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The Ovine Sexually Dimorphic Nucleus, Aromatase, and Sexual Partner Preferences in Sheep

C.E. Roselli[1] and **F. Stormshak**[2]

[1]Department of Physiology and Pharmacology, Oregon Health and Sciences University, Portland, OR 97239

[2]Department of Animal Sciences, Oregon State University, Corvallis, OR 97331

Abstract

We are using the domestic ram as an experimental model to examine the role of aromatase in the development of sexual partner preferences. This interest has arisen because of the observation that as many as 8% of domestic rams are sexually attracted to other rams (male-oriented) in contrast to the majority of rams that are attracted to estrous ewes (female-oriented). Our findings demonstrate that aromatase expression is enriched in a cluster of neurons in the medial preoptic nucleus called the ovine sexually dimorphic nucleus (oSDN). The size of the oSDN is associated with a ram's sexual partner preference, such that the nucleus is 2 -3 times larger in rams that are attracted to females (female-oriented) than in rams that are attracted to other rams (male-oriented). Moreover, the volume of the oSDN in male-oriented rams is similar to the volume in ewes. These volume differences are not influenced by adult concentrations of serum testosterone. Instead, we found that the oSDN is already present in late gestation lamb fetuses (~ day 135 of gestation) when it is ~2-fold greater in males than in females. Exposure of genetic female fetuses to exogenous testosterone during the critical period for sexual differentiation masculinizes oSDN volume and aromatase expression when examined subsequently on day 135. The demonstration that the oSDN is organized prenatally by testosterone exposure suggests that the brain of the male-oriented ram may be under-androgenized during development.

Keywords

estrogen; androgen; behavior; sexual differentiation; preoptic area

1. Introduction

Nearly 40 years ago Naftolin first demonstrated that hypothalamic and limbic brain tissues are able to aromatize testosterone and other C19 steroids into estrogens [1,2]. Numerous studies have since shown the activity, regulation, and distribution of cytochrome P450 aromatase (CYP 19) in the central nervous system of several species of vertebrates, including humans. Aromatase is thought to amplify and diversify the actions of circulating testosterone in androgen target cells of the brain. Based on studies in rats, it was hypothesized originally (i.e.,

Correspondence: Dr. Charles E. Roselli, Department of Physiology & Pharmacology L334, Oregon Health & Science University, 3181 S.W. Sam Jackson Park Road, Portland, OR 97201-3098, rosellic@ohsu.edu.

aromatase hypothesis) that testosterone secreted by the fetal and neonatal testis is aromatized to estradiol in the brain to initiate the process of male-typical brain sexual differentiation. The aromatase hypothesis was later applied to adults when it was discovered that brain aromatization was involved in the activation of male sexual behavior. The aromatization hypothesis has been examined in various neural tissues from several species under both physiological and pathological conditions [3]. It is now evident that the local synthesis of estrogen in the brain is a dynamic and regulated process that varies with age, sex, and physiological status. Moreover, the functional importance of brain aromatase differs among the endpoints examined and the species studied. In our laboratory, we have used the domestic ram to study the role of aromatase for the sexual differentiation of the brain in this long gestation species and, in particular for the development of male-typical sexual partner preferences. This review will present a synopsis of recent research on this interesting and novel animal model.

2. The male-oriented ram model of sexual partner preference

Unlike other mammalian models that are in use currently, variations in sexual attraction occur spontaneously in domestic ram populations [4]. Most domestic rams are sexually attracted to and active with estrous ewes and, thus, can be referred to as female-oriented rams. However, it is estimated that as many as 8% of rams exhibit a sexual partner preference for other males classifying them as male-oriented rams [5,6]. Male-oriented rams can be identified through a combination of performance tests conducted with estrous females and preference tests in which the animals are presented with a choice of either an estrous ewe or unfamiliar male as a sexual partner [6].

Zenchak et al. [4] were the first to systematically study the behavior of male-oriented rams. They observed that the occurrence of this trait was not related to a ram's social dominance. Subsequent studies failed to identify any environmental or social variables of rearing that can alter a ram's preference for an estrous ewe [7,7]. Reports that wild male Bighorn sheep display male-oriented sexual partner preference suggest that this trait may not be the result of selective breeding or husbandry [8]. Most domestic rams that are reared in unisexual group after weaning develop a sexual preference for females by 9 months of age [5]. Although rams that perform sexually with estrous ewes occasionally mount other rams, the male-oriented rams selected for our studies are never observed to mount ewes. Indeed, this is our selection criterion. Likewise, we select female-oriented rams for our studies that sexually interact exclusively with estrous ewes. Once established, these behavioral phenotypes appear to be stable throughout adulthood suggesting that sexual partner preferences are organized during an early period of life, probably during fetal development when sexual differentiation of the brain occurs.

3. The organizational hormone theory of brain sexual differentiation

Male and female mammals have different sets of sex chromosomes, and sexual differentiation results from a series of events that result from the expression of genes on these chromosomes. Expression of the Sry gene on the Y chromosome interacts with the X chromosome genes, Sox9, and autosomal genes to cause the undifferentiated fetal gonad to become a testis instead of an ovary [9]. Hormones secreted by the developing testis direct the differentiation of masculine characteristics while suppressing feminine traits. Thus, the principal pathway by which sexual differentiation proceeds begins with gonadal differentiation, which leads to testicular hormone production, then to genital, neuroanatomical, and behavioral differentiation.

The basic sexual form in mammals is female [10]. This means morphogenic processes are adapted to produce female endpoints in sexual differentiation more easily than they produce male endpoints. This concept is valuable because it implies that if the mechanisms that produce a male trait fail, the female trait emerges instead. The converse is not true: that if a female process is blocked, a male characteristic emerges. Thus, it is now evident that male

differentiation requires two specific and separate processes commonly referred to as masculinization and defeminization. Masculinization imposes male like characteristics on the developing organism, whereas defeminization suppresses female like characteristics that would otherwise arise. These processes are involved whether the endpoints are anatomical (i.e. gonadal differentiation or brain morphology), physiological (i.e. gonadotropin secretion), or behavioral (i.e. copulatory versus receptive behaviors). An important corollary is that it is possible for the same individual to express both masculine and feminine traits.

Although it is apparent that testicular hormones play a dominant and critical role in sexual differentiation recent observations suggest that genes residing on the sex chromosomes, which are asymmetrically inherited between males and females, may influence sexual differentiation directly or interact with sex hormones to determine sexually dimorphic brain structure and function [10,11]. In addition, exogenous hormones, nutrients, environmental endocrine disruptors, and other chemical substances that enter the fetal circulation via the mother can produce permanent changes that alter sexual differentiation of brain structure and function [12].

4. Sexual differentiation of the sheep brain

Sexual differentiation in sheep occurs from approximately days 30 to 100 of the 145 day gestation [13]. During the critical period fetal male lambs experience exposure to significantly higher levels of testosterone, though there is not an apparent difference between the sexes in DHT and androstenedione [14,15]. As in other mammals, testosterone secreted by the fetal lamb testes masculinizes and defeminizes the genitalia and the brain areas controlling gonadotropin release and coital behavior. A fully masculinized adult male shows a tonic pattern of gonadotropin secretion and male-typical copulatory behavior. In a fully defeminized ram, female-typical characteristics are suppressed. This means that female-typical courtship and receptive sexual behaviors and cyclic gonadotropin secretion cannot be elicited after estrogen treatment of the adult. Prenatal exposure of female lamb fetuses to testosterone during the critical period defeminizes GnRH feedback controls by reducing the ewe lamb's postnatal sensitivity to estrogen negative feedback thus advancing the timing of puberty and abolishing the estrogen-induced GnRH surge mechanism necessary for normal cyclic ovarian function in adulthood [13].

5. Sexual partner preference (sexual orientation)

Sexual attraction between opposite-sex individuals is essential to successful reproduction and the propagation of all mammalian species. Sexual partner preferences are highly sexually dimorphic in almost all animals. Males typically prefer female sex partners while females typically prefer male sex partners. Extensive animal studies performed over the past several decades have demonstrated that the fundamental principles of the organizational hormone theory of sexual differentiation apply to the development of sexually dimorphic mate preferences (see [16,17] for review). An animal's sexual partner preference (sexual orientation) can only be judged by administering tests that allow subjects to freely approach and attempt to mate with either a female or a male conspecific. In rats, neonatal treatment of genetic females with testosterone or castration of genetic males will completely and permanently reverse their adult sexual partner preference. In other species such as hamsters and ferrets, both fetal and neonatal exposure to testosterone is required to masculinize/defeminize adult partner preferences. In pigs the organization of the brain mechanisms controlling sexual partner preference occur as late as 3 months postnatally.

For the most part sexual differentiation of partner preferences in existing animal models cannot be dissociated from early hormone effects on the genitalia and copulatory behavior patterns. In contrast, the male-oriented ram is a unique animal model in which the animals' genitalia,

copulatory behavior and neuroendocrine function are masculinized, but not their sexual partner preference. It is well established that coital behavior and gonadotropin secretion in sheep are masculinized by prenatal testosterone exposure [13,18]. Thus, the dissociation among these functions and mate preferences in the male-oriented ram can only be explained by the organizational hypothesis if different molecular mechanisms and/or critical periods are required for the differentiation of sexual partner preferences.

6. Role of androgen and estrogen receptors in the organization of sexual partner preferences

The involvement of estrogen and androgen receptors for the organization of sexual partner preferences differs across species. Aromatization does not appear to be involved in all species and, in particular, does not appear to be obligatory in long gestation animals such as primates. Studies on rats suggest that many, though not all of the organizational actions of testosterone on the differentiation of male-typical sexual partner preference actually result from the neural actions of estradiol formed via local aromatization of testosterone in the developing male brain [19]. Early studies showed that neonatal administration of the aromatase inhibitor, 1,3,6-androstatriene-3,17-dione (ATD), duplicated the long-lasting effects of neonatal castration on the partner preference profile of male rats [20-22]. When tested in adulthood gonadally intact males exposed to ATD neonatally preferred to approach and interact with a stimulus male rather than an estrous female. Additional studies indicated that complete virilization probably requires both prenatal and neonatal exposure to testosterone-derived estrogen [20,22,23], but failed to find a direct prenatal role for androgen [24].

The possible contributions of estrogen and androgen receptor signaling to male-typical partner preferences have been assessed more recently in several genetic mouse models. Male mice in which the estrogen receptor-α gene has been knocked out (ER-α KO) show no preference for an estrous female over a male, whereas wild-type males strongly preferred to approach estrous females [25]. However, it is not clear from this study whether the disruption of estrogen signaling reflected a perinatal (organizational) or adult (activational) action. Study of sexual preferences in the aromatase knock out (ArKO) mouse suggest that prenatal estrogen exposure is needed to masculinize sexual partner preferences since male ArKO mice show no distinct preference for either male or estrous female conspecifics even after adult estrogen replacement [26].

Masculinization of partner preference also appears to require a functional androgen receptor during development since male mice carrying the testicular feminization (Tfm) non-functional mutation of the androgen receptor exhibit female-typical partner and odor preferences [27]. However, this conclusion has been challenged recently by the demonstration that conditionally mutant mice lacking androgen receptor in the nervous system exhibit male-typical olfactory preferences and neuronal activation in response to non-volatile odors derived from soiled bedding [28].

The male-typical preference of male ferrets to seek out an estrous female is organized by the perinatal actions of testosterone over an extended period of time beginning during fetal life and ending ~20 days after birth [29]. Like other aspects of sexual differentiation in ferrets, it believed that estrogen derived from brain aromatization initiates masculinization and that testosterone acting through androgen receptors after birth completes this process [30].

Androgen receptor activation and not aromatization is essential for sexual differentiation of copulatory behavior and gonadotropin secretion in guinea pigs and nonhuman primates [31], but no studies have analyzed the contribution of perinatal steroid hormones to the differentiation of male-typical partner preferences in these long gestation species.

In humans, the main mechanism for sexual differentiation of the brain appears to involve the direct effects of testosterone acting through androgen receptors in the developing brain. The small amount of clinical evidence suggests that estradiol plays little or no perinatal role in male-typical psychosexual differentiation in man. The few men found to have inactivating mutations of estrogen receptor-α [32] or aromatase [33,34] are heterosexual. There is some evidence of increased incidence of bisexual orientation in female offspring of mothers who had been given the synthetic estrogen, diethylstilbestrol (DES), to prevent spontaneous abortions [35]. However, these effects appeared to be minor in comparison to the degree of masculinization of sexual orientation observed in women with the salt wasting form of congenital adrenal hyperplasia that were exposed to excess androgens during fetal development [36].

Aromatization was presumed to be obligatory for defeminization of the sheep brain and behavior because prenatal treatment with testosterone, but not dihydrotestosterone, blocks the development of the LH surge mechanism and decreases the capacity of females to show receptive behaviors [37]. We performed studies in which fetal ram lambs were exposed to the aromatase inhibitor ATD throughout the critical period and found that this treatment does not block the development of female-oriented partner preferences and male-typical copulatory behaviors, nor does it feminize the LH surge mechanism [38]. Rather, testosterone acting on androgen receptors, not its estrogenic metabolite, could be the agent responsible for organizing this behavior in male sheep brain, similar to what has been suggested in humans [17].

7. Neuroanatomical correlates of male-typical sexual partner preference

Sex differences in behavior are thought to derive, in part, from structural or morphological differences (i.e. sexual dimorphisms) in the central nervous system. The first demonstration of a sexual dimorphism in the brain was the discovery by Raisman and Field [39] that the number of synapses in the preoptic area was greater in male rats than in females. Gorski et al. [40] subsequently described an easily studied, sexually dimorphic nucleus (SDN) of the medial preoptic area/anterior hypothalamus (MPOA/AH) that is 3 –4 times larger in volume in male than in female rats. There is also a sexually dimorphic male nucleus of the MPOA/AH in certain strains of mice [41-43], ferrets [44], gerbils [45], guinea pigs [46], sheep [47], macaques [48], and humans [49]. It is generally believed that these are homologous structures across species, but this conclusion is based more on positional similarities than on any critical phenotypic or functional analysis.

Studies in ferrets and rats have linked the male preference to seek out a female as opposed to another male to the function of a male-typical MPOA/AH. Bilateral lesion of the sexually dimorphic MPOA/AH of male ferrets results in a shift in sexual attraction from females to males [50] reflecting an attraction to male body odors that correlates with an increased ability of soiled male bedding to elicit a Fos response in the MPOA [51]. Similar results were observed in rats after bilateral electrolytic lesions [52] were placed in the male sexually dimorphic MPOA/AH. Administering MPOA/AH lesions to either female ferrets [50] or rats [52] had no effect on their male-oriented partner preference. These results suggest that male-typical sexual partner preference depends on an intact sexually dimorphic MPOA/AH. One possibility is that the male-typical MPOA/AH suppresses female-typical responses to male body odors or other sensory input. With destruction of the MPOA/AH female-typical attractions are expressed.

Early support for the idea that male-typical neuroanatomical features (e.g., larger volume of the nucleus) of the sexually dimorphic MPOA/AH contributed to male-typical sexual preferences was the report by Houstmuller et al. [53] who found a positive association between the size of the SDN-POA in male rats and their preference for female versus males conspecifics when they were given tests of sexual partner preference. These animals were, however, perinatally treated with ATD in order to alter their adult behavior.

Our studies in sheep produced the first evidence in an unmanipulated animal model showing a strong association between the size of the male-typical MPOA/AH and a preference for female versus male sexual partners. We identified a group of sexually dimorphic aromatase-expressing neurons that occupy the central component of the MPOA/AH of the sheep brain and called it the ovine sexually dimorphic nucleus (oSDN) [47]. We found that the volume of the oSDN is 2- to 3-fold greater in female-oriented rams than in male-oriented rams, and similar in size in male-oriented rams and ewes. Thus, the preference of individual rams for male versus female sexual partners correlates directly with the volume of the oSDN suggesting that the size of oSDN and number of neurons it contains contribute to sexual attraction in rams. Taken together, with the functional studies in ferrets and rats, the correlation between oSDN volume and sexual preference suggests that the male-typical preference for an estrous female depends on sexually differentiated characteristics of intact oSDN neurons, perhaps related to hormone sensitivity or connectivity, that appear to be essential for sexually dimorphic processing of sensory cues

Several structural differences in the brain have been described in relation to sexual orientation in humans [54]. However, only the observation by LeVay [55], that a region of the sexually dimorphic MPOA/AH called the third interstitial nucleus of the anterior hypothalamus (INAH-3), which is significantly larger in heterosexual men than in homosexual men and heterosexual women, seems to be anatomically related to mate preferences. Unfortunately, this observation has never been fully replicated although Byne [56] did show a trend for INAH-3 volume to be greater in heterosexual as opposed to homosexual men.

8. The oSDN is organized prenatally by testosterone

The discovery that male-oriented rams have a smaller (female-typical) oSDN than female-oriented rams raises the question of whether the number of neurons that comprise this nucleus and their connections determine sexual preferences or whether the preference behavior somehow influences the size of the nucleus. This is a difficult question to answer and can only be approached indirectly at this time. Our data suggest that the differences found in oSDN size among ewes, male-oriented rams and female-oriented rams are not the result of variations in adult levels of serum testosterone, because the volume differences are apparent even in gonadectomized animals that were given equivalent doses of testosterone [57]. Thus, it is plausible to suggest that the differences in the size of oSDN between male-oriented rams and female-oriented rams results from the organizational effects of prenatal testosterone.

The size of the sexually dimorphic nucleus of the preoptic area (SDN-POA) in rats described originally by Gorski [40] is perhaps the best known example of organizational action exerted by testosterone during perinatal development. After combined pre- and post-natal treatment with testosterone proprionate the SDN-POA of female rats becomes enlarged and equivalent in size to males rats. Perinatal treatment of females with the estrogen diethylstilbestrol also masculinizes the adult SDN-POA [58]. These results, among many more, demonstrate that gross morphological differences in SDN-POA are completely controlled by the hormonal environment conforming to the aromatization hypothesis during the critical period for sexual differentiation.

We are not able to study the development of the oSDN in relation to same-sex partner preference because we cannot predict which male lamb fetuses will as adults express male versus female sexual partner preferences. However, we can evaluate oSDN volume in relation to sex and prenatal hormone exposure as a test of whether oSDN volume is organized prior to birth. Because sexual differentiation occurs during midgestation in sheep, we hypothesized that the oSDN develops before birth and is organized by exposure to testosterone. In support of this hypothesis, we found a cluster of aromatase mRNA-expressing cells in the caudal

preoptic area of late gestation fetal lambs [59]. The volume of this nucleus was ~2-fold greater in males than in females, suggesting that it is the fetal equivalent to the adult oSDN. We provided additional evidence that the oSDN is organized prenatally by demonstrating that testosterone exposure *in utero* promoted the growth of the oSDN to a size in females that resembled males. These results demonstrate that T exposure during the midgestation critical period is sufficient to morphologically masculinize the oSDN.

Despite the abundant aromatase mRNA expression within the oSDN, preliminary studies found that oSDN size was not altered in rams treated prenatally with the aromatase inhibitor ATD (our unpublished observations). These results indicate that aromatization is unlikely to account for brain masculinization of the oSDN and are consistent with observation that prenatal ATD exposure does not affect adult sexual partner preferences. Instead, sexual differentiation of the oSDN and partner preferences in sheep are most likely controlled through androgen receptor mechanisms. Further studies are needed to test this hypothesis by determining whether pharmacological treatments which block androgen receptors during fetal development can disrupt male-typical sexual differentiation in rams.

9. Conclusions

The domestic ram is a unique animal model that exhibits exclusive same-sex sexual partner preferences. Male-oriented rams actively court other rams using male-typical sexual behaviors, while completely ignoring estrous ewes. Yet, with respect to their responses to gonadal hormones and capacity to exhibit sex-typical consummatory behaviors, male-oriented rams show male-typical mounting, not estrogen-induced receptivity and LH surge secretion. These observations can be interpreted to suggest that male-oriented rams, like female-oriented rams, are masculinized and defeminized with respect to mounting, receptivity, and gonadotropin secretion, but are not defeminized for sexual partner preferences. This is one of few examples, other than humans and nonhuman primates [60], where sexual behaviors and sexual partner preferences are dissociated suggesting that these behaviors may be programmed differently. Together with their female-typical mate preference, male-oriented rams have a small, i.e., female-typical, oSDN. This observation reinforces the notion that there are aspects of brain structure and function which are also not completely defeminized in male-oriented rams. Although the exact function of the oSDN is not yet known, it has been implicated in sexual preference [61] and, as such, a dimorphism in its volume and number of cells could bias the processing of sexually relevant sensory cues involved in sexual partner choice. Finally, our research suggests that the oSDN develops prenatally and is controlled by testosterone. Despite the abundant expression of aromatase mRNA in the oSDN, we have no evidence that aromatization plays an obligate role in male-typical development of oSDN or adult sexual partner preference. In this way, sheep appear similar to other long gestation mammals and may rely more on androgen receptor mediated mechanisms for sexual differentiation of reproductive behaviors. Nonetheless, this still leaves unanswered the question of what aromatase is doing in the oSDN. Thus, more research is needed to understand the requirements and timing of its development and ultimately whether and how prenatal hormones effect the expression of sexual partner preferences in adults.

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