54.0	62.4	Y07508
<i>M. musculus</i>	S65878	46.6	63.5	D00659
<i>D. rerio</i>	AF014926	55.7	62.2	AF004521

^aSequence alignments were performed using MatGAT version 2.01. Identity at each aligned cDNA base pair position was scored whereas similarity values were calculated for the corresponding deduced amino acid positions.

Table 3

Spearman's rho correlation coefficients for CYP19 and SF-1 mRNA transcripts in the brain. Both 1 µg/L and 100 µg/L concentrations were combined for analysis for DEA or ATZ. The asterisk denotes significance.

Treatment	Correlation Coefficient	p Value	n
IPA	0.464	0.071	16
EE ₂	0.048	0.911	8
T ₃	0.403	0.097	18
ATZ	0.526	0.017*	20
DEA	0.368	0.121	19