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Arginine vasotocin V1a2 receptor and GnRH-I co-localize in preoptic neurons of the sex changing grouper, *Epinephelus adscensionis*

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ABSTRACT

The arginine vasotocin/vasopressin (AVT/AVP) and gonadotropin releasing hormone (GnRH) systems are known to control sexual behaviors and reproduction, respectively, in different vertebrate groups. However, a direct functional connection between these two neuroendocrine systems has not been demonstrated for any vertebrate species. Therefore, the objective of this research was to test the hypothesis that AVT acts on the GnRH system via an AVT V1a receptor in a sex changing grouper species, the rock hind, *Epinephelus adscensionis*. AVT V1a2 receptors were co-localized with GnRH-I on neurons in the preoptic anterior hypothalamus identifying a structural linkage between the AVT system and GnRH-I. Transcripts for *avt*, *gnrh-I*, and two AVT receptor subtypes (*v1a1* and *v1a2*) were isolated and characterized for *E. adscensionis* and their expression was measured in males and females by q-RT-PCR. Translation of V1a-type cDNA sequences revealed two distinct forms of the AVT V1a receptor in *E. adscensionis* brain similar to those reported for other species. The observation of significantly higher *gnrh-I* mRNA in the POA + H of rock hind males as compared to females suggests differential regulation of the *gnrh-I* transcripts in the two sexes of this protogynous species. In male *E. adscensionis*, but not in females, a negative relationship was seen between plasma 11-ketotestosterone (11-KT) and the *v1a1* receptor mRNA levels in the POA + H, while a positive trend was observed between 11-KT and *v1a2* receptor mRNA levels, indicating that these receptor forms may be differentially regulated.

1. Introduction

The neuropeptide hormones arginine vasotocin/vasopressin (AVT/AVP) and gonadotropin releasing hormone (GnRH) are both found in the preoptic area and anterior hypothalamus (POA + H), an important brain area controlling many aspects of reproduction and behavior in all vertebrates (Foran and Bass, 1999). Although a link between these two hormones has been proposed in several vertebrate classes (D'Hondt et al., 2000; Funabashi et al., 2000), a functional relationship between a hormone receptor and its target neuron(s) has not been demonstrated.

The coupling of sexual behaviors and reproductive development is evident in many vertebrate groups where commensurate rises in aggression and courtship occur with the progression of gonadal development (Liley and Stacey, 1983). In the special case of protogynous hermaphrodite fishes that change sex from female to male, AVT associated sexual behavior can be de-coupled from the reproductive axis. For example, in the bluehead wrasse, *Thalassoma bifasciatum*, behavioral sex change occurs even in the absence of gonadal input (Godwin et al., 1996), suggesting the involvement of one or more central factors in the control of behavioral sex change. AVT is one of the important central factors implicated in the control of behavioral sex change (Lema and Nevitt, 2004; Semsar and Godwin, 2003, 2004; Semsar et al., 2001) and the GnRH originating in the preoptic area is the primary neuroendocrine regulator of pituitary gonadotropins that control reproduction (Kah et al., 1993; Mohamed et al., 2005; Senthilkumaran et al., 1999). Considering the close proximity of the AVT and GnRH-I in the preoptic area in fishes (Maruska et al., 2007; Saito et al., 2003), it is possible that AVT could act on GnRH-I neurons in the preoptic area of sex changing fishes.

AVT and the mammalian homolog AVP have been associated with several physiological processes such as seasonal and daily rhythms, osmoregulation, metabolism in general and the vasopressor response (see reviews by Balment et al. (2006) and Hasunuma et al. (2013)). Moreover, the linkage of the AVT/AVP system with

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behavioral functions has received considerable attention, especially in relation to the control of male-specific behaviors such as courtship and aggression (Goodson and Bass, 2000; Grober et al., 2002; Lema and Nevitt, 2004; Salek et al., 2002; Semsar et al., 2001). In general, sexually dimorphic AVT/AVP neuron populations have been associated with social behavior in many species from fish (Foran and Bass, 1998) to birds (Jurkevich et al., 1996) and mammals (De Vries and Panzica, 2006). Among the sex changing species, both the abundance of AVT producing neurons in the halfspotted goby, *Asterropteryx semipunctata* (Maruska et al., 2007) and mRNA expression in bluehead wrasse (Godwin et al., 2000), are associated with sexual phenotype. In addition, treatments of AVT have yielded immediate changes in calling behavior and territoriality in birds (Maney et al., 1997), frogs (Tito et al., 1999) and fish (Salek et al., 2002), and central administration directly to the brain has been successful in several studies (Boyd, 1991; Goodson and Bass, 2000; Maney et al., 1997) demonstrating that the behavioral effect likely originates from the brain rather than other areas of the body.

Three main types of AVT/AVP receptors have been characterized in vertebrates see review by Hasunuma et al. (2013). These receptors are named V1a, V1b and V2 and differ in their tissue expression and major known functions (Balment et al., 2006). However, two V1a-type and V2-type receptors have been identified in bony (Actiopterygiian) fish (Konno et al., 2009, 2010; Lema, 2010), but no V1b-type has been identified to date (Hasunuma et al., 2013). Most behavioral effects of the AVT/AVP system have been attributed to the V1a receptor sub-type due to its high expression in the brain, particularly in the POA + H, and also due to the observed behavioral changes that occur when a V1a specific antagonist is used (Goodson and Bass, 2000; Propper and Dixon, 1997; Salek et al., 2002). Presently, only two of the major forms, V1a and V2 have been identified in fish and two cDNA sequences coding for two distinct forms of the V1a receptor have been identified in rock hind (Kline et al., 2011b) and described in pupfish (*Cyprinodon nevadensis amargosae*) (Lema, 2010), and bluehead wrasse (Lema et al., 2012). The mRNA expression of the two V1a forms in pupfish and bluehead wrasse shows overlapping distributions in the forebrain, midbrain, cerebellum and hindbrain (Lema, 2010; Lema et al., 2012).

In fishes, multiple forms of GnRH are present and one or more of these have been implicated in the control of reproductive behaviors (Ogawa et al., 2006; Propper and Dixon, 1997). The GnRH variant from the preoptic area released from the nerve terminals in the teleostean pituitary and median eminence in mammals, is considered the key hormone controlling reproductive function in all vertebrates (Gore, 2002) and is typically identified as GnRH-I (Lethimonier et al., 2004; Miranda et al., 1999; Mohamed et al., 2005; Senthilkumaran et al., 1999). In groupers (Epinephelinae), three forms have been identified for orange-spotted grouper (*Epinephelus coioides*) (Shi et al., 2010). However, no published accounts regarding the distribution of GnRHs in the brain of any grouper species can be found in the literature.

AVT and GnRH expression have been shown to co-vary across sexual phenotypes in the halfspotted goby (Maruska et al., 2007). Similarly, Parhar et al. (2001) have shown differences in the soma area and optical density of immunolabeled AVT and GnRH cells according to the reproductive stage in the goldfish, *Carassius auratus*. In addition, studies in fishes have noted the close proximity of some populations of AVT and GnRH, particularly in the parvocellular region (Maruska et al., 2007; Saito et al., 2003). In chicken, AVT is hypothesized to stimulate release of GnRH-I due to the close proximity of neurons in the preoptic area (D'Hondt et al., 2000). In female rat, AVP in the presence of estrogen increases GnRH release *in vitro* from preoptic area cell cultures (Funabashi et al., 2000). Moreover, blockage of V1a receptor by central administration of an antagonist in the preoptic area causes inhibition of LH secretion, implying a stimulatory influence of AVP on GnRH in mammals (Miller et al., 2006). Alternatively, evidence in rainbow trout (*Oncorhynchus mykiss*) and goldfish (*C. auratus*) suggests that GnRH may modulate AVT release due to the proximity of AVT and GnRH fibers and increases in Ca^{2+} signaling of AVT-containing fibers when GnRH treatment is applied (Saito et al., 2003). In any case, a mechanism involving specific receptor mediation has not been described.

The behavioral and gonadal sex change in protogynous hermaphroditic fishes makes them excellent models to examine the relationship of AVT and GnRH systems between female and male phenotypes. The rock hind (*Epinephelus adscensionis*) is a protogynous hermaphroditic species that is commonly found around submerged structures in the Eastern and Western Atlantic, Caribbean and the Gulf of Mexico (Polovina and Ralston, 1987). Previous work by Kline et al. (2011a) on captive rock hind has demonstrated that change from female to male sexual phenotype can be elicited in 32 ± 2 days by removal of the largest male in the social group. The relatively small size, sexual plasticity as adults, and previous work on the distribution of the AVT V1a2 receptor in the brain of rock hind (Kline et al., 2011b) makes it a convenient model to examine the roles of AVT and GnRH and their possible interactions in the control of behavioral and gonadal sex change in this species.

The objective of this research was to test the hypothesis that AVT acts on GnRH-I neurons via a vasotocin V1a receptor in rock hind. The expression of *avt*, *gnrh-I* and two V1a-type receptor mRNAs were compared between male and female phenotypes to provide further support for a possible relationship between the AVT system and GnRH-I. Finally, *avt* and *gnrh-I* expression was evaluated in relation to circulating sex steroids (e.g., 11-ketotestosterone [11-KT] and 17beta-estradiol [E2]) concentrations in male and female rock hind. 11-KT and E2 are the predominant sex steroids in reproductive fish (Devlin and Nagahama, 2002), and plasma concentrations of these hormones coincide with reorganization of the gonad and sex-specific coloration in sex-changing groupers and wrasses (Higa et al., 2003; Nakamura et al., 2007; Semsar and Godwin, 2004).

2. Methods

2.1. Animals and tissue sampling

All rock hind were sampled from socially stable, captive groups with a single habitat structure as described in Kline et al. (2011a). Fish were anesthetized in a bath of clove oil and seawater (0.1 ml/l) for body weight and total length measurements. Seven male rock hind with mean total length 323 ± 6 mm and weight 791.3 ± 19.8 and ten female rock hind with mean TL 228 ± 9 mm and weight 287.5 ± 23.4 g were sampled for blood, brain and gonadal tissues. Plasma extractions were unsuccessful in two males resulting in five male and ten females compared for plasma steroid levels. All mRNA transcript comparisons were made with the POA + H region only. 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.

Blood was drawn from the caudal vein by a heparinized syringe and plasma samples separated by centrifugation at 2500×*g* for 20 min at 4 °C. Samples were stored at −20 °C until use in steroid hormone assays. The fish were killed by decapitation, brains removed and placed in RNA Later solution (Ambion, Austin, TX) on ice, stored at 4 °C overnight and then stored at −20 °C until processing. Gonads were examined to verify sex and weighed to the nearest gram. For co-localization experiments, rock hind were anesthetized and cardially perfused with ice cold Zamboni's fixa-