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The distribution of an AVT V1a receptor in the brain of a sex changing fish, *Epinephelus adscensionis*☆

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ABSTRACT

The present study describes the distribution of an arginine vasotocin (AVT) V1a receptor (AVTr) throughout the brain of a sex-changing grouper, rock hind *Epinephelus adscensionis*. The objectives of this study were to describe the AVTr distribution in the brain of rock hind for potential linkages of the AVT hormone system with sex-specific behaviors observed in this species and to examine sex-specific differences that might exist. An antibody was designed for rock hind AVTr against the deduced amino acid sequence for the third intracellular loop. Protein expression, identified with immunohistochemistry showed high concordance with mRNA expression, identified with *in situ* hybridization. AVTr protein and mRNA expression was widely distributed throughout the brain, indicating that AVT may act as a neuromodulator via this V1a receptor subtype. AVTr protein and mRNA were present in regions associated with behavior, reproduction and spatial learning, as well as sensory functions such as vision, olfaction and lateral line sensory processing. We observed high AVTr expression in granular cell formations in the internal cellular layer of olfactory bulbs, torus longitudinalis, granular layer of the corpus cerebellum, valvula of the cerebellum, nuclei of the lateral and posterior recesses, and granular eminence. High protein and mRNA expression was also observed in the preoptic area, anterior hypothalamus, and habenular nucleus. No obvious sex differences were noted in any region of the rock hind brain.

1. Introduction

Arginine vasotocin (AVT) and its mammalian homologue arginine vasopressin (AVP) influence many behavioral and physiological processes such as sexual and social behaviors, seasonal and circadian rhythms, stress response, as well as metabolic, cardiovascular and osmotic processes (see review by Balment et al., 2006). Major functions of the AVT/AVP system in the brain include control of behaviors such as social approach, courtship and aggression, with these behaviors typically attributed to males. Most notably, research with voles has revealed interspecies differences in monogamous and polygamous species attributed to differential expression of the AVP V1a receptor in specific brain regions (Insel et al., 1994; Nair and Young, 2006).

The AVP system in mammals has received the most attention, and three receptor subtypes have been identified that differ in their tissue distribution and pharmacological characterization. The three receptors, designated V1a, V1b and V2, are membrane-associated, G protein-coupled receptors that are expressed in numerous tissues throughout the body with considerable overlap (Birnbaumer, 2000). However, differences are seen in their function through second messenger systems, and major effects are discerned through pharmacological experiments. There is a clear linkage between V1a receptor expression in the brain and sex specific behaviors in mammals, birds, amphibians and fish (Baeyens and Cornett, 2006; Hasunuma et al., 2007; Insel et al., 1994; Semsar et al., 2001), while the V1b receptor mediates the vasopressor response through an action in the pituitary where it is linked with adrenocorticotropic hormone (ACTH) function (Jurkevich et al., 2005; Tanoue et al., 2004). The V2 receptor, on the other hand, regulates water uptake via aquaporins in the kidney (Hayashi et al., 1994). Both V1 receptor forms utilize the inositol phosphate second messenger system and mobilize intracellular Ca^{2+} whereas the V2 subtype is linked to the adenylate cyclase

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pathway (Birnbaumer, 2000). In mammals, the V1 type receptors can be further distinguished from one another based on differing agonist and antagonist affinities (Manning and Sawyer, 1993; Serradeil-Le Gal et al., 2002). However, similar research on specific AVT receptor agonists and antagonists is currently lacking for non-mammalian vertebrates.

Although the three major subtypes have been well characterized in mammals, the function and presence of homologous receptors are less clear in fish. Mahlmann et al. (1994) first isolated an AVT receptor in white sucker (*Catostomus commersonii*) and characterized it as pharmacologically and functionally similar to the mammalian V1a type receptor. Other investigators have shown the V1 type receptor associated with osmoregulation in the kidney is widely distributed in other fish tissues such as brain, pituitary and gill (Lema, 2010; Warne, 2001).

Recently, three forms of AVT receptor mRNA have been isolated in newt and identified by amino acid homologies and mRNA tissue distribution as V1a, V1b and V2 subtype receptors (Hasunuma et al., 2007). The two proposed V1 subtypes are highly expressed in the brain and pituitary whereas the proposed V2 subtype is highly expressed in the kidney but absent from the pituitary, similar to the distribution of the AVP system in mammals (Birnbaumer, 2000; Ostrowski et al., 1994). In pupfish (*Cyprinodon nevadensis amargosae*), Lema (2010) has isolated mRNA sequences for three AVT receptor subtypes and identified them by their mRNA tissue distribution and amino acid homologies as V1a1, V1a2 and V2 receptors. He has reported the presence of two distinct forms of the V1a subtype, with overlapping mRNA distributions in the forebrain, midbrain, cerebellum and hind brain. This study did not identify a V1b receptor in pupfish, although its existence cannot be discounted based on the data presented.

The presence of three receptor subtypes in teleost fish and their range of functions in relation to their mammalian counterparts are unclear. Numerous effects of AVT have been noted and some similar functions have been attributed to receptor subtypes as characterized in mammals, such as changes in social behavior (Semsar et al., 2001), osmoregulation (Warne et al., 2005) and smooth muscle contraction (Conklin et al., 1999). Behavior experiments in fish and amphibians have implicated a V1a type receptor based on the effects of an AVP V1a antagonist, Manning compound. With these behavioral effects attributed to the V1a receptor and the recent identification of multiple V1a type receptors by Lema (2010) with overlapping expression in the brain, there is a need for localization studies to understand receptor distribution and to infer potential function(s).

Neuroanatomical distribution of AVT binding sites has been described in only a few fish and amphibian species. In one study, fluorescently labeled AVT was administered directly to the brain of newt and specific cell populations in the reticular formation of the hind brain were labeled and identified as areas of AVT action (Lewis et al., 2005). Similarly, only one study in fish has reported identification of AVT binding sites in the pituitary and several generalized brain regions in the seabass, *Dicentrarchus labrax* (Moons et al., 1989). However, the receptor subtypes and detailed examination in specific neuronal populations could not be established using this method.

In the only published immunohistochemistry (IHC) study of an AVT receptor in fish, Warne (2001) has cloned and characterized a V1a type receptor and developed an antibody against a 15 amino acid sequence of the third intracellular loop (Warne et al., 2005). This study focused on osmoregulation in the European flounder, *Platichthys flesus* and IHC experiments with the AVTr V1a antibody revealed strong staining in kidney tissue as well as functional evidence of its role in osmoregulation. Though this study noted mRNA expression of the V1a receptor in the brain tissue, no information regarding its distribution in specific brain regions was provided.

As in mammals, sex specific behavior has been documented in several fish species and correlated with changes in AVT hormone expression in specific cell populations in the preoptic area–anterior hypothalamus (POAH). This difference has been documented in several species of sex changing fish such as wrasse, goby, and anemone fish where sex specific behavior has been correlated with AVT cell populations in the POAH that vary in relation to sexual state (Godwin et al., 2000; Grober and Sunobe, 1996; Iwata et al., 2008). The AVT V1a type receptor has been implicated in the control of certain male behaviors in different vertebrate groups because administration of an AVP V1a specific antagonist can disrupt these behaviors. The reported effects range from delayed onset of aggression and marking behavior in mammals (Albers et al., 1986; Winslow et al., 1993), reduced courtship and aggression in fish (Semsar et al., 2001) to the inhibition of clasping behavior in amphibians (Moore and Miller, 1983).

Aside from the effects of AVP V1a antagonists and sexual dimorphism of specific AVT producing cell types and a single published study on AVT binding sites listed above, little is known regarding the function and distribution of vasotocin receptors in the brain of fish. The objective of this research was to describe the distribution of an AVT receptor using an antibody designed against the deduced amino acid sequence from the AVT V1a2 receptor cDNA sequence in rock hind. In addition, the AVT V1a2 receptor mRNA expression was localized by *in situ* hybridization (ISH) to confirm specificity of the immunoreaction. Rock hind behavior and gonadal sex can be altered via manipulations of the social environment (Kline et al., 2011) and ongoing research on sex specific behaviors and AVT prompted this study on the distribution of the AVT V1a2 receptor in this species. Of particular interest are the sites of action in the brain, especially the POAH and other areas that might affect sexual behavior and have potential for downstream effects on the reproductive system.

2. Methods

2.1. Animals

Nine rock hind were captured from oil platforms in the Gulf of Mexico near Port Aransas, TX, and used immediately for IHC, ISH and Western blot studies. Sex was determined by gross examination of the gonads after the collection of brain samples. Microscopic examination showed that these fish were not in reproductive season with testis and ovaries in the early stages of development. All rock hind used in this study were treated in compliance with a protocol approved by the University of Texas at Austin Animal Care and Use Committee.

2.2. AVT receptor antiserum

Antibodies to the rock hind V1a2 subtype vasotocin receptor were raised in rabbit against a region corresponding to the predicted protein sequence (GenBank accession no. HQ141396) of the third intracellular loop of the receptor and affinity purified (Fig. 1). The two forms of the V1a receptor for rock hind differ substantially in the antigenic site of the third intracellular loop and for other vasotocin receptor forms identified in fish (Fig. 1). Although no other AVT receptor forms are available for rock hind and no V1b form has been identified in any fish, the third intracellular loop region differs substantially between V1a and V1b receptor forms in newt (Hasunuma et al., 2007).

To test the specificity of the AVTr antibody, Western blot was performed to determine which proteins were identified by the antibody. One male and one female rock hind were killed by an overdose of MS-222 (1 g l^{-1}) and whole brain removed. Brain tissue was extracted using the Q-proteome cell compartment extraction kit to isolate membrane, cytosolic and nuclear fractions (Invitrogen, USA). Membrane protein (15 μg) from each cell compartment fraction was loaded and run on a 10% sodium dodecyl sulfate–polyacrylamide electrophoresis (SDS–PAGE) gel. Following overnight transfer to polyvinylidene fluoride (PVDF) membrane, the membranes were washed 3 times for 5 min with PBS-T (20 mM phosphate base, 150 mM NaCl, 0.1% Tween-20, pH 7.6) and immersed in blocking buffer (5% normal goat serum and 0.5% porcine gelatin in PBS-T) for 1 h at room temperature. Membranes were then rinsed in PBS-T and incubated overnight at 4 °C with AVTr antibody or antibody pre-absorbed overnight with 1 μg of antigen peptide to 1 μl antibody at a final dilution of 1:1000 in PBS-T. Following primary