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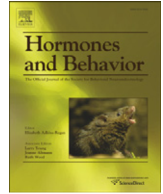

Evidence that sex chromosome genes affect sexual differentiation of female sexual behavior

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ABSTRACT

Female receptivity including the immobile hormone-dependent lordosis posture is essential for successful reproduction in rodents. It is well documented that lordosis is organized during the perinatal period when the actions of androgens decrease the males' ability to display this behavior in adulthood. Conversely the absence of androgens, and the presence of low levels of prepubertal estrogens, preserve circuitry that regulates this behavior in females. The current study set out to determine whether sex chromosomal genes are involved in the differentiation of this behavior. An agonadal mouse model was used to test this hypothesis. The SF-1 gene (*Nr5a1*) is required for development of gonads and adrenal glands, and knockout mice are consequently not exposed to endogenous gonadal steroids. Thus contributions of sex chromosome genes can be disassociated from the actions of estrogens. Use of this model reveals a direct genetic contribution from sex chromosomes in the display of lordosis and other female-typical sexual behavior patterns. It is likely that the concentrations of gonadal steroids present during normal male development modify the actions of sex chromosome genes on the potential to display female sexual behavior.
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Introduction

Female sexual behaviors facilitate and enable copulation and therefore ensure successful mating and consequently reproductive success. One of the best documented indicators of receptivity in rodents is lordosis, an arched back immobile reflex posture exhibited when males attempt to mount and mate. This posture has been used to map the reflexive circuits in the central nervous system that are regulated by the steroid hormones required for its expression (Kow and Pfaff, 1977; Pfaff and Sakuma, 1979). The lordosis reflex is sexually differentiated and is displayed to a much greater extent by females as the male brain is normally defeminized (Blaustein and Erskine, 2002). The sex differences in circulating androgens during the perinatal period mold brain sex differences via a number of factors, including steroidogenic enzymes, receptors and neurotransmitters including but not limited to: testosterone, aromatase enzyme, estradiol, estrogen receptors, and glutamate receptors (Kudwa et al., 2006; Schwarz et al., 2008). The ability of neonatal testosterone to block adult expression of these “female-typical” behavior patterns in rodents is one of the cornerstone arguments for the primary role that gonadal steroids play in brain sexual differentiation (Phoenix et al., 1959).

In addition to the organizing roles that steroid hormones play, sex differences can also be influenced in the brain by genes on the sex chromosomes that are expressed at different levels in male and female brains. For example, genes on the Y-chromosome that are not represented on X-chromosome, and/or X-chromosome genes that escape X-inactivation might favor differentiation in male or female directions (De Vries et al., 2002). There are several mouse models currently available that can be used to test this hypothesis (Majdic and Tobet, 2011).

Steroidogenic factor 1 (SF-1) is a transcription factor that regulates the expression of many genes involved in development and function of the reproductive axis (Parker and Schimmer, 1997). Mice lacking the *Nr5a1* gene (SF-1 KO) are born without gonads and adrenal glands and are therefore not exposed to endogenous sex steroid hormones (Ingraham et al., 1994). As such, sex differences between chromosomal males (XY) and females (XX) may be caused by genes located on sex chromosomes in contrast to differences in gonadal steroid hormones during development (Budefeld et al., 2008). In addition to gonadal and adrenal agenesis, these mice have a disorganized ventromedial hypothalamus (VMH) and the male knockouts have feminine external genitalia (Dellovade et al., 2000; Parker and Schimmer, 1997). The SF-1 KO model has provided evidence that developmental exposure to gonadal steroids is not required for adult display of aggression (Grgurevic et al., 2008) while

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brain specific female knockout mice have reduced levels of lordosis (Kim et al., 2010).

The aim of the present study was therefore to examine female-typical sexual behaviors and immunoreactive progesterone receptors in SF-1 KO mice to determine a contribution from sex chromosomal genes to the development of female biased brain circuitry and behavior. It is well established that progesterone, acting through the estrogen-induced receptors in the VMH area are an important component of the functional circuitry needed for the appropriate display of female sex behavior (Rubin and Barfield, 1983). In the current study, immunoreactive progesterone receptors were examined to assess the functional capacity of this component of the behavioral circuitry.

Material and methods

Animals

Mice with the disrupted SF-1 allele were backcrossed for more than 10 generations to C57BL/6J mice to produce a congenic line. All mice were housed under standard laboratory conditions at the University of Ljubljana Veterinary Faculty in a 12:12 light/dark cycle (lights off at 1300 h) with phytoestrogen free food (Harlan mouse chow 2916, Harlan Teklad, Milano, Italy) and water ad libitum. All animal experiments were done according to ethical principles and in accordance with EU directive (86/609/EEC). Animal experiments were approved by the Veterinary commission of Slovenia and the Animal Care and Use Committee at Colorado State University.

SF-1 heterozygous mice were mated to produce homozygous SF-1 KO and wild type (WT) offspring. To ensure survival of SF-1 KO mice, all newborn pups were injected daily for 6–7 days with 50 μl of a corticosteroid cocktail in corn oil (s.c. 200 μg/ml hydrocortisone, 400 ng/ml dexamethasone, and 500 ng/ml fludrocortisone acetate; all from Sigma, Steinheim, Germany). Mice were genotyped by PCR assay of tail DNA on day 6 or 7 after birth as previously described (Ingraham et al., 1994). Female WT littermates or female pups from other C57BL/6J litters born within 3 days were used as a source of adrenal transplants; these techniques have been published (Majdic et al., 2002). After weaning, mice were initially group housed and then separated at 30 days of age and remained isolated until behavioral tests. C57BL/6J mice were used for controls, which were gonadectomized at 21–25 days of age and housed under the same conditions as SF-1 KO animals. We tested mice in each of these four groups; SF-1 KOF (n = 7), SF-1 WTF (n = 7), SF-1 KOM (n = 6), and SF-1 WTM (n = 7). Behavioral testing started at the age of 65 days, and mice were sacrificed by perfusion fixation with 4% paraformaldehyde 7 days after the last test, around 90 days of age.

Behavioral testing

All behavioral tests were conducted by NG who was blinded to genotype and chromosomal sex of the experimental mice at the time of the testing. For stimulus animals, sexually experienced C57BL/6J males were used. All subject mice received estradiol benzoate (EB; 0.5 μg/0.15 ml s.c.) 48 h prior to test followed by progesterone (P; 1.6 mg/0.2 ml s.c.) approximately 4–9 h prior to testing. Tests were started at least 1 h after the dark portion of the light/dark cycle under the red light illumination.

Testing was performed in a clear glass aquarium (26 × 42 cm) without bedding, food and water. A mirror was placed under the aquarium to facilitate counting intromissions. Tested animals were placed in the aquaria for at least 1 h to habituate before male partners were added. Behavior was evaluated during 20 min tests or until ejaculation by a stimulus male. If no receptivity was observed, the test ended after 15 mount attempts. If the stimulus males did not try to mount the subject, they were replaced with different stimulus males after 5 min of testing. Each mouse was tested 7 successive times with at least 3 days and no more than 6 days between tests to mimic the natural female cycle.

During testing, the numbers of attempted and successful mounts, and the numbers of times the subject stood during these mounts were scored. Mounts were counted when the tested mice had all four limbs on the floor. Lordosis was scored from 1 to 5 regarding to dismounts, movement and vocalization of the tested animal (Dominguez-Salazar et al., 2004). These data were used to calculate lordosis quotients (LQ) by the following formula: number of times the female stood for mounts / total number of mounts and mount attempts × 100. In addition, number of intromissions, number of thrusts, latency to intromit and number of ejaculations were scored.

Immunocytochemistry on floating sections

Brains were embedded in 5% agarose (Sigma) and sectioned at 50 μm in cold 0.05 M PBS using a vibrating microtome (Integraslice 7550 MM, Campden Instruments, UK). Sections were incubated in 0.1 M glycine (Sigma) in 0.05 M PBS for 30 min followed by incubation in 0.5% sodium borohydride (Sigma) for 15 min at 4 °C. Glycine and sodium borohydride were washed out with 15 min and 20 min washes in 0.05 M PBS. Sections were blocked in 5% normal goat serum (Chemicon, Temecula, CA, USA) containing 0.5% Triton X-100 (Sigma) and 1% H_2O_2 (Merck, Darmstadt, Germany) for 30 min at 4 °C. Rabbit primary antibodies against progesterone receptor (PR, 1:1000, Dako, Glostrup, Denmark) were diluted in 0.05 M PBS containing 1% bovine serum albumin (Sigma) and 0.5% Triton X-100. Sections were incubated with primary antibodies over 3 nights at 4 °C with shaking. Sections were then washed in 0.05 M PBS containing 1% normal goat serum and 0.02% Triton X-100 four times for 15 min at room temperature. Biotinylated secondary antibodies (Jackson Immunoresearch, West Grove, PA, USA) against primary rabbit antibodies were diluted 1:500 in 0.05 M PBS containing 1% normal goat serum and 0.5% Triton X-100. Sections were incubated with secondary antibodies for 2 h, followed by 4 washes (15 min each) in 0.05 M PBS buffer containing 0.02% Triton X-100. Streptavidin–HRP complex (Jackson Immunoresearch) was diluted 1:2000 in 0.05 M PBS solution containing 0.5% TritonX-100. Sections were incubated with Streptavidin–HRP for 1 h at room temperature and then washed in Tris-buffered saline (0.05 M Tris–HCl/0.9% NaCl; pH 7.5; Sigma) for 1 h at room temperature. Antigen–antibody complexes were visualized as a black reaction product by incubating sections in 0.025% 3′3′-diaminobenzidine/ammonium nickel(II) sulfate substrate (Sigma) in Tris-buffered saline (pH 7.5) containing 0.02% H_2O_2 for 5 min at room temperature. After mounting, sections were dried and coverslipped using hydrophobic medium (Pertex, Burgdorf, Germany). Immunocytochemical controls included omission of the primary antiserum and validation of immunoreactivity with patterns of distribution from prior publications.

Data collection and analyses

Digital images of ventromedial region of hypothalamus were obtained using a Nikon Eclipse 80i microscope with Nikon DS-Fi1 camera. Images were enhanced for contrast using an Adobe Photoshop software package (Version 8.0). The number of neurons that were immunoreactive for progesterone receptor (PR) was analyzed in two coronal sections containing the ventrolateral part of the VMH 1.55 mm and 1.7 mm caudal from bregma according to stereotaxic coordinates (Paxinos and Franklin, 2001) in WT gonadectomized mice and the corresponding ventromedial hypothalamic region in SF-1 KO mice. Digital images were taken under 100 × magnification and the third ventricle and base of the brain were considered as reference boundaries. Due to the possibility of asymmetry in antigen detection between the left and right sides of the brain, the side with more immunoreactive cells or fibers was always chosen for analysis. As

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