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Research report

Volumetric analysis of sexually dimorphic limbic nuclei in normal and sex-reversed whiptail lizards

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Abstract

Sex differences in the size of key limbic nuclei have been found in many species. In some of these species, steroid hormones have been implicated in both the development and the maintenance of the sex difference. However, the possible role of sex-specific genes has not been examined, in part due to lack of an appropriate model system. In this study we measured the size of the ventromedial hypothalamus and preoptic area–anterior hypothalamus in normal female whiptail lizards and in genetic female whiptails that had been sex-reversed by treatment early in development with the aromatase inhibitor fadrozole. We found no difference in the size of these two nuclei between females and the sex-reversed animals. These results suggest that either the sex-reversing treatment itself interfered with the masculinization process, or that a male genome is required to produce a male-like limbic phenotype.

Keywords: Reptile; Sex-reversed; Preoptic area; Ventromedial hypothalamus

1. Introduction

In male vertebrates, the preoptic area and anterior hypothalamus (POAH) are involved in the integration of male sex behavior [7,22,28]. The ventromedial hypothalamus (VMH) appears to play an important role in role in female sex behavior [21,26,29]. These nuclei have been shown to be sexually dimorphic in size in a wide range of vertebrate species [1,6,8,13,16,23,33]. In males, the POAH or regions within this complex are typically larger than in females, while in the VMH the direction of the sexual dimorphism is not consistent. The hormonal basis for the sex difference in POAH size has been examined in several of the species in which it occurs. In gerbils [6], ferrets [33], and whiptail lizards [38], testosterone administered to males castrated in adulthood will increase the size of the POAH. The only avian species where sex differences in the size of limbic nuclei have been extensively studied is the Japanese quail. The dimorphisms in the POAH of the quail do not become apparent until puberty, but can be induced in males or females by treatment with testosterone at that time [25,31]. However, in whiptails and ferrets, adult females are unresponsive to androgen treatment, suggesting an organizational sex difference in sensitivity to androgens. In rats, testosterone does not seem to affect the size of the POAH in either sex in adulthood, but perinatally administered aromatizable androgens and estrogens will increase the size of a region of the POAH in females to a male-like level [2,11,13,20]. The same developmental effect of androgens or their metabolites has been found in ferrets [32] and gerbils [34,35,44], although in some cases the affected brain regions do not reach the size of those in control males.

What has not been examined to date is the possibility of direct genetic control over the development of sexually dimorphic limbic nuclei. Using traditional animal models, it is difficult to rule out the effects of gonadal secretions. We have developed an animal model in which questions like this can be addressed. The desert-grassland whiptail lizard *Cnemidophorus uniparens* is an obligate parthenogen; all individuals are genetically and phenotypically

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female. However, application of fadrozole, a non-steroidal aromatase inhibitor, to eggs from the parthenogen soon after they are laid causes the treated embryos to develop as males [41,42]. These ''created males'' have fully developed hemipenes and vasa deferentia, and their testes are capable of producing motile sperm [41]. Phenotypically the created males closely resemble genetic males of a related sexually-reproducing species *C. inornatus*, but little is yet known about the sexual differentiation of their brains. Intact created males display male-typical sexual behavior when presented with receptive females and fail to show female-like receptive behavior when presented with other males [41]. However, this behavior may represent a purely activational effect of testosterone; female parthenogens given exogenous androgen will also readily mount and court other females but will not allow themselves to be mounted [15]. In the sexually-reproducing species, males have much larger POAHs and smaller VMHs than do either conspecific females or parthenogens. The goal of the current research is to use these previously-established markers for a masculine whiptail lizard brain — a large POAH and a small VMH — to examine the sexual differentiation of the created males' brains. Does the brain of the created males match their gonadal sex or their genetic sex?

2. Materials and methods

2.1. Animals

Adult females were collected in and around Portal, AZ and transported to the University of Texas at Austin. There they were maintained in groups of four to five as described previously [14]. The males used in the study were lab-reared and had been treated in ovo on day 5 of incubation with 1 to 25 μg of fadrozole, a non-steroidal aromatase inhibitor that causes the embryos to develop as males [41,42]. The doses of fadrozole varied because the males used in this study were originally part of a study examining the effect of dosage on sex determination. The dosage study established that 100% of embryos treated with 1 μg of fadrozole or more develop as males, while embryos treated with 0.1 μg of fadrozole or less are female; no intersexes were produced [41]. It has proved very difficult to raise whiptail hatchlings to adulthood; we were unable to obtain a complete cohort from any single dosage group. The males had been maintained in the laboratory since the time of hatching (12 to 18 months). The females were wild-caught and therefore of unknown age; they had been held in the lab for approximately 8 months at the time of sacrifice. All animals used in the study were sexually active adults and were housed in breeding cages with three to four female conspecifics. The females used in the study had developing follicles or corpora lutea; the males courted receptive females, had enlarged testes, and showed secretion from a row of pores on the hindlimb called femoral pores. All the latter measures are indicative of stimulation by circulating androgens.

2.2. Brain removal and histology

Animals were sacrificed by rapid decapitation and their heads were placed in Kolmer's fixative for 24 h to decalcify the skull and fix the brain. The brains were then removed from the skulls, rinsed in distilled water, dehydrated in a series of ethanols, and embedded with paraffin. The brains were sectioned coronally at 25 μm and then stained with Cresyl violet.

2.3. Morphometric analysis

The perimeters of the POAH and VMH were traced on one side of the brain using NIH Image image analysis software. The brains were coded to prevent bias in measuring. The analysis system captured each brain image from the slide onto the computer monitor through a camera attached to a Zeiss microscope. The images were traced on the screen with the use of a mouse. The images on the screen were visually compared by the measurer to the actual sections through the microscope as needed for clarification. The images were viewed through the scope at 40 × magnification for the POAH and VMH and at 20.5 × for whole brain measurements.

The volumes of the POAH and the VMH were measured according to procedures described previously [8,37]. Briefly, measurements of the POAH began where the nucleus appears as a small, darkly stained oval 50–75 μm rostral to the first appearance of the third ventricle. Measurements continued caudally between the third ventricle and the lateral forebrain bundle (LFB), including the cell-dense medial POA and the less dense lateral POA, but excluding the very sparsely populated cell groups nearest the LFB. The boundary between the anterior hypothalamus (AH) and the POA is not clearly distinct in the whiptail; measurements continued through the AH until the POA was no longer decipherable from the tissues dorsal to it. This point generally coincided with the posterior end of the anterior commissure.

Measurements of the VMH began where it appears as a small bean-shaped group of densely packed cells on either side of the third ventricle. Measurements continued through the VMH until it separated into a medial band of cells and a lateral ovoid cell group. Once the groups separated, the measurements continued through the lateral portion only until the densely packed cell group disappeared.

The cross-sectional areas for the POAH and VMH were determined in every fifth section on one side of the brain. Sequential pairs of cross-sectional areas were averaged and multiplied by the distance between these areas. These

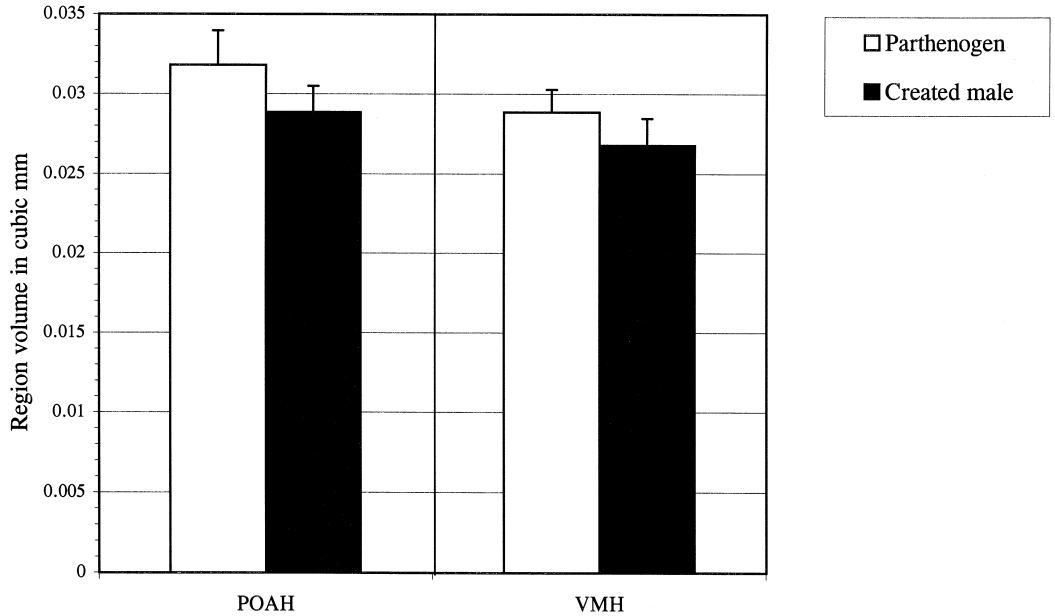

Fig. 1. Mean volumes of POAH and VMH in adult created male ($n = 5$) and normal female ($n = 6$) parthenogenetic whiptail lizards. Created males were produced by administration of 1 μg ($n = 2$), 10 μg ($n = 2$), or 25 μg ($n = 1$) of fadrozole as described in the text. Error bars are ±1 S.E.M.

volumes were then summed for each brain region to obtain the total volume measurement for that region. An estimate of relative brain size was determined by measuring the area of one-half of the brain at the most anterior portion of the POAH and again 55 sections later, which roughly corresponds to the end of the VMH. The volume of this area was then calculated as if it were a cone frustum.

2.4. Statistical analysis

Regional measurements were compared between females and created males with an independent means *t*-test using the SigmaStat v. 2.0 statistical software package on a Dell 450/ME computer. *P*-values of 0.05 or less were considered significant.

3. Results

A regression analysis examining the relationship between dosage of fadrozole and size of the created males' POAH and VMH revealed no significant correlations (POAH $r^2 = 0.624$, $p = 0.112$; VMH $r^2 = 0.364$, $p = 0.282$). The data from the created males were therefore treated as a single group.

The created males had POAH and VMH volumes very similar to those of normal, unmanipulated females (Fig. 1). There was no statistically significant difference between males and females in the volume of either the VMH (male VMH = 0.0268 $mm^3 \pm 0.0017$, female VMH = 0.0289 $mm^3 \pm 0.0014$, $p = 0.365$) or the POAH (male POAH $x = 0.0289$ $mm^3 \pm 0.0016$, female POAH = 0.0318 $mm^3 \pm 0.0021$, $p = 0.318$). In addition, there was no sex difference in estimated whole brain volume (male $x = 4.71$ $mm^3 \pm .104$, female $x = 4.36$ $mm^3 \pm 0.171$, $p = 0.102$).

4. Discussion

Despite complete sex reversal of urogenital and gonadal phenotype as well as external appearance, parthenogenetic whiptail lizards treated early in development with a non-steroidal aromatase inhibitor have POAH and VMH volumes indistinguishable from those of unmanipulated females. This result is unexpected, since the hybrid parthenogen shares two-thirds of its genome with the sexually reproducing *Cnemidophorus inornatus* [43]. Males of the sexually reproducing species have substantially larger POAHs and smaller VMHs than either female *C. inornatus* or females of the parthenogenetic *C. uniparens* [8]. Thus, we may reasonably expect that parthenogens who have had testicular development induced from the earliest stages of embryonic development would show a pattern of sexual differentiation of the brain similar to males of the sexually reproducing species. However, our results indicate that this is not the case.

It does not seem parsimonious to hypothesize that while the hybrid whiptails have retained the genetic instructions to produce male-typical gonads and secondary sexual characters, they have lost the ability to produce a masculine limbic morphology. Why then might the created male whiptails have a structurally female brain sex despite having functional testes? There are two critical differences between the created males and normal males of the related

sexually reproducing species: (i) blockade of estrogen synthesis early in development and (ii) genetic sex.

Studies of the sexually dimorphic nucleus of the preoptic area (SDN-POA) in rats may provide a point of comparison with regard to hormonal control of sexual differentiation of brain structures. The SDN-POA is a subdivision of the medial preoptic area, to which the whiptail POAH roughly corresponds [8,22]. Males have larger SDN-POAs than do females, and several lines of evidence indicate that the aromatization of testicular androgens is necessary for masculinization of this nucleus. Treatment of female rats with estrogen or aromatizible androgens will increase SDN-POA volume [11,13,19] while treatment with aromatase inhibitors [18] or estrogen antagonists [10] will decrease it. In the latter study, treatment of normal females with the antiestrogen actually caused the SDN-POA to be smaller than in unmanipulated females, suggesting that ovarian estrogens play a role in normal development of the female SDN-POA as well. More recent evidence indicates that there are differential rates of apoptosis in the preoptic area of neonatal male and female rats [9]. In that study, testosterone inhibited apoptosis in neonatally castrated males; estrogen treatment has a similar effect [3], suggesting that prevention of programmed cell death is a possible mechanism of action for the previously observed effects of aromatizable androgens on SDN-POA volume.

Based on this evidence, there is reason to suspect that aromatization may also play a role in masculinization of the whiptail POAH and possibly also the VMH. If this is the case, then perhaps fadrozole treatment-though it induced testicular development-blocked masculinization of the these brain areas by preventing the formation of estrogen. This would suggest that normal sexual differentiation of the POAH and VMH must take place during the time that fadrozole is present in the embryo.

Previous studies have found no sex difference in POAH or VMH volume in thirty-day old hatchling whiptails. This is not due to lack of circulating sex hormones; unlike the case in adults, treatment with E, T, or DHT has no effect on brain morphology in the young animals [38]. This evidence implies that the brains of hatchlings must still have changes to undergo before they assume the adult phenotype. This presents a problem for the hypothesis that the fadrozole treatment in this study blocked masculinization of the brain by blocking estrogen synthesis. Eggs were treated with fadrozole on the fifth day after they were laid. Even if one assumes that the fadrozole was present throughout embryonic development, after the animals hatched and used up any stored yolk (no more than one week after hatch, K. Wennstrom pers. obsv.), the effects of the compound should dissipate rapidly. Studies in rats indicate that normal estrus cycles return within a few days after cessation of treatment with fadrozole [24]. If this is the case, then it is unclear why fadrozole treatment during embryonic development should have caused the observed disjunction between brain sex and gonadal sex in the created males. One possibility is that the process of sexual differentiation involves long-lasting changes in the affected tissues' sensitivity to gonadal steroids [5,40]. Just such a two-stage process, wherein early exposure to gonadal steroids sensitizes the brain such that it responds to subsequent lower levels of hormone has been demonstrated in ferrets [4] and in rats [17]. Perhaps lack of estrogen in ovo affects how the brains of individual lizards respond to their own hormones post-hatching, after the fadrozole itself has been metabolized and excreted.

An alternative explanation is that masculinization of the POAH and VMH in whiptail lizards is not primarily controlled by gonadal hormones, but instead is driven by the genetic sex of the individual. While the idea that sexual differentiation of the brain might be genetically determined is relatively new, there is growing evidence for such a mechanism [3]. For example, genetic sex appears to play a role in the differentiation of monoaminergic activity in the limbic system of mice. In studies where explants of embryonic day 14 mouse diencephalon were grown in vitro in the absence of sex steroids, researchers observed sex differences in the size of tyrosine hydroxylase (TH) immunoreactive cells, TH activity, and endogenous dopamine content (see Ref. [27] for review). A similar set of studies in zebra finches indicates that sexual dimorphisms in androgen receptor expression in the song system also develops independently of sex steroids [12]. Further, genetic female zebra finches treated in ovo with fadrozole develop functional testicular tissue, but fail to develop masculine song systems [36,39] or song behavior [30]. However, their genetic male counterparts developed normal male song systems, indicating that fadrozole treatment itself did not block song system growth.

At this point, though the current results are intriguing, it is too early to say whether there is a direct effect of genetic sex on development of sexual dimorphisms in limbic structures in whiptails. Further research will be necessary to determine whether an aromatase inhibitor treatment regime can be found that will produce an animal with both testes and a male-like POAH and VMH. It would also be useful to investigate the effect of aromatase inhibitors on genetic males-such studies could determine whether fadrozole treatment itself prevents masculinization. Treatment of female embryos with aromatase inhibitor after gonadal differentiation takes place would also shed light on the question of when and how masculinization occurs.

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