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Sexually dimorphic androgen and estrogen receptor mRNA expression in the brain of túngara frogs

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ABSTRACT

Sex steroid hormones are potent regulators of behavior and they exert their effects through influences on sensory, motor, and motivational systems. To elucidate where androgens and estrogens can act to regulate sex-typical behaviors in the túngara frog (*Physalaemus pustulosus*), we quantified expression of the androgen receptor (*AR*), estrogen receptor alpha (*ERα*), and estrogen receptor beta (*ERβ*) genes in the brains of male and females. To do so, we cloned túngara-specific sequences for *AR*, *ERα*, and *ERβ*, determined their distribution in the brain, and then quantified their expression in areas that are important in sexual communication. We found that *AR*, *ERα*, and *ERβ* were expressed in the pallium, limbic forebrain (preoptic area, hypothalamus, nucleus accumbens, amygdala, septum, striatum), parts of the thalamus, and the auditory midbrain (torus semicircularis). Males and females had a similar distribution of *AR* and *ER* expression, but expression levels differed in some brain regions. In the auditory midbrain, females had higher *ERα* and *ERβ* expression than males, whereas males had higher *AR* expression than females. In the forebrain, females had higher *AR* expression than males in the ventral hypothalamus and medial pallium (homolog to hippocampus), whereas males had higher *ERα* expression in the medial pallium. In the preoptic area, striatum, and septum, males and females had similar levels of *AR* and *ER* expression. Our results suggest that sex steroid hormones have sexually dimorphic effects on auditory processing, sexual motivation, and possibly memory and, therefore, have important implications for sexual communication in this system.

Introduction

Steroid hormones regulate a variety of physiological functions, including reproduction, and influence the expression of sex-typical behaviors by modulating sensory, motor, and motivational systems. The relationship between gonadal steroid hormones and expression of male- and female-typical sexual behaviors is well established in vertebrates. In general, gonadectomy abolishes sexual behavior, which can then be reinstated by administration of androgens and/or estrogens (Adkins et al., 1980; Wallis and Luttge, 1975). Androgens and estrogens exert their effects by acting through steroid receptors in the brain. The classical mechanism of androgen and estrogen action is mediated by nuclear receptors that function as ligand-dependent transcription factors that regulate transcription of target genes, although gonadal steroids can also exert effects through membrane-bound receptors (see review by Björnström and Sjöberg, 2005). In some vertebrates, the neuroanatomical distribution of nuclear sex steroid receptors in the brain is conserved between the sexes (e.g. Balthazart et al., 1989; Rhen and Crews, 2001; Rosen et al., 2002) but there is variation in steroid receptor expression among brain regions, between sexes, among seasons, and across species, all of which can influence the expression of sex-typical behaviors (Young and Crews, 1995). Therefore, a detailed examination of the neuroanatomical distribution of steroid receptors is important for understanding sexually dimorphic, hormone–behavior relationships.

As in other vertebrates, anurans display sex-typical behaviors when plasma steroid hormone levels are high (see reviews by Arch and Narins, 2009; Moore et al., 2005; Wilczynski et al., 2005). Typically, male anurans produce advertisement calls to attract females and females, who do not typically produce advertisement calls, express mating preferences by differential phonotaxis toward the male of choice. Steroid hormones regulate advertisement calling in males (Burmeister and Wilczynski, 2001; Wetzel and Kelley, 1983) and phonotaxis in females (Chakraborty and Burmeister, 2009; Kelley, 1982; Schmidt, 1984). Furthermore, parts of the neural pathways controlling communication concentrate androgens and estrogens (Kelley, 1980; Kelley et al., 1975; Morrell et al., 1975), although the distribution of sex steroid hormone receptors has rarely been addressed directly. To our knowledge, only androgen receptors have been localized in the brain of any anuran (Guerriero et al., 2005). Thus, our understanding of the neural targets of sex steroid hormones in anurans is incomplete.

To identify the neural targets of sex steroid hormones that may contribute to sexually dimorphic behaviors in anurans, we localized and quantified expression of androgen and estrogen receptor genes in the

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brains of male and female túngara frogs (*Physalaemus pustulosus*), an important model species in sexual selection studies (Endler and Basolo, 1998; Ryan, 1991). Communication in túngara frogs is typical of many anurans: males produce mating calls to attract females while females, who do not vocalize, initiate mating by approaching a calling male (Ryan, 1985). We used PCR to clone túngara-specific sequences for *AR*, *ERα*, and *ERβ* and *in situ* hybridization to localize and quantify expression of the *AR*, *ERα*, and *ERβ* genes. We found *AR*, *ERα*, and *ERβ* expression in areas that have been reported to contain steroid concentrating cells in other anurans and we identified new sites of steroid action, including the pallium, posterior tuberculum, and locus coeruleus. Although there were no sex differences in the distribution of *AR* and *ER* expression, we found that expression levels differed in the auditory midbrain, (*AR*, *ERα*, and *ERβ*), the ventral hypothalamus (*AR*), and the medial pallium (*AR*, *ERα*), but not in the preoptic area, striatum, or septum. These sex differences in steroid receptor gene expression provide a substrate for sex-specific hormonal effects on behavior. To our knowledge, ours is the first study to provide a detailed description of the neuroanatomical distribution of *AR* and *ER*s in an anuran, and the first to report a sexual dimorphism in steroid receptor gene expression in the brain of an amphibian.

Materials and methods

Identification of túngara-specific receptor sequences

In order to examine sex differences in receptor expression patterns, we first needed to identify the túngara-specific sequences for the *AR*, *ERα*, and *ERβ* genes. To do so, we used degenerate PCR to amplify partial cDNA sequences of gene that we later used to generate probes for *in situ* hybridization (see Table 1 for primers). We extracted total RNA from ovaries (*AR* and *ERβ*) or liver (*ERα*) of adult females from a laboratory stock that was originally derived from natural populations in Gamboa, Panama, and synthesized cDNA from 10 μg of RNA using an anchored poly-dT primer and SuperScript II reverse transcriptase (Invitrogen, Carlsbad, CA). For *AR*, we amplified a 641-base pair fragment using the following PCR conditions: denaturation at 95 °C for 2 min followed by 35 cycles of denaturation at 93 °C for 30 s, annealing at 55 °C for 30 s, and elongation at 65 °C for 1 min. For *ERβ*, we amplified a 877-base pair fragment using the following PCR conditions: denaturation at 94 °C for 1 min followed by 35 cycles of denaturation at 94 °C for 30 s, annealing at 55 °C for 30 s, and elongation at 65 °C for 1 min. For *ERα*, we amplified two overlapping fragments, one of 923-base pairs (primer pair 1; Table 1) and a second of 414-base pairs (primer pair 2; Table 1). To generate the 923-base pair fragment, we used the following PCR conditions: denaturation at 94 °C for 2 min followed by 20 cycles of denaturation at 94 °C for 30 s, annealing at 60–51 °C (decreasing 2 degrees per cycle) for 30 s, and elongation at 65 °C for 90 s, followed by 35 cycles of denaturation at 94 °C for 30 s, annealing at 50 °C for 30 s, elongation at 65 °C for 90 s, and a final elongation at 65 °C for 7 min. To generate the 414-base pair fragment, we used the following PCR conditions: denaturation at 94 °C for 2 min, followed by 35 cycles of denaturation at 94 °C for 30 s, annealing at 58 °C for 30 s, elongation at 65 °C for 1 min, and a final elongation at 65 °C for 7 min. We subcloned the PCR products into a TOPO TA cloning Vector TOP 10 (Invitrogen), sequenced the inserts, and confirmed our results by aligning the predicted amino acid sequences with that of other reported receptors using BLAST.

Neuroanatomical distribution of AR, ERα, and ERβ expression

Because we were interested in dimorphisms underlying sex-typical behaviors, we examined expression of *AR* and *ER*s in the brains of reproductively active males and females. We collected five mating pairs at breeding ponds on the Osa Peninsula, Costa Rica in July 2007. We captured pairs in a mating clasp (amplexus) between 20:00 and 24:00 h and brought them back to the laboratory at the Osa Biodiversity Center where we rapidly decapitated them. After decapitation, we opened the skull in order to fix the brains (10 min in 4% paraformaldehyde) before removing them. We then rinsed the brains in phosphate buffered saline for 10 min before freezing them in liquid nitrogen in 2 ml tubes containing Tissue-Tek OCT Compound (Sakura, Finetek, Torrance, CA). We kept the brains on dry ice during transportation to University of North Carolina where we stored them at −80 °C until further processing. The University of North Carolina Institutional Animal Care and Use Committee (IACUC) approved our experimental procedures and Costa Rica's Ministerio del Ambiente Y Energia (MINAE) and Sistema Nacional de Áreas de Conservación (SINAC) permitted tissue collection and export.

We sectioned brains in the transverse plane at 16 μm in 3 series on a cryostat. To localize *AR*, *ERα*, and *ERβ* mRNA, we used radioactive *in situ* procedures previously described Burmeister et al. (2008) with some modifications. Briefly, we generated radioactively labeled sense and antisense probes from *in vitro* transcription of 641-, 414-, and 877-base pair subclones for *AR*, *ERα* and *ERβ*, respectively. We linearized the plasmids with *Eco*RV or *Hin*dIII (New England BioLabs Inc., Ipswich, MA). We prepared the S^{35}-labeled RNA by *in vitro* transcription with Sp6 or T7 polymerase using a MAXISCRIPT kit (Ambion, Austin, TX), and we removed the unincorporated nucleotides by using NucAway spin columns (Ambion). Before hybridization, we fixed the tissue for 10 s in 4% paraformaldehyde before washing in phosphate-buffered saline, triethanolamine, acetic anhydride, 2× SSC, and a series of ethanols. We hybridized the tissue with 90 μl of 3.0×10^5 cpm/ml of hybridization buffer at 65 °C overnight and removed unbound probe with a series of 65 °C washes, first in 50% formamide and 2× SSC (1.25 h) followed by two washes in 0.1× SSC (30 min each). We visualized the bound riboprobe as silver grains by exposing the slides to NTB emulsion diluted 1:1 in distilled water for 30 days at 4 °C, and we visualized the cell bodies by staining the tissue with thionin. Tissue incubated with the sense probe showed no significant binding above background (Fig. 1). We used darkfield and brightfield illumination on a compound microscope to qualitatively examine the neuroanatomical distribution of *AR*, *ERα*, and *ERβ* expression. We focused our attention on brain areas involved in sexual communication, areas previously reported to bind sex steroids, and areas previously reported to express steroid receptors.

Sex differences in receptor mRNA levels

Since we did not observe any obvious sex differences in *AR* or *ER* distribution, we quantified levels of mRNA expression focusing on a subset of brain regions involved in sexual communication. We

Table 1
Primers (5' to 3') used to generate cDNA sequences.

		Forward	Reverse	Genbank No. of cDNA
AR		GCS AGC AGR AAY GAY TGY AC[1]	GCY TTC ATG CAS AGG AAY TC[1]	DQ320626
ERβ		ATI TGY CCI GCI ACI AAY CA[2]	ARR TGY TCC ATI CCY TTR TT[2]	HM217201
ERα	Pair 1	GGD CAY AAY GAY TAY ATG TG[3]	TCC ATK CCY TTR TTR CTC AT[3]	HM439763
	Pair 2	GTA TCA GGA ARG AYC GSA GRG	CGC CAA ATT AAD CCR ACC ATW	

[1]Chattopadhayay et al. (2003); [2]Wu et al. (2003); [3]Ko et al. (2008).