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Pattern of aromatase mRNA expression in the brain of a weakly electric fish, *Apteronotus leptorhynchus*

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ABSTRACT

Aromatase is a steroidogenic enzyme involved in the conversion of testosterone into estradiol. Teleosts are unique among vertebrates in possessing two distinct aromatase genes that show different expression patterns within the body. Since the brain is the essential organ underlying the control of behavior, an understanding of the expression pattern of aromatase in the brain can help to identify neural circuits and behaviors that are most likely to be affected by aromatase activity. In addition, identifying species differences in aromatase expression in the brain can further our understanding of divergence in behaviors regulated by local estradiol production and estrogen signaling. *Apteronotus leptorhynchus* is a species of weakly electric fish in which little is known about sex steroid expression within the brain and its role in electric signaling behavior. The goal of this study was to identify the mRNA expression pattern of aromatase in the brain of *A. leptorhynchus*. Aromatase mRNA was detected in several parts of the forebrain and in the pituitary gland; however, no aromatase expression was detected in the midbrain or hindbrain. These findings in *A. leptorhynchus* support a role for aromatase activity in reproduction, but no direct role in electric signaling behavior in non-breeding fish. The findings of this study help to broaden the basis for making phylogenetic comparisons of aromatase expression across teleost lineages as well as different signaling systems, and provide information on behaviors and neural circuits that are potentially affected by local estradiol production in *A. leptorhynchus*.

1. Introduction

The actions of locally produced sex steroids in the brain have come to the forefront in neuroethology research, because these steroids provide a mechanistic pathway to explain rapid and transient changes in neural activity and behavior (Remage-Healey, 2012). A first step towards understanding the function of locally produced steroids in the brain is to determine where these steroids are produced, i.e. where in the brain steroidogenic enzymes are expressed.

Aromatase is a steroidogenic enzyme in the cytochrome P450 superfamily that enables the conversion of the aromatizable androgens, testosterone and androstenedione, into the bioactive estrogens, estradiol and estrone, respectively (Naftolin et al., 1974). Aromatase activity is believed to play an important role in vertebrate physiology and behavior, because its expression has been conserved across vertebrate lineages (Castro et al., 2005). Teleosts are unique among vertebrates in possessing two distinct aromatase genes, termed cyp19a1a and cyp19a1b, that are hypothesized to have arisen following the whole genome duplication event in the common ancestor of teleosts (Pellegrini et al., 2005). Over time, the cyp19a1a and cyp19a1b gene sequences diverged and their expression was restricted predominantly to the ovary and brain, respectively (reviewed in Diotel et al., 2010). Identifying the expression pattern of brain aromatase can help us to understand how its activity affects neural circuits and behaviors.

To date, the mRNA expression pattern of brain aromatase in teleosts has been identified in several species including killifish *Fundulus heteroclitus* (Dong and Willett, 2008), plainfin midshipman *Porichthys notatus* (Forlano et al., 2001), zebrafish *Danio rerio* (Goto-Kazeto et al., 2004; Menuet et al., 2005), guppies *Poecilia reticulata* (Hallgren, 2009), the Japanese eel *Anguilla japonica* (Jeng et al., 2012), rainbow trout *Oncorhynchus mykiss* (Menuet et al., 2003), the Asian stinging catfish *Heteropneustes fossilis* (Mishra and Chaube, 2016), and pejerrey *Odontesthes bonariensis* (Strobl-Mazzulla et al., 2008). Among these teleosts there is a high degree of regional conservation in the neural expression pattern of aromatase, with highest levels seen in forebrain regions, most notably the peripheral layer of the telencephalon, the forebrain

Abbreviations: EOD, electric organ discharge; EODf, electric organ discharge frequency; GnRH, gonadotropin releasing hormone; JAR, jamming avoidance response; PM, pacemaker nucleus; PPn-C, prepacemaker nucleus- chirp; SPPn, sublemniscal prepacemaker nucleus

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ventricles, preoptic area, and the ventral hypothalamus. Much lower expression levels have been documented in the midbrain and hindbrain regions with many differences observed between species. The neural expression pattern of aromatase can be informative in identifying potential behaviors that might be under local control by estrogen, as well as provide insights into potential mechanisms underlying individual differences in these behaviors. For example, in the plainfin midshipman fish aromatase is expressed in forebrain regions involved in the vocal-acoustic circuit used for call production (Forlano and Bass, 2011). Differences in the level of aromatase expression have been identified between males and females, as well as between male reproductive morphs, and are hypothesized to underlie individual differences in calling behavior during social interactions (Forlano and Bass, 2005). It is therefore interesting to extend the study of aromatase in the brain to other systems in which the neural circuitry underlying signal production has been well characterized to see if aromatase expression might also be important for regulating local estrogen production involved in other signaling systems.

Apteronotus leptorhynchus is a South American weakly electric fish. These fish use active electrosensation as their main sensory modality for both navigation in their environment (Heiligenberg and Bastian, 1986) and social communication (Hopkins, 1974; reviewed in Lorenzo et al., 2006). They use emissions of a quasi-sinusoidal electric organ discharge (EOD) signal to create an electric field around the body. Electroreceptors that line the body surface detect objects in the environment and the EODs of conspecifics via distortions created in the electric field. In aggressive and courtship interactions, fish produce slow and rapid frequency modulations of their EOD, called the jamming avoidance response (JAR) and chirps, respectively (Bullock et al., 1972a,b; Zakon et al., 2002).

The neural circuitry involved in the production of the EOD, its modulations, and in the sensory processing of communication signals has been well characterized (Bell and Maler, 2005; Chacron et al., 2011; Krahe and Maler, 2014; reviewed in Metzner, 1999). Pacemaker cells located in the pacemaker nucleus (PM) in the hindbrain synapse onto relay cells that project their axons down the spinal cord to eventually synapse onto the electromotor neurons in the spinal cord. Axons of these electromotor neurons make up the electric organ in the fish's tail and the rate of the action potentials of these cells, entrained by the PM, determines the frequency of a fish's EOD. Modulations of the EODf, such as the JAR and chirps, are produced by changes in the firing rate of neurons in the sublemniscal (SPPn) and chirp (PPn-C) prepacemaker nuclei located in the mesencephalon and diencephalon, respectively. Neurons from the SPPn and PPn-C project to the PM to ultimately induce changes in the EODf (Heiligenberg et al., 1996). Electrosensory input reaches the electrosensory lateral line lobe (ELL) via primary afferents from the electroreceptors in the skin. The ELL output neurons target the rhombencephalic nucleus praeeminentialis and the midbrain torus semicircularis, from where electrosensory information is relayed to the tectum opticum and the diencephalic nucleus electrosensorius. The latter projects to SPPn and thus closes the sensory-motor loop. Though the neural circuits involved in electric signaling behavior and electrosensory processing have been well studied, little is known about local steroid production in the brain and its effects within these circuits on signal production.

To date, three studies have indicated an effect of aromatase activity on behavior in weakly electric fish. Firstly, Dulka and Maler (1994) demonstrated that testosterone implants feminize the baseline EODf in *A. leptorhynchus*, while also increasing female chirping behavior. The authors suggested that aromatization was likely responsible for these observed effects, because they were similar to those seen following estradiol administration in a sister species, *Apteronotus rostratus* (Meyer et al., 1987). Zucker (1997) showed that when fadrozole, an aromatase inhibitor, was administered alongside testosterone implants there was no effect on baseline EODf, effectively confirming the previous hypothesis that testosterone was being aromatized. This study did not, however, assess if this were also true for chirping behavior. Finally, Jalabert et al. (2015) showed that fadrozole injections in another species of weakly electric fish, *Gymnotus omarorum*, decreased the physical aggression of dominant fish; however, there was no comment of an effect on electric signaling behavior. Together these studies provide evidence to suggest an effect of aromatase activity in weakly electric fish, though it is currently unclear where aromatase is expressed in the neural circuit to affect these behaviors.

The goal of this study was to identify the expression pattern of cyp19a1b mRNA in the brain of *A. leptorhynchus* to: 1) determine whether aromatase mRNA is expressed in brain nuclei underlying electrocommunication behavior, and 2) compare the mRNA expression pattern in a species of weakly electric fish with that of non-electrogenic teleosts.

2. Material and methods

2.1. Animal subjects

Male and female brown ghost knifefish, *Apteronotus leptorhynchus*, were obtained from a local tropical fish supplier. Fish were individually housed in a temperature controlled room on a 12:12 light:dark cycle with water conditions maintained at 26–28 °C, pH 7–8.5, and conductivity 200–300 μS. Housing tanks were supplied with artificial plants and sections of polyvinyl chloride tubes to serve as shelters. Fish were fed daily with frozen bloodworms. Male and female fish were identified by a combination of measurements including morphology (as described by Hagedorn and Heiligenberg, 1985), EOD frequency (males > 800 Hz, females < 800 Hz; Zakon et al., 2002), chirping behavior (as described by Zupanc and Maler, 1993), and confirmed by post-mortem gonad inspection. GSI calculations were used to determine the reproductive state of each fish. GSI was calculated as (weight of the gonads)/(weight of the body) × 100%. For all females, GSI values were ≤0.25% and for all males ≤0.20%; indicating that fish had regressed gonads and were not in breeding condition during the experiment. Brain aromatase mRNA expression was assessed in five fish, two male and three female. All animal care and experimental procedures were approved by McGill's Animal Care Committee.

2.2. RNA probe generation

The RNA probe generation, histological processing, and in situ hybridization techniques used in this project were based on the protocols used previously in a study of muscarinic acetylcholine receptor mRNA distribution in *A. leptorhynchus* (Toscano-Márquez et al., 2013). Briefly, total RNA was extracted from the brain of *A. leptorhynchus* using TRI reagent solution (Ambion, Austin, TX) and homogenized for use in RT/PCR reactions. Total RNA was reverse transcribed to cDNA using a SuperScript III kit (Invitrogen, Carlsbad, CA). Degenerate primers for binding aromatase cDNA were designed based on known regions of conserved sequence identity in the channel catfish, *Ictalurus punctatus* (Accession No. NM_001200163.1), common carp, *Cyprinus carpio* (Accession No. EU375456.1), goldfish, *Carassius auratus* (Accession No. AB009335.1), Japanese medaka, *Oryzias latipes* (Accession No. AB591736.1), Nile tilapia, *Oreochromis niloticus* (Accession No. NM_001279590.1), and zebrafish (Accession No. AF226619.1). Ovarian aromatase in the channel catfish (Accession No. S75715.1) was used as an outgroup for comparison to brain aromatase sequences. The aromatase sequences were obtained from the Genbank database (National Center for Biotechnology Institute, http://www.ncbi.nlm.nih.gov/genbank/). Nucleotide and predicted protein sequences were analyzed to identify evolutionarily conserved regions using Geneious sequence alignment editor version 10.0.7 (Biomatters Ltd., Auckland, New Zealand). The degenerate primers used in PCR reactions to obtain a 382 basepair partial fragment of the aromatase sequence from *A. leptorhynchus* cDNA were as follows: forward 5′- GGT GAT YGC WGC