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Seasonal aromatase activity in the brain of the male red-sided garter snake

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ABSTRACT

We investigated regional and seasonal variations in neural aromatase activity (AA), the enzyme that converts androgens into estrogens, to examine a possible indirect role of testosterone (T) in mediating spring reproductive behavior of red-sided garter snakes, a species exhibiting a dissociated reproductive pattern. Neural AA in male snakes varied significantly among brain regions. Additionally, there were significant interactions between brain region and season. In the spring, actively courting males had greater AA in the olfactory region (O) compared to the septum/anterior-hypothalamus preoptic area (S/AHPOA), nucleus sphericus (NS) and midbrain (Mb). Fall animals collected as they returned to the den prior to winter dormancy had significantly greater AA in the S/AHPOA compared to all other regions. These findings were consistent using either regional (gross) dissection or punch microdissection, which allowed us to separate the S and AHPOA. There were no significant differences in AA production between the S and AHPOA. This study provides the first documentation of seasonal and regional variations in AA in a snake brain and suggests that aromatization of androgens may play a role in regulating reproduction in red-sided garter snakes. During spring mating, elevated AA in the O may activate pathways essential for detection of courtship pheromones, while increased AA in the S and AHPOA of fall animals suggests that circulating androgens play an indirect role in programming critical neural pathways involved in reproduction. Thus, as in many other vertebrates, estrogenic metabolites of testosterone may be a critical hormonal component regulating reproductive behavior in this dissociated breeder.

Introduction

The majority of seasonally breeding vertebrates display an associated reproductive pattern where gamete maturation and elevated levels of sex steroid hormones immediately precede or coincide with the breeding season (Crews et al., 1984; Licht, 1984). In species exhibiting this type of associated reproductive pattern, castration eliminates reproductive activity while administration of exogenous sex steroids restores sexual activity in castrates and will initiate courtship in non-breeding individuals (Crews, 1991). In contrast, a small number of vertebrate species including some turtles, snakes and bats exhibit a dissociated reproductive pattern, mating at a time when their gonads are inactive and circulating levels of sex steroid hormones are reported to be low (see reviews in Licht, 1984 and Woolley et al., 2004). In these species, castration does not eliminate sexual activity, nor does the administration of exogenous sex steroids initiate reproductive behavior in non-courting individuals (Woolley et al., 2004).

The red-sided garter snake (*Thamnophis sirtalis parietalis*) is a well-studied example of a species exhibiting a dissociated reproductive pattern, mating at a time when the gonads are quiescent. Subsequently, spermatogenesis and steroidogenesis are not initiated until the breeding season has ended, with sperm being stored during winter dormancy in the ductus deferens until the following spring mating period.

In the adult male red-sided garter snake, initiation of courtship behavior and mating has been reported to be independent of testicular or pituitary hormone control (Camazine et al., 1980). Systemic administration of sex steroids, hypothalamic or pituitary hormones, or implantation of sex steroid hormones directly into the hypothalamic region fails to induce reproductive behavior in non-courting individuals (Camazine, et al., 1980; Crews et al., 1984; Friedman and Crews, 1985a). Moreover, males continue to exhibit courtship behavior for up to 3 years following castration (Crews, 1991).

Initial studies reported the level of circulating androgens to be low or absent upon emergence from winter dormancy (Garstka et al., 1982). However, subsequent investigations found that circulating androgens, elevated in the fall prior to winter dormancy, remain elevated throughout low temperature dormancy (LTD; Krohmer et al., 1987; Lutterschmidt and Mason 2009) and are not basal upon emergence in the spring (Krohmer et al., 1987; Moore et al., 2000,

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2001). More recently, Lutterschmidt and Mason (2009) demonstrated that androgen concentrations are elevated during the fall and decline significantly faster in snakes hibernated at warmer temperatures (i.e., 10 versus 5 °C). Thus, androgen concentrations appear to decline during winter dormancy via metabolic clearance. These results indicate that the observed annual variation in spring androgen levels may be related to a variation in environmental conditions, particularly temperature profiles (Lutterschmidt and Mason, 2009). To date, the only known stimulus capable of initiating courtship behavior in the red-sided garter snake is a prolonged period of LTD (Camazine et al., 1980; Garstka et al., 1982; Bona-Gallo and Licht, 1983; Whittier et al., 1987). Similar to the suggestions of Crews (1991) and Saint Girons et al. (1993), our current hypothesis is that elevated androgen concentrations during the fall and winter dormancy period, in combination with low temperature exposure, induce the changes in neuroanatomy and neurophysiology necessary to elicit reproductive behavior in the spring (Krohmer et al., 1987; Lutterschmidt and Mason, 2009).

Studies of the neural pathways regulating sexual behavior in male vertebrates have shown that the anterior-hypothalamus preoptic area (AHPOA) is a major integrative region for the coordination of internal and external stimuli (Crews and Silver, 1985; Ingle and Crews, 1985). These neural pathways contain sexually dimorphic sex steroid concentrating nuclei (regions) in the AHPOA, bed nucleus of the stria terminalis (BNST), nucleus sphericus (NS), septum (S) and ventromedial nucleus of the amygdala (AMY) (Tobet et al., 1986; Cherry et al., 1990; Aste et al., 1993; O'Bryant and Wade, 2002; Beck et al., 2008). Moreover, both androgens and estrogens can cause hypertrophy of these sex steroid concentrating nuclei (Balthazart and Adkins-Regan, 2002; Panzica et al., 1996). Similar to species exhibiting an associated reproductive pattern, the AHPOA also plays an important role in regulating reproduction of male red-sided garter snakes. Lesions placed in the AHPOA of male snakes eliminates all courtship behavior (Friedman and Crews, 1985b; Krohmer and Crews, 1987a), while lesions limited to the anterior portion of the preoptic area (POA) affect thermoregulatory capabilities but do not automatically affect courtship behavior and mating (Krohmer and Crews, 1987a). In the male red-sided garter snake, the neural regions that comprise the pathways regulating reproductive behavior (i.e., POA, NS, S and hypothalamus) also contain sex steroid concentrating nuclei (Halpern et al., 1982) that are responsive to both androgens and estrogens (Baleckaitis and Krohmer, unpublished data). However, unlike the majority of seasonally breeding vertebrates, where sex steroids play a critical role in the initiation of courtship behavior, the role of sex steroid concentrating nuclei in an animal exhibiting a dissociated reproductive pattern remains unknown, as does the importance of sex steroid hormone metabolism within these neural regions.

One component of sex steroid hormone metabolism critical to regulating sexual behavior in associated breeders is aromatase, the enzyme that catalyzes the conversion of androgens to estrogens. Since the initial characterization of aromatase in the brains of several vertebrate species (Naftolin et al., 1975), aromatase activity has been found in all major vertebrate groups (Callard et al., 1978a,b). The importance of estrogens in the control of reproductive behavior has been investigated in great detail in both mammals and birds (e.g., Balthazart et al., 2009; Wallen and Baum, 2002). Specifically, it has been shown that aromatization of testosterone (T) in the POA can mediate the activation of many aspects of reproductive behavior in a variety of vertebrate species (e.g., Balthazart, 1989; Ball and Balthazart, 2002, 2004; Baum, 2003; Naftolin et al., 1997; Wallen and Baum, 2002). However, only a few studies have examined the role of aromatase in the control of courtship behavior and mating in reptiles (Callard, 1983; Beck and Wade, 2009a,b; Rosen and Wade, 2001; Winkler and Wade, 1998; Wade, 1997). Using an antibody developed for quail (QR1; Foidart et al., 1995), Krohmer et al. (2002) documented that aromatase enzyme is present in all regions of the male red-sided garter snake forebrain. This study identified aromatase enzyme in two morphologically distinct cell types. Type II neurons, characterized as small neurons with a weakly staining cell body and few, if any, visible processes are found scattered throughout the entire forebrain. In contrast, the large, deeply staining Type I neurons are concentrated in areas containing sex steroid concentrating nuclei that are associated with the regulation of courtship and mating (Halpern et al., 1982; Krohmer et al., 2002). Thus, elevated circulating levels of testosterone during fall and winter dormancy, in association with sex steroid concentrating nuclei within the pathways that control courtship behavior and mating, suggest that testosterone may play a role in regulating reproduction indirectly through its neural aromatization to estrogens. To better understand the importance of neural sex steroid hormone metabolism to dissociated breeders, we examined the seasonal variation in aromatase activity (AA) among different regions of the male red-sided garter snake forebrain.

Material and methods

Animal and tissue collection

Male red-sided garter snakes (*T. sirtalis parietalis*) were collected from dens located in the Interlake Region of Manitoba, Canada during the spring mating season and in the fall as animals were returning to the dens in preparation for winter dormancy (Experiment 1, $n = 10$/season; Experiment 2, $n = 24$/season). All animals were returned to the field lab in Chatfield, Manitoba and, when possible, processed within 4 h or maintained in outdoor testing arenas (Moore and Mason, 2001) under natural conditions for no more than 5 days. Briefly, the snout-vent length (SVL) and body mass of each animal was measured and a lethal dose of sodium brevital (methohexitol) was administered (Jones Pharma Inc., St. Louis, MO, Wang et al., 1977). Once anesthetized, the heart was exposed, 0.2 ml of 1% heparin (Sigma, St. Louis, MO) was injected into the ventricle and animals were perfused through the heart with cold buffered saline (pH 7.2) until the return flow was clear (approximately 100 ml). Following perfusion, the brain was removed from the cranium, cryoprotected in 20% sucrose in 0.1 M phosphate buffer solution (pH 7.2) at 4 °C overnight, snap frozen on dry ice and stored at −70 °C until processed. The Saint Xavier University IACUC adheres to the principles set forth by NIH and the PHS policy on Humane Care and Use of Laboratory Animals. This study was conducted in accordance with the guidelines adopted by the Saint Xavier University Institutional Animal Care and Use Committee (IACUC).

Brain dissections

Experiment 1. Regional dissection

Brains ($n = 10$/season) were removed from −70 °C, placed on a glass plate seated over ice, allowed to warm slightly to prevent shattering and dissected into four regions: olfactory (O), NS, septum/anterior-hypothalamus preoptic area (S/AHPOA), and midbrain (Mb) (Fig. 1A). All neural tissue anterior to the optic chiasm was identified as O. Subsequently, the optic chiasm formed the anterior-most extent of the S/AHPOA while the blood sinus containing the pineal gland at the junction of the telencephalon and midbrain marked the posterior border (Halpern, 1980; Krohmer and Crews, 1987a,b; Krohmer et al., 2002). The border between the S/AHPOA and NS was distinguished by the lateral extent of the optic tracts. Each region was placed into a separate conical tube containing cold stabilizing medium (50 mM potassium phosphate, 0.1 mM EDTA, 20% glycerol and 1.0 mM dithiothreotol, all purchased from Sigma, St. Louis, MO, USA) and maintained on ice until assayed.

Experiment 2. Specific area punch microdissection

Specific area punch microdissection of the brains was performed using a modification of the procedure of Palkovitz (1973). The specific