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Research report

The effects of partial and complete masculinization on the sexual differentiation of nuclei that control lordotic behavior in the male rat

Santiago Segovia, Alicia Garcia-Falgueras, Carmen Perez-Laso, Helena Pinos, Beatriz Carrillo, Paloma Collado, Francisco Claro, Antonio Guillamon*

Departamento de Psicobiología, UNED, c/Juan del Rosal, 10, 28040 Madrid, Spain

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ABSTRACT

Male rats, under certain experimental conditions, may show lordosis, the typical expression of female sexual receptivity. This work studies the sexual morphological pattern of facilitatory and inhibitory structures that control lordosis. Three groups of males were neonatally subjected to a gradient of androgen exposure (castrated plus injected oil (GxM + oil); castrated plus androstenedione treated (GxM + AND); and sham operated [CM]); a group of control females (CF) was also added. Lordotic response after these different hormonal and neonatal surgical treatments, as well as the volume or number of neurons in facilitatory (ventromedial nucleus of the hypothalamus [VMN]) and inhibitory (the intermediate region of the lateral septum [LSi] and accessory olfactory bulb [AOB]) nuclei involved in lordosis was studied in adults. The inhibition of lordosis in the males seems to be associated to the neonatal presence of testosterone and the consequent masculinization of the VMN, VMNvl, LSi and AOB. It is suggested that one of the functions of the sex differences consistently seen in these structures might be to inhibit the lordosis response in the male.

1. Introduction

The lordosis response, the typical expression of female receptivity, is a complex phenomenon regulated by excitatory and inhibitory neural systems in the brain [28]. Within the facilitatory system, the ventromedial nucleus of the hypothalamus (VMN) plays an important role. Lesion of this nucleus inhibits lordosis in female rats primed with estrogens [27] while electrical stimulation facilitates it [26].

However, the septum (S) is reported to belong to the inhibitory network of the lordotic response in female rats. Septal lesions lowered the estrogen threshold for the induction of lordotic behavior in these rats [23]. The same effect was found with a bilateral ventrolateral cut in the ventrolateral septum [48], while electrical stimulation of this structure shortened the duration of lordosis in female hamsters [49]. The role of the Lateral septum (LS) in lordosis seems to be estrogen-dependent, since implants of dihydrotestosterone propionate in this structure inhibits lordosis in females [42], while estrogen implants releases the inhibition of lordotic behavior [32].

These structures, which facilitate or inhibit female lordosis, show sex differences. Females show a smaller VMN volume than male rats [22]. These results have been confirmed by Madeira et al. [21] for the volume of the whole VMN and extended for the volumes of the anterior, dorsomedial, central and ventrolateral subdivisions of the VMN. However, these authors did not find sex differences in the number of neurons in the whole VMN, or in any of its subdivisions [21]. Moreover, sex differences were found in neurons of the intermediate region of the lateral septum (LSi), neurons which project axons to midbrain central gray matter. In this subdivision, the right LSi has more fluoro-gold (FG) immunoreactive cells in female than in male rats [45].

These facts indicate that the structures that facilitate or inhibit lordosis in the female present the following morphological (volume and/or number of neurons) patterns of sexual differentiation: female < male for the facilitatory VMN, and female > male in the inhibitory LSi.

Although lordosis is not the males typical sexual behavior, it is very well known that they can express it under adequate hormonal

* Corresponding author. Tel.: +34 91 398 6272; fax: +34 91 398 6287.
E-mail address: aguillamon@psi.uned.es (A. Guillamon).

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conditions (castration plus estradiol and progesterone treatment) and that some males can show lordosis in response to manual stimulation [38]. The fact that males can show lordosis means that this sex has the necessary neural networks to show this behavior. In the female, the expression of lordosis is controlled by facilitatory or inhibitory structures and there are data in the literature signaling that those structures, mentioned above, have a similar facilitatory or inhibitory role in the male.

It has been reported that lesions in the VMN inhibit lordosis in the male rat [5]. However, lesions and cuts in the LS facilitate the lordosis response in adult castrated male rats [20,44]. In addition, dorsal differentiation of the preoptic area that may cause a transection of the ventral output from the LS facilitates lordosis in males [47] and, in light of this, it was suggested that a direct connection from the intermediate part of the LSi to the periaqueductal gray matter would have an inhibitory function in regulating lordosis in male rats [44]. In addition, a vomeronasal olfactory structure, the accessory olfactory bulb (AOB), has also been described to play an inhibitory role of lordosis in the male. Schaeffer et al. [33] reported that AOB ablation in estrogen–progesterone treated males increased the number of these rats responding with lordosis to male mounts as compared to controls and sham-treated males.

These structures that facilitate (VMN) or inhibit (LSi and AOB) lordosis in male rats undergo sexual differentiation during the perinatal period [2,21,22,31,34,35,43]. These sex differences suggest that the inhibition of lordosis exhibited by the male in normal hormonal conditions might be associated to the masculinization of the neural networks involved in the control of lordosis in this sex.

This study was designed to elucidate if the masculinization of these facilitatory or inhibitory structures (AOB, VMN and LSi) underlies the inhibition of lordosis in the male. For that purpose we studied lordosis in both male and female rats as well as the existence of sex differences in the volume or the number of neurons of these structures. A gradient of androgen exposure for the males during the early postnatal period was applied: absence of testosterone (castrated males), partial neonatal androgenization (castrated males treated with androstenedione (AND), an androgen with weak biopotency [30]), and control (sham operated) males (full presence of testosterone). There was also a female control group.

2. Materials and methods

2.1. Subjects

18 female Wistar rats were maintained in an automatically controlled room programmed at a temperature of 22 ± 2 °C under a 12 h light/12 h dark cycle (lights on at 08:00 h), with food (Purina chow) and water *ad libitum*. Throughout the experimental study, animal care and handling were approved by Local Committees and were in accordance with the European Community Council Directive, 1986 (86/609/EEC). For mating, a male was placed in a cage with two females. Sperm-positive females were placed individually in plastic maternity cages with wood shavings as nesting material. On the day of birth (D1), pups were randomly assigned to a group according to sex and experimental treatment: control male (CM) ($n = 11$), control female (CF) ($n = 8$), castrated male treated with oil (GxM + oil) ($n = 12$) and castrated male treated with AND (GxM + AND) ($n = 12$).

2.2. Experimental procedures

On the day of birth, and under hypothermia, neonatal gonadectomy was performed on the animals of the GxM + oil and GxM + AND groups. The subjects in the control groups were sham operated the same day. Control animals were not given injections. GxM + oil groups received injections of 0.05 ml sesame oil and the GxM + AND group received injections of 0.025 mg of androstenedione (Sigma, Madrid, Spain) dissolved in 0.05 ml of sesame oil. The treated subjects received injections every other day, for a total of 10 injections until the 19th day of life after birth [11]. Living conditions were the same for all study groups.

Control females (CF) were ovariectomized at 65 days of age. At the age of 90 days, subjects were submitted to a blinded lordosis behavioral test in which the experimenter was ignorant of the experimental group to which the animals belonged. The lordosis test was performed as follows. All subjects in the four experimental groups were primed with estradiol benzoate (Sigma, Madrid, Spain) (30 μg/animal, 48 h prior to test) and progesterone (Sigma, Madrid, Spain) (1 mg/animal, 8 h before test). Each subject was tested in two consecutives sessions separated by a minimum of 1 week. In each session, a stud male was placed in a plexiglass cage (20 cm × 40 cm × 60 cm) for 3 min before adding the test subject to the cage, at which time the session was considered to start. Each testing session was stopped after 10 male mounts, changing the stud male if necessary (e.g., if he did not mount enough).

2.3. Measures of sexual behavior

Standard measures of female sexual behavior were recorded using the SBR software [3]. To calculate a measure of receptivity we used the Lordosis Quotient (LQ) defined as: LQ = (L + HL × 0.5)/NM; L: number of lordosis responses; HL: number of half-lordosis (defined as a not full lordosis); NM: number of mounts. In order to do the statistical analysis we calculated the arithmetical mean of the LQ for both sessions: LQ = (LQ1 + LQ2)/2.

2.4. Morphometrical study

A randomly selected sample was taken from a pool of the behavioral subjects. Rats were sacrificed using an anesthesia overdose and perfusion. The subjects were anesthetized by an i.p. injection of tribromoethanol (250 mg/kg), followed by transcardiac perfusion of a saline solution (0.9%) and then 4% paraformaldehyde (PAF) in phosphate buffer (PB, 0.1 M, pH 7.4).

The brains were removed and stored in a freshly prepared PAF solution for two hours at 4 °C, followed by several washings in PB. Finally, they were stored in a 30% sucrose solution in PB at 4 °C. The brains were then frozen in dry ice and serially sectioned in the coronal plane at thicknesses of 40 μm (AOB) and 50 μm (LS and VMN). Sections were directly collected on glass slides and stained with cresyl violet (Fluka, Barcelona, Spain, 0.1% solution, pH 4).

VMN volume was estimated using a Diaplan Leitz microscope equipped with a motor-driven stage controlled by a computer software system (Multicontrol 2000, Märzhäuser Wetzlar, Germany) and provided with a special rotating device to rotate the slides 360° independently of the x–y axis movements. The stereological software package (GRID, Interactivision, Denmark) used the classic Cavalieri method [15], generating a set of points systematically placed over each section. References to locate the VMN and the other nuclei were obtained from the atlas of Paxinos and Watson [24].

VMN was divided into four subdivisions: anterior (VMNa), dorsomedial (VMNdm), central (VMNc) and ventrolateral (VMNvl). All section in the VMNa and every third section in the VMNdm, VMNc, and VMNvl subdivisions were counted. The numbers of points coinciding with the surfaces of VMNa, VMNdm, VMNc, and VMNvl were counted at a magnification of ×473. Total volume was estimated by multiplying the number of points by the area associated with each point (VMNa: a(p): (3643 μm^2); VMNdm, VMNc and VMNvl a(p): (13.825 μm^2)) and by the average distance between the two sections examined (obtained by multiplying with the section thickness (50 μm) for the sampling interval). The total volume of the VMN was obtained as the sum of the volumes of its subdivisions.

To obtain a direct unbiased estimation of the total number of neurons in the AOB, LS and VNM (divided into its four subdivisions), we used the optical fractionator, which combines the optical disector and fractionator techniques [13,14,39]. The sections were cut with a cryostat to a thickness of 40 μm for the AOB and 50 μm for the LS and VNM, although their actual width after using the microcator varied between 20 and 25 μm. The optical disector was used as follows: at ×186 magnification, frames (disectors) were generated over the area with horizontal (h) and vertical (v) steps (AOB mitral neurons: h: 120 μm, v: 80 μm; granular neurons: h: 150 μm, v: 120 μm; LS neurons: h: 200 μm, v: 160 μm.; VMN neurons: horizontal step of 90 μm and a vertical step of 90 μm for the VMNa and a horizontal step of 150 μm and a vertical step of 150 μm for the VMNdm, VMNc and VMNvl subdivisions). All of the frames that included the nuclear surface were considered. The profiles (cell nuclei) that were completely enclosed in the test frame ($A(f)$ = 1040 μm^2 for mitral AOB cells, $A(f)$ = 289 μm^2 for AOB granule cells, $A(f)$ = 1040 μm^2 for LS and $A(f)$ = 842 μm^2 for VMN cells) and those intersected by the inclusion edges at ×4323 magnification were considered. The height of the disector was 12 μm except for granule cells for which it was 10 μm. Finally, the total number of neurons was obtained by applying the fractionator formula:

$$\sum Q = \frac{1}{ssf} \times \frac{1}{asf} \times \frac{1}{hsf}$$

In this formula, $\sum Q$ is the total number of cell nuclei counted; *ssf* is the section sampling fraction; *asf* is the area sampling fraction and *hsf* is the height sampling fraction. Neurons were differentiated from glial cells by their large unstained nucleus with a prominent nucleolus, in contrast to the relatively smaller intensely stained nucleus of glial cells. The counting unit was the nucleolus. The total number of LS and VMN neurons was computed as the sum of the total estimated in each subdivision. To differentiate the light and the dark granule cells in the AOB we followed Struble and Walters criteria [41].