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

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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.

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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 release 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
2.2. Experimental procedures

The experiment 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 consecutive sessions separated by a minimum of 1 week. In each session, a stud male was placed in a Plexiglas cage (20 cm × 40 cm × 60 cm) for 3 min before adding the test subject to the cage, at which time mating behavior was considered to be initiated. 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 SRR software [3]. To calculate a measure of receptivity we used the Lordosis Quotient (LQ) defined as: LQ = (1+HL × 0.5)/NM; N: number of lordosis responses; HL: number of half-lordosis (defined as a full not lordosis); NM: number of mounts. In order to do this, the standardised analytical measure 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. Ss were sacrificed using an anesthesia overdose and perfusion. The subjects were anesthetized by i.p. injection of tribromoethanol (250 mg/kg), followed by transcerebral perfusion of a saline solution (0.9%) and then 4% paraformaldehyde (PAF) in phosphate buffer (pH 7.4).

The brains were removed and stored in a freshly prepared PAF solution for four 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 sectioned using a Vibratome (RJ Joyce Loops, Bar, UK). Sections were collected on a glass slide and stained with cresyl violet (Fluka, Barcelona, Spain; 0.1% solution, pH 4).

VMN volume was estimated using a Digital Leitz microscope equipped with a motor-driven stage controlled by a computer software system (Multicontrol 2000, Mühlhäuser 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 (VMNV). All section in the VMNA and every third section in the VMNDM, VMNC, and VMNV subdivisions were counted. The numbers of points coinciding with the surfaces of VMNA, VMNDM, VMNC, and VMNV 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 μm²); VMNDM, VMNC, and VMNV a(p): (13.825 μm²)) 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 LS and VMN (divided into its four subdivisions), we used the optical fractionator, which combines the optical dissector and fractionator techniques [13,34,39].

The sections were cut with a cryostat to a thickness of 40 μm for the AOB and 50 μm for the LS and VMN, although their actual width after using the microtome varied between 20 and 25 μm. The optical dissector was used as follows: at ×186 magnification, frames (dissectors) were generated over the area with horizontal (h) and vertical (v) steps (AOB: 100 μm × 100 μm; LS: 50 μm × 50 μm; VMN: 25 μm × 25 μm). All of the frames that included the nuclear surface were considered. The profiles (cell nuclei) that were completely enclosed in the test frame (A(T)/f = 1040 μm² for mitral AOB cells, A(T)/f = 289 μm² for AOB granule cells) were counted for LS and VMN (A(T)/f = 842 μm² for VMN cells) and those intersected by the inclusion edges at ≥4323 magnification were considered. The height of the dissector 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:

\[ \sum Q = \frac{1}{s} + \frac{1}{a} + \frac{1}{h} \]

In this formula, \( \sum Q \) is the total number of cell nuclei counted; \( s/2 \) is the section sampling fraction; \( a/2 \) is the area sampling fraction and \( h/2 \) is the height sampling fraction.

Neurons were differentiated from glial cells by their large unstained nucleus and lack of neurofilaments. Neurons were differentiated from glial cells by their large unstained nucleus, and the fraction of glial cells was calculated. The total number of glial per slide was determined by applying the method of grid points with a dissector of 400 μm × 400 μm (AOB) and 500 μm × 500 μm (LS and VMN). The fractions of glial cells were calculated for AOB, LS and VMN.
with respect to CF; \( p < 0.05 \) with respect to CM; \( p < 0.05 \) with respect to GxM + oil; CM vs. GxM + AND, \( p < 0.05 \). As seen in Fig. 3A CM have a larger total VMN volume than CF (\( p < 0.009 \)), GxM + oil (\( p < 0.014 \)) and GxM + AND (\( p < 0.014 \)). The CF group does not differ from GxM + oil and GxM + AND.

Moreover, no differences were found between the GxM + oil and GxM + AND groups.

With respect to the VMNvl (Fig. 3B), CM have a larger volume than CF (\( p < 0.009 \)), GxM + oil (\( p < 0.014 \)) and GxM + AND (\( p < 0.014 \)). The CF group did not differ from the GxM + oil group but did differ from the GxM + AND group (\( p < 0.05 \)). Moreover, no differences were found between the GxM + oil and GxM + AND groups.

### 3. Results

#### 3.1. Study of the lordosis response

ANOVA analysis of the means for lordosis quotient (LQ) showed a significant effect of group (\( F_{3,40} = 29.965, p < 0.001 \)). Post hoc analyses performed with the Student–Newman–Keuls test showed statistically significant differences between females and all the male groups (\( p < 0.05 \)); females had a higher lordosis response. With respect to males, CM had the lowest lordosis response and there were significant differences between all the male groups: CM vs. GxM + oil, \( p < 0.05 \); CM vs. GxM + AND, \( p < 0.05 \); GxM + oil vs. GxM + AND, \( p < 0.05 \) (Fig. 1).

#### 3.2. Morphological study

##### 3.2.1. Ventromedial hypothalamus

No sex differences were found in the number of neurons in the whole VMN or in its anterior, central, dorsomedial and ventrolateral subdivisions (Fig. 2 illustrates the VMN subdivisions). However, sex differences were found with respect to volume measurements. Results are given with respect the total volume of the VMN and the VMNvl subdivisions, of which the latter is related to lordosis behavior (Pfaff, 1980). The volumes of the total VMN and the VMNvl subdivisions present statistically significant differences (VMN total: \( H(3) = 12.174, p < 0.007 \), VMNvl \( H(3) = 12.932, p < 0.005 \)). As seen in Fig. 3A CM have a larger total VMN volume than CF (\( p < 0.009 \)), GxM + oil (\( p < 0.014 \)) and GxM + AND (\( p < 0.014 \)). The CF group does not differ from GxM + oil and GxM + AND.

Moreover, no differences were found between the GxM + oil and GxM + AND groups.

#### 3.2.2. Lateral septum

With respect to the number of neurons no differences were found in the dorsal or the ventral parts of the LS. However, the intermediate part of this structure did show statistically significant differences between groups (\( H(3) = 9.451, p < 0.037 \)). As can be seen in Fig. 4, CF had more neurons than CM (\( p < 0.014 \)) or GxM + AND (\( p < 0.028 \)). Moreover, GxM + oil also had more LSi neurons than CM (\( p < 0.049 \)) but did not differ significantly from the GxM + AND which, in turn, did not differ from the CM. Fig. 5 illustrates sex differences in the LSi.

#### 3.2.3. Accessory olfactory bulb

The Kruskal–Wallis analyses showed statistically significant differences between groups with respect to the number of neurons in the AOB (\( H(3) = 11.789, p = 0.008 \)). As shown in Fig. 6, CM had a higher number of mitral, light and dark granule cells than CF (\( p < 0.006 \), in all cases); GxM + oil had a lower number of mitral, light and dark granule cells than CM (\( p < 0.009 \), in all cases) and did not differ from CF (\( p > 0.05 \), in all cases). GxM + AND did not differ from the GxM + oil or the CF. However, these other male groups did have less mitral, light and dark granule cells than the CM (\( p < 0.004 \), in all cases).

### 4. Discussion

The aim of the work was to elucidate the role of masculinization in the inhibition of lordosis in male rats. For that purpose three groups of males were neonatally subjected to a gradient of androgen exposure. Their lordotic response was studied as well as the volume and/or number of neurons in facilitatory (VMN)
and inhibitory (LSi and AOB) structures that control this behavior. Although there are many previous data in the literature about the hormonal and the neural control of lordosis behavior in male and female rats, to our knowledge, this is the first study in which the expression of lordosis and sexual differentiation of structures that control this behavior have been taken together.

With respect to lordosis, our results show that the inhibition of the male lordotic response depended on neonatal exposure to androgens, a well known fact in the literature (see [1] for review). Neonatally castrated and oil injected males showed a higher lordosis quotient than the neonatally castrated males treated with androstenedione and the control males. However, neonatally castrated and oil injected males showed significantly less lordosis than the control females. It is well known that the first step in the inhibition of lordosis in the male occurs prenatally since prenatal administration of antiandrogens increases the expression of lordosis in males [9,10]. These reports could explain why the expression of lordosis by the neonatally castrated and oil injected males was significantly lower than the lordosis shown by the control females. Androstenedione, which is also secreted by the testes, is considered an androgen of weak biological activity [30] (that is why it was used in our study); however, lordosis was markedly inhibited but still present in these animals. Males neonatally treated with androstenedione present significantly less lordosis than control females and neonatally castrated males treated with oil; nevertheless, they retain their capacity to show lordosis since they differ significantly from the control males. There are reports in the literature stating that the administration of androstenedione in neonatally castrated males causes an incomplete masculinization since the subjects retain the capacity to exhibit lordosis [11,40]. These results indicate that, as the gradient of neonatal androgen exposure increases, the capacity to show lordosis in the adult males decreases under an appropriate hormonal treatment.

The effect of the neonatal androgenization gradient on the expression of male lordosis seen in the behavioral test is the framework for discussing the effects of this hormonal gradient on the sexual differentiation of nuclei that are implicated in the lordosis in the male.

The VMN facilitates the lordosis response as demonstrated by lesions and electrical stimulation studies in male and female rats [5,26,27]. In this nucleus, the ventrolateral subdivision seems to play a crucial role in the generation of the lordotic response [25]. Like other authors [21], we have not found sex differences in the number of neurons in this nucleus. However, we did find sex differences with respect to the total and the VMNvl volume as previously reported in the literature [21,22]. Neonatal castration induced a feminine VMN in the male, as was previously reported by others [22]. In addition, we found that neonatal treatment in castrated males with androstenedione was unable to masculinize the volume of the total VMN or the VMNvl. It seems that the masculinization of this facilitatory nucleus for lordotic behavior depends on the full action of testicular androgens.

The LS plays an inhibitory role in the expression of lordosis in males [44]. Sex differences have been reported with respect to the fluro-gold (FG)-immunoreactive cells in the right side of the intermediate part of the LS [44]. Our study has found sex differences in the LSi using Nissl staining; control females showed more neurons than control males. Neonatal male castration increased the number

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**Fig. 3.** Graphs show (A) the total volume of the ventromedial nucleus of the hypothalamus (VMN). In B the volume of the ventrolateral subdivision of VMN is showed. * At least $p < 0.05$ with respect to CM; $+$ at least $p < 0.05$ with respect to CF (Kruskal–Wallis non-parametric test with Mann–Whitney U post-test). Results are expressed as median and semi-interquartile range. CF: control females, CM: control males; GxM + oil: castrated males treated with oil; GxM + AND: castrated males treated with androstenedione.

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**Fig. 4.** Graphs show the number of neurons in the intermediate part of the lateral septum. * At least $p < 0.05$ with respect to CF; $+$ at least $p < 0.05$ with respect to GxM + oil. Kruskal–Wallis non-parametric test with Mann–Whitney U post-test were used. Results are expressed as median and semi-interquartile range. CF: control females, CM: control males; GxM + oil: castrated males treated with oil; GxM + AND: castrated males treated with androstenedione.
Fig. 5. Photomicrographs showing illustrative Nissl-stained sections of the sexual dimorphism found in the intermediate lateral septum. (A) Control male; (B) control female; bar for A and B = 300 μm; C and D show magnified details of the number of neurons in the LSi of control males (C) and control females (D); bar = 30 μm. LSd: lateral septum, dorsolateral part; LSi: lateral septum, intermediate part; LSv: lateral septum, ventral part.

of neurons in the LSi, indicating that the presence of postnatal androgens is necessary to masculinize this region. Similar findings of sexual dimorphism in which females show greater morphological patterns (volume, number of cells, synapses, etc.) than males, using Nissl staining, have been reported for the parastrial nucleus [7], the medial anterior and the lateral anterior subdivisions of the nucleus of the stria terminalis [6,12] and the locus coeruleus [29]. However, androstenedione partially masculinizes the LSi because these animals presented a significantly smaller number of neurons than control females but they did not differ from the castrated and oil injected and control male groups in the number of LSi neurons.

The AOB is a sexually dimorphic olfactory structure that belongs to the sexually dimorphic vomeronasal system, a system that is implicated in the control of reproductive behaviors (for review see [4,8,16–18,36,37]). The AOB also has an inhibitory function since lesions in this structure facilitate the expression of lordosis in the male [33] and in virgin female rats [19]. In our study, we found sex differences with respect to the number of AOB mitral and light and dark granule cells. Control males showed significantly higher numbers of these AOB cells than control females, confirming previous results in the literature [31,34,35,37,46]. Neonatal treatment with androstenedione in castrated males did not masculinize the number of mitral and light and dark granule cells in the AOB. Nevertheless, as previously reported [31,34,35,37,46], neonatal castration induced a feminine number of AOB mitral and light and dark granule cells, because no differences in the number of these cells were found between the neonatally castrated and oil injected males and the control females. It seems that the masculinization of this
inhibitory nucleus for lordotic behavior in the male depends on the full action of testicular androgens.

With respect to the three structures, VMN, LSi and AOB, that belong to facilitatory and inhibitory networks of lordosis, the sexually morphological dimorphic pattern associated to lordosis in the female is: (a) in the facilitatory VMN and VMNvl, a smaller volume than in male rats; (b) in the inhibitory LSi, females have a greater number of neurons than males; (c) in the also inhibitory AOB, females have a smaller number of mitral and granule cells than males. This morphological sexually dimorphic pattern is associated to the expression of lordosis in the female.

If we compare the sexually dimorphic patterns found in the males exposed to a gradient of postnatal androgens, to the pattern we have just described above for the normal female, we find that: (a) neonatal gonadectomy of males facilitated the expression of lordosis and is associated to a pattern of sex differences in the VMN, VMNvl, AOB and LSi that is similar to pattern in the female; (b) the partially androgenized males, that is, the neonatally castrated males treated with androstenedione, showed a higher LQ than control males but a lower LQ than the neonatally castrated males. Their expression of lordosis is associated to: a smaller volume of the VMN and VMNvl, fewer neurons in the AOB than the control males but a similar number of LSi neurons to the control males. Finally, the control males that show a inhibition of lordosis coincides with a completely inverted pattern of sexual dimorphism with respect to the control females and the neonatally castrated males in the VMN, VMNvl, AOB and LSi.

The above mentioned findings point out that greater expression of lordosis in the male is related to the feminization of both the facilitatory (VMN, VMNvl) and inhibitory (LSi and AOB) structures controlling this behavior. However, the inhibition of lordosis in the male is associated to the masculinization of these facilitatory and inhibitory structures.

We have seen among the male groups that a greater expression of lordosis is associated with the loss of androgens resulting from neonatal castration and, as a consequence, the feminization of the structures that facilitate or inhibit lordosis. However, partially androgenized males, those neonatally castrated and androstenedione treated are significantly in between the neonatally castrated males and the control males with respect to their lordosis response, and show a feminization of the VMN and the AOB while the LSi remains masculinized.

In contrast to the above groups, the inhibition of lordosis shown by the control males seems to be associated to the neonatal presence of testosterone and the masculinization of the VMN, VMNvl, LSi and the AOB.

In relation to sexual behavior, both male and female rats share the same structures for the facilitation and inhibition of lordosis. In that context, and taking into account our results, it could be suggested that the morphological pattern of sex differences in the VMN, VMNvl, LSi and the AOB is related to the inhibition of lordosis in the male. Moreover, the morphological sex differences consistently seen in these structures might be functionally related to the inhibition of lordosis response in the male, as we have previously suggested [36].

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