Physiology & Behavior 102 (2011) 532–537

Contents lists available at ScienceDirect

Physiology & Behavior

journal homepage: www.elsevier.com/locate/phb

Brain nuclei in actively courting red-sided garter snakes: A paradigm of neural trimorphism

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ARTICLE INFO

Article history:
Received 6 April 2010
Received in revised form 15 December 2010
Accepted 21 December 2010

Keywords:
Anterior hypothalamus
Brain nuclei
External nucleus of the optic tract
Preoptic area
Red-sided garter snake
Sexual dimorphism
She-male
Thamnophis sirtalis parietalis

ABSTRACT

During the breeding season, two distinct male phenotypes are exhibited by red-sided garter snakes (*Thamnophis sirtalis parietalis*), with courtship behavior being directed not only toward females, but also toward a sub-population of males called she-males. She-males are morphologically identical to other males except for a circulating androgen level three times that of normal males and their ability to produce a female-like pheromone. As in other vertebrates, limbic nuclei in the red-sided garter snake brain are involved in the control of sexual behaviors. For example, an intact anterior hypothalamus pre-optic area (AHPOA) is essential for the initiation and maintenance of reproduction. To determine if brain morphology varies among the three behavioral phenotypes (i.e., males, she-males, and females) during the breeding season, we examined the volume, cell size and cell density of the AHPOA as well as a control region, the external nucleus of the optic tract (ENOT). We used Luxol Fast Blue and Ziehl's Fuchsin to visualize neurons and glial cells, respectively. No significant differences were observed among the three behavioral phenotypes in the volume, cell size or density in the control region. In contrast, the volume, cell size and density of the AHPOA of she-males were significantly greater than those of both male and female snakes. While the volume of the AHPOA was significantly greater in females compared to males, no differences were observed in cell size or density. These differences in brain morphology suggest a possible underlying mechanism for phenotypic-specific behavioral patterns.

1. Introduction

At the northern limit of the species' range, male red-sided garter snakes (*Thamnophis sirtalis parietalis*) emerge from low temperature dormancy (LTD) en masse, while females emerge singly or in small groups. As a result, females are actively pursued by a large number of males, creating a pile of snakes referred to as a "mating ball". Upon emergence, attractive females possess a non-volatile, lipid-based pheromone on their dorsal and lateral surfaces. Newly emerged adult males utilize this pheromonal cue to determine a female's attractivity, which initiates courtship behavior and mating [1–3]. Courtship in the red-side garter snake is a collection of stereotypic behaviors that can be easily quantified to analyze intensity and duration [4]. Briefly, males recognize females using visual and olfactory cues. As a male detects an attractive female, its tongue-flick rate increases. Once recognized, males exhibit chin rubbing behavior, a behavior exhibited only in the context of courtship and mating. As courtship proceeds, the male travels along the female's body in an attempt to align his body parallel to the female. After alignment, the male initiates muscle contractions and tail searching behavior, in which the male attempts to maneuver his tail under the female to achieve intromission [4].

These robust behaviors have been used successfully to define the parameters of the neural pathways controlling reproductive behaviors in the red-sided garter snake. Similar to other vertebrate species, the integrity of the anterior hypothalamus pre-optic area (AHPOA) in the male red-sided garter snake is critical for the activation and maintenance of courtship and mating [5,6]. Lesions placed in the AHPOA following emergence from [5] or prior to LTD [6] extinguished all courtship behavior, while bilateral lesions in the septum or nucleus sphericus prior to LTD facilitated courtship behavior [7].

Interestingly, male courtship behavior is sometimes directed toward a subset of the male population that appears to mimic sexually attractive females [1,2]. These female mimics, or "she-males", have been described as morphologically and physiologically identical to other males except for two rather unique exceptions. First, she-males have been found to produce a female-like pheromone that

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doi:10.1016/j.physbeh.2010.12.022

attracts normal males, resulting in misdirected courtship efforts. Second, the level of circulating androgens in she-males has been reported to be as much as three times greater than levels found in normal males [8].

Differences in plasma androgen concentrations between male and she-male snakes are particularly intriguing because red-sided garter snakes exhibit a dissociated reproductive pattern, mating at a time when spermatogenesis and steroidogenesis are inactive and sex steroid hormones are reportedly low [9,10]. Although subsequent research has shown that sex steroid hormone levels can be elevated at the beginning of the breeding season [11–13], initiation of courtship behavior and mating in this species appears to be independent of sex steroid hormones [14–16].

Moore [17] proposed the relative plasticity hypothesis as a basis for understanding differences in behavioral and morphological characteristics observed among alternate male phenotypes. This hypothesis proposes that fixed differences between alternate phenotypes are due to organizational actions of steroid hormones, whereas more plastic differences are due to activational influences of these hormones. Subsequently, several model systems exhibiting specific, quantifiable examples of dimorphism linked to a behavior or behaviors that differ either between or within the sexes have been used to better understand sex differences in the brain [18–20].

The existence of two distinct male behavioral phenotypes in the red-sided garter snake offers a unique opportunity to further examine the biological principles that cause natural variation in brain structure and function within the same sex. Therefore, the purpose of this study was to examine the forebrains of male, female and she-male red-sided garter snakes during the breeding season to determine possible morphological differences within the pathways regulating courtship behavior and mating. Of specific interest is the AHPOA, a known sex steroid concentrating region [21] and critical component of the pathway regulating courtship behavior and mating [5,6]. Halpern et al. [21] describes this region as consisting of a cell-dense POA, confluent with the anterior portion of the hypothalamus that is composed of the dorsal, intermediate and ventral nuclei. In addition, the volume of the external nucleus of the optic tract (ENOT), an area not associated with reproduction in the red-sided garter snake, was evaluated as a control region.

2. Materials and methods

2.1. Animals and tissue collection

Animals were collected during the breeding season in the spring of 2001 from dens located in the Interlake Region of Manitoba, Canada. On days when animals were actively courting, mating balls were examined to determine if the animal being courted was an attractive female or male. Suspected she-males, actively courting males and attractive females collected on the same day were returned to the field lab in Chatfield, Manitoba. Snakes were placed in outdoor testing arenas [12,13], maintained under natural conditions and tested for courtship behavior (males) and attractivity (females and she-males) daily for three consecutive days. Only males that continued to exhibit intense courtship behavior and females and she-males that remained attractive to courting males for all three testing days were used in this study.

All brains (n = 8/group) were collected within 2 h of the final courtship trial. Briefly, animals were weighed, measured, and then deeply anesthetized with an overdose of 1% Brevital Sodium (0.0015 μl/kg body weight) [22]. Once anesthetized, the heart was exposed and 0.5 ml of 1% heparin (Sigma) was injected into the ventricle. Animals were perfused through the heart with cold saline until the return flow was clear (~100 ml) followed by approximately 150 ml cold 4% paraformaldehyde and 0.1% glutaraldehyde in 0.1 M phosphate buffer, pH 7.2. Brains were removed from the cranium, cryoprotected in phosphate buffer containing 20% sucrose overnight at 4 °C, snap frozen on dry ice and stored at −70 °C until sectioned.

This study was conducted in accordance with the guidelines adopted by the Saint Xavier University Institutional Animal Care and Use Committee (IACUC). 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.

2.2. Histology and data collection

Brains were cut coronally at a thickness of 30 μm on a Leitz 1720 cryostat. All sections were collected directly onto gelatin-coated slides in the order of sectioning (anterior to posterior) and allowed to dry overnight. Tissues were stained with Luxol fast blue, resulting in myelinated nerve fibers and the phospholipids in the cell membrane appearing blue [23,24]. The tissues were then counter stained with Ziehl's Fuchsin, rendering glial and other support cells a reddish-purple. Tissues were then dehydrated in progressive alcohols, cleared in xylene and cover slipped using the Permount (Fisher) covering medium.

It should be noted that the stains we used to differentiate between neurons and glial cells do not provide absolute specificity. Therefore, in addition to differential staining, morphological criteria were used to distinguish neurons from glial cells. Thus in the most conservative approach, the results presented here represent the morphological analysis of cells with an apparent neuronal phenotype. For ease of communication, we simply refer to these cells as neurons throughout the manuscript. Future studies using immunohistochemistry for specific cell markers (i.e., NeuN) would be helpful in confirming the phenotype of these cells.

In the garter snake brain, the anterior boundary of the POA begins as the optic tracts extend laterally from the optic chiasm, corresponding with the initial appearance of the third ventricle (Fig. 1). As the optic tracts cut dorsally into the brain the interface of the POA and anterior hypothalamus (AH) is indicated by the appearance of the stria medullaris at the dorsal end of the optic tracts. At its caudal boundary the AHPOA becomes continuous with the bed nucleus of the stria terminalis (Bst) [25,26] and terminates at the anterior aspect of the supraoptic nucleus and rostrolateral hypothalamic area [21].

Similar to animals exhibiting an associated reproductive pattern, specific regions (nuclei) within the brain of red-sided garter snakes have been shown to concentrate sex steroid hormones [21]. These regions can be distinguished visually by morphological differences in the size of the region of interest as well as the number and density of neurons within these regions [27, Fig. 2]. Therefore, the AHPOA and external nucleus of the optic tract (ENOT) were assessed visually by comparison with the surrounding tissues. All sections comprising the rostrocaudal extent of the AHPOA from each brain were examined with a Nikon Labphot-2 light microscope fitted with a camera lucida. Using the camera lucida, the volume of each of the two regions was estimated by tracing the areas of interest in both hemispheres. All slides were coded to circumvent possible bias and each animal was drawn independently by two observers.

The area of each outlined region was determined by overlaying a grid containing units of area standardized to the magnification of the drawings (40×) and summing the number of squares contained within the outline. Using standard stereological procedures, partial boxes were not counted on the upper and left extent of the outline while partial boxes were counted on the lower and right side [28]. The volume of each section was computed by multiplying the area outlined by the thickness of the section (30 μm). The total volume of each brain nucleus could then be calculated by summing the volumes of all sections [6,27].

For each animal, the diameters of 25 and 10 neuronal cell bodies within the AHPOA and ENOT, respectively, were measured using a

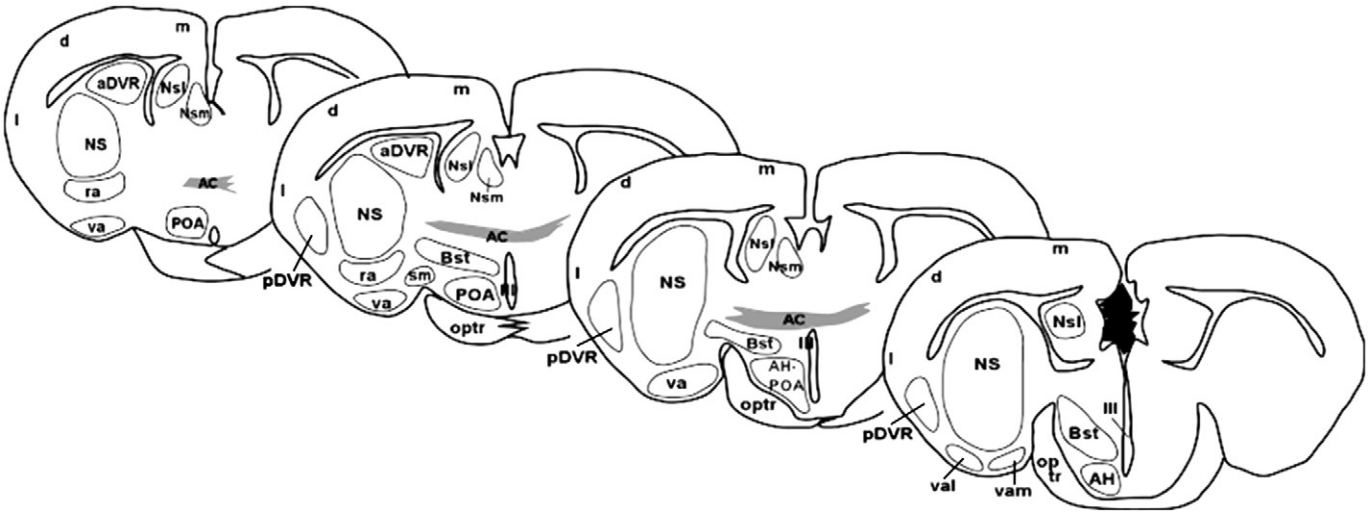

Fig. 1. Diagram of sections through the forebrain of the red-sided garter snake depicting the extent of the AHPOA. Abbreviations: III, third ventricle; aDVR, anterior dorsal ventricular ridge; AC, anterior commissure; AH, anterior hypothalamus; Bst, bed nucleus of the stria terminalis; d, dorsal cortex; l, lateral cortex; m, medial cortex; NS, nucleus sphericus; Nsl, lateral septal nucleus Nsm, medial septal nucleus; optr, optic tract; POA, preoptic area; pDVR, posterior dorsal ventricular ridge; ra, rostral amygdaloid nucleus; sm, strial medullaris; va, ventral amygdaloid nucleus. val, ventral amygdaloid nucleus, lateral portion; vam, ventral amygdaloid nucleus, medial portion.

calibrated ocular micrometer. Also, for each animal, cell density was assessed by counting the number of cell bodies contained within an area of 2000 μm^2 (AHPOA) or 300 μm^2 (ENOT). Analysis of changes in cell density was determined by the Delesse–Sorby principle, which asserts that on average, the fractional area of a feature on histological sections taken from a solid tissue will be directly proportional to the fractional volume of that feature in the original tissue [28].

Photomicrographs of representative sections were digitized with a Zeiss Axio Imager. D1 microscope equipped with an AxioCam MRm Camera. Images were captured on an HP Compac Computer and recovered with Adobe Photoshop 7.0. All graphs and drawings were created with Canvas 8.0 (Deneba) and saved as TIFF files.

2.3. Statistical analysis

The volume of the AHPOA and ENOT nuclei and the mean diameter of the cell bodies contained within each region were analyzed using a one-way analysis of variance (ANOVA). Due to unequal variance in cellular density among behavioral phenotypes, we used a Kruskal–Wallis one-way ANOVA to examine differences in cellular density in the areas of interest. All pairwise multiple comparisons were made using the Student–Newman–Keuls post-hoc method. SigmaStat® 3.5 (Systat 2006) and SPSS® 17.0 (SPSS 2008) were used for all statistical analyses. For all statistics, the significance level was set at $p<0.05$.

3. Results

No significant differences were found among the behavioral phenotypes in the volume ($F_{(2,23)}=0.25$; $p=0.781$), cell body diameter ($F_{(2,23)}=0.168$; $p=0.846$) and cellular density ($H_{(2)}=3.015$; $p=0.221$) of the ENOT (Table 1).

The volume of the AHPOA of red-sided garter snakes differed significantly ($F_{(2,23)}=9.306$; $p=0.001$) among the three behavioral phenotypes (Figs. 2 and 3A). Post hoc analysis revealed that the volume of the AHPOA in she-males was significantly larger than the volume of the AHPOA in either males ($p<0.001$) or females ($p=0.04$). Moreover, the volume of the AHPOA in females was significantly larger ($p<0.04$) than the corresponding region in males.

The mean cell body diameter of neurons within the AHPOA was significantly different ($F_{(2,23)}=15.355$; $p<0.001$) among the behavioral phenotypes (Fig. 3B). Post hoc analysis revealed that the mean cell body diameter in the AHPOA of she-males was significantly larger than that of both males and females (both $p<0.001$). However, no significant difference in mean neuronal cell body diameter was observed between males and females ($p=0.747$).

The mean cell density within the AHPOA was also found to be significantly different ($H_{(2)}=7.594$; $p=0.022$) among the behavioral phenotypes (Fig. 3C). Post hoc examination revealed that cell density in the AHPOA of she-males was significantly greater than that of either males or females (both $p<0.05$). However, as with cell body diameter, no significant difference in mean cellular density was observed between males and females ($p>0.05$).

4. Discussion

During the spring breeding season, red-sided garter snakes exhibit three distinct behavioral phenotypes. In the current study, we examined the morphology of two regions of interest and found there to be significant differences in the AHPOA among the three phenotypes. There were no significant morphological differences in a control region, the ENOT, among male, she-male, or female snakes. Comparisons of brain morphology revealed that the volume of the sex steroid concentrating nucleus, neuronal cell body size and cellular density within the AHPOA of she-males were significantly greater than those of either males or females. While the AHPOA nucleus of females was significantly larger than the corresponding region in males, neither the mean cell body diameter nor cellular density was significantly different between males and females.

The majority of sex differences in brain structure appear to occur in regions that are closely associated with the hypothalamus. Hypothalamic and limbic nuclei have been found to play critical roles in the regulation of species-typical and, in a number of cases, sexually dimorphic behaviors [29]. An area such as the AHPOA is essential for the integration of sensory and motor events associated with specific behaviors; it has been shown to be sexually dimorphic in size, fluctuating in volume in response to reproductive condition [30]. Moreover, many hypothalamic nuclei are interrelated and contain a large quantity of sex steroid hormone receptors, suggesting that these regions are involved in the control and regulation of behaviors influenced by gonadal steroids [31]. For example, hormone dependence of the hypothalamus has been reported in gerbils, [32,33] Japanese quail, [34–36] rats, [37–39] ferrets, [40–42] midshipman fish, [43] and whiptail lizards [44,45].

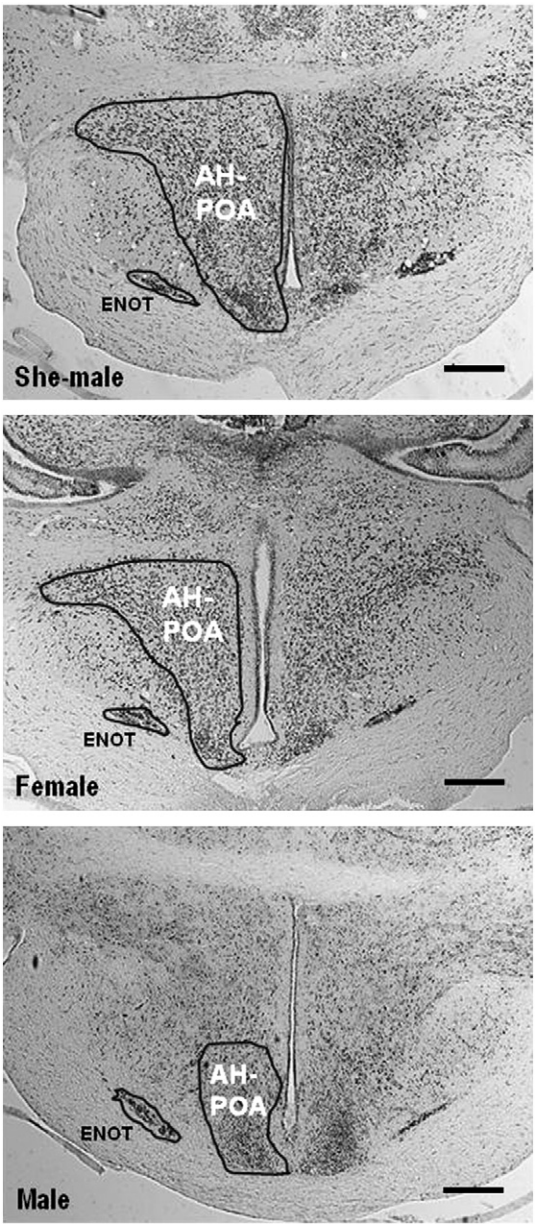

Fig. 2. Photomicrographs of coronal sections at the level of the anterior hypothalamus preoptic areas (AHPOA) from the brains of she-male, female and male red-sided garter snakes. These photomicrographs depicting each forebrain at the same general level and magnification represent examples of the apparent size differences of the sex steroid concentrating nuclei (outlined). bar = 600 μm.

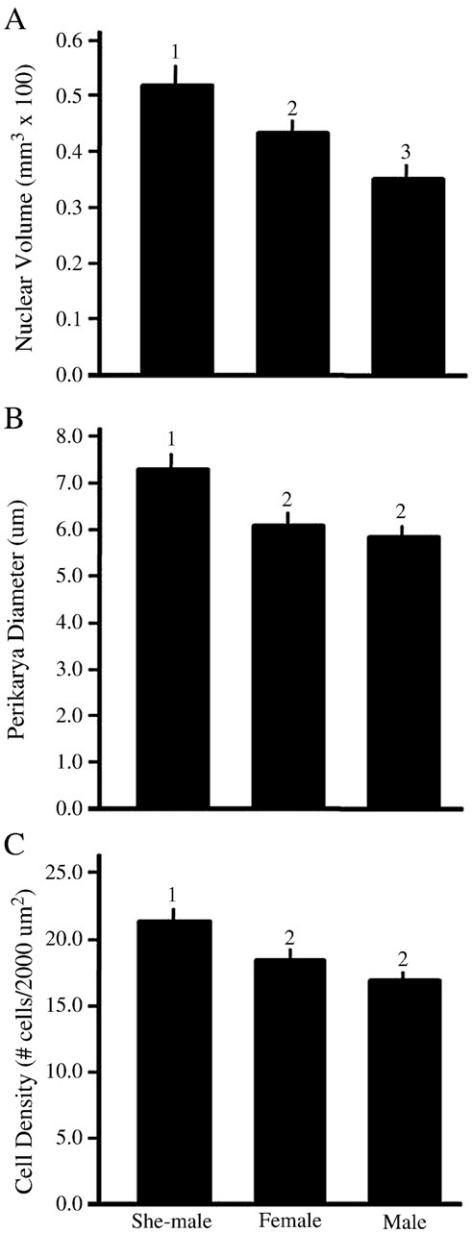

Fig. 3. A. Mean volume (±SE) of the sex steroid concentrating nucleus within the AHPOA of the three reproductive behavioral phenotypes (she-male, female, male) found in red-sided garter snakes. The volume of the sex steroid concentrating region in she-males is significantly larger than that of either females or males. In females the volume is significantly larger than in males. Numbers represent statistical differences among the behavioral phenotypes. B. Mean diameter (±SE) of neuronal cell bodies within the sex steroid concentrating regions of the three reproductive behavioral phenotypes (she-male, female, and male) found in red-sided garter snakes. The size of neuronal cell bodies within the sex steroid concentrating region in she-males was significantly larger than that of either females or males. No significant differences were found between females and males. Numbers represent statistical differences among the behavioral phenotypes. C. Mean cell density (±SE) within the sex steroid concentrating regions of the three reproductive behavioral phenotypes (she-male, female, and male) found in red-sided garter snakes. The mean cell density within the sex steroid concentrating region in she-males was significantly greater than the density in either females or males. No significant differences were found between females and males. Numbers represent statistical differences among the behavioral phenotypes.

Crews et al. [27] examined possible seasonal and sexually dimorphic variations in the ENOT, medial forebrain bundle (MFB), POA and ventral medial nucleus of the hypothalamus (VMH) in male and female red-sided garter snake brains. Sexually dimorphic differences were observed in only two regions: 1) the POA of females placed in low temperature dormancy (LTD) was significantly smaller than that of males under the same treatment regimen; and 2) the nucleus sphericus (NS) of females was significantly smaller than that of males in animals not placed into LTD [27]. Furthermore, Crews et al. [25] found no seasonal or hormonally induced variation in the brains

Table 1
Average volume, cell body diameter and cell density (±SE) of the external nucleus of the optic tract (ENOT) in she-male, female and male red-sided garter snakes during the breeding season. No significant differences were found among the behavioral phenotypes ($p > 0.05$).

	Volume ($mm^3 \times 100$)	Diameter (μm)	Density (#cells/200μm^2)
She-male	0.078 ± 0.03	6.3 ± 0.14	19.05 ± 0.23
Female	0.075 ± 0.03	6.3 ± 0.18	17.96 ± 0.53
Male	0.075 ± 0.04	6.2 ± 0.08	18.57 ± 0.29

of males, while females exhibited significant seasonal fluctuations in both the POA and VMH.

The expression of sexually dimorphic behaviors appear to be regulated, for the most part, by sexually dimorphic morphological and/or neurochemical sex differences in the brain [46–50]. For example, in species exhibiting an associated reproductive pattern, where elevated levels of sex steroid hormones are required for the initiation and maintenance of reproductive behaviors [9,10], sex steroids appear to be essential for the organization of sexually dimorphic nuclei in the male preoptic area [34,41,48,51]. In addition, organization of neural pathways responsible for the regulation of reproductive behaviors in some species appears to depend largely on the presence of estrogenic metabolites and not the direct action of circulating androgens [52–57]. Furthermore, in associated reproductive patterns, estrogens have been found to be essential for the activation and regulation of reproductive behaviors in the adult male of some species [58–62].

The red-sided garter snake is probably the most studied species exhibiting a dissociated reproductive pattern, mating at a time when spermatogenesis and steroidogenesis are inactive [9,10]. Although the seasonal physiological and hormonal cycles have been well documented [4,11,13–15], initiation of courtship behavior and mating appears to be independent of sex steroid hormones. In fact, the only known stimulus/cue identified to date that will initiate courtship behavior and mating in male red-sided garter snakes is a prolonged period of LTD followed by exposure to warm temperatures [14–16,63]. Therefore, it is quite interesting to note that, similar to species exhibiting an associated reproductive pattern, the forebrain of red-sided garter snakes contain sex steroid concentrating regions within the neural pathways vital for the control of reproductive behaviors [5–7,21].

Although she-males are morphologically identical to normal males, she-males do exhibit three-fold higher levels of circulating testosterone as compared to males [1,3]. These higher androgen concentrations would provide a larger amount of substrate to be aromatized to estrogens than found in normal males. For example, aromatase-immunoreactive (ARO-ir) cells are found in all regions of the garter snake forebrain, with the highest density of immunoreactive cells occurring within sex steroid concentrating regions [26] similar to both mammals and birds [59,60,64–67]. In addition, seasonally elevated levels of circulating estrogen would be available to affect brain morphology. The variable availability of estrogen to steroid sensitive regions of the red-sided garter snake brain suggests a type of hormonal (estrogen) gradient that may affect the volume of sex steroid concentrating nuclei, with she-males exhibiting the greatest hypertrophy and males the least.

The current study identifies morphological variations within the AHPOA of male, female and she-male red-sided garter snakes. The observed morphological differences in the AHPOA offer evidence of a possible neuroendocrine mechanism controlling the various behavior phenotypes in the red-sided garter snake. While we know the AHPOA is important for the control of courtship behavior [5,6], further research is necessary to determine if the sexually dimorphic steroid concentrating region within the AHPOA is involved in regulating pheromone production and attractivity of females and she-males.

The alternative mating strategy described for the red-sided garter snake is not unique among vertebrates. Variations in reproductive tactics have been described for certain species of fish [18,43,68–71] and lizards [72–77]. In each of these species, the internal hormonal environment has been shown to play an extremely important role during development and/or as adults. While the hormonal environment of adult red-sided garter snakes has been well documented, the hormonal environment of fetal snakes should be investigated further to determine if an organizational effect of sex steroid hormones on the developing brain contributes to the three distinct behavioral phenotypes described here.

In memoriam

Geno A. DeMarchi (1981–2010), a great student and good friend.

Acknowledgements

Research funded in part by the Center for Educational Practice, Dean's Fund, College of Arts and Sciences and the Office of the Vice President for Academic Affairs, Saint Xavier University. We would also like to thank Bill Watkins, Department of Natural Resources, Manitoba, Canada and Al and Gerry Johnson, Chatfield, Manitoba, Canada.

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