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Research Report

Sexual dimorphism in the vomeronasal system of the rabbit

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ABSTRACT

Studies have shown that the vomeronasal system (VNS), an olfactory neural network that participates in the control of reproductive physiology and behavior, is sexually dimorphic in the rat. These works have also shown two main characteristics of brain sexual dimorphism: (a) dimorphism appears in neural networks related to reproduction and (b) it can present two morphological patterns: one in which males present greater morphological measures than females (male > female) and another in which the opposite is true (female > male). The present work extends the hypothesis to the rabbit, as a representative species of Lagomorpha. In addition, the locus coeruleus (LC), which is known to send rich noradrenergic projections to VNS structures, was also studied. Sex differences were found in: (a) the number of mitral, and dark and light granule cells (female > male) of the accessory olfactory bulb (AOB); (b) the medial amygdala (Me) and its dorsal (Med) and ventral (Mev) subdivisions, males showing greater values than females in volume and number of neurons, while in the posteromedial cortical amygdala (PMCo or C₃), females show greater density of neurons than males and (c) the posteromedial division of the bed nucleus of the stria terminalis (BSTMP) in which males have more neurons than females. No sex differences were seen in the bed nucleus of the accessory olfactory tract (BAOT) and the LC. These results evidence that, as it was observed in rodents, sex differences are also seen in the VNS of Lagomorpha and that these sex differences present the two morphological patterns seen in Rodentia. Differences between orders are discussed with respect to the species-specific physiological and behavioral peculiarities.

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1. Introduction

The dual olfactory system hypothesis, which proposes the existence of two separate olfactory pathways: the main and vomeronasal pathways (Scalia and Winans, 1975, 1976; Winans and Scalia, 1970), has generated a huge amount of research and has helped us to understand important aspects

of vertebrate reproductive behaviors. Starting with Powers and Winans (1975) who demonstrated that the vomeronasal organ (VNO) participates in the control of copulatory behavior of male hamsters, many studies have appeared in the literature suggesting that the VNS mediates the action of pheromones implicated in the expression of masculine and feminine behavior, maternal behavior and physiological primer

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pheromone mechanisms that influence puberty and estrous cycle (see for review [Del Cerro, 1998](#); [Halpern, 1987](#); [Halpern and Martinez-Marcos, 2003](#); [Wysocki, 1979](#)).

At the beginning of the eighties, and working with rats, we found that the rat VNO is a sexually dimorphic chemosensory structure differentiated by gonadal hormones early after birth ([Segovia and Guillamon, 1982](#)) and we suggested that the whole vomeronasal system (VNS) could be sexually dimorphic (see for review [Guillamon and Segovia, 1993, 1996, 1997](#); [Segovia and Guillamon, 1986, 1993, 1996, Segovia et al., 1999](#)). The structures that receive vomeronasal input, such as the medial amygdala (Me), medial preoptic area (MPA), the ventromedial hypothalamus (VMH) and premammillary nucleus (PMV) have androgen and estrogen receptors ([Simerly et al., 1990](#)) and present sexual dimorphism ([Bleier et al., 1982a](#); [Dörner, 1976](#); [Gorski et al., 1978, 1980](#); [Matsumoto and Arai, 1983](#); [Nishizuka and Arai, 1981, 1983](#); see also for review [Arnold and Gorski, 1984](#); [Guillamon and Segovia, 1993, 1996, 1997](#); [Segovia and Guillamon, 1986, 1993, 1996](#); [Segovia et al., 1999](#)). As it occurs in the SDN-POA ([Döhler et al., 1986](#)), we have demonstrated, also in the rat, the existence of sex differences, controlled by sex steroids shortly after birth, in other structures of the vomeronasal pathway like the AOB ([Segovia et al., 1984, 1986](#); [Valencia et al., 1986](#)), the BAOT ([Collado et al., 1990, 1993, 1998](#)), the BST ([Del Abril et al., 1987](#); [Guillamon et al., 1988a](#)) and the C₃ ([Vinader-Caerols et al., 1998, 2000](#)).

Sex differences in structures that receive VNO input have been found in several species; for instance, differences in the sexually dimorphic nucleus of the medial preoptic area (SDN-POA) were reported first in rats ([Gorski et al., 1978, 1980](#); see for review [Arnold and Gorski, 1984](#)) and confirmed in hamsters ([Greenough et al., 1977](#)), polygamous montane voles ([Shapiro et al., 1991](#)), gerbils ([Yahr et al., 1994](#)), ferrets ([Cherry et al., 1990](#)), guinea pigs ([Bleier et al., 1982b](#); [Hines et al., 1985](#)), sheep ([Roselli et al., 2004](#)), rhesus monkeys ([Byne, 1998](#)), humans ([Hofman and Swaab, 1989](#); [Swaab and Fliers, 1985](#)) and also in quails ([Viglietti-Panzica et al., 1986](#)) and doves ([Steimer and Hutchison, 1990](#)). Similarly, sex differences in the medial region of the BST have been reported in some species of birds ([Panzica et al., 2001](#)) as well as in Wistar and Long Evans rats ([Del Abril et al., 1987](#); [Garcia-Falgueras et al., 2005](#); [Guillamon et al., 1988a](#)), guinea pigs ([Hines et al., 1985](#)) and humans ([Allen and Gorski, 1990](#); [Zhou et al., 1995](#)). All these findings support the hypothesis that the VNS might be a sexually dimorphic network in vertebrates.

Rats and rabbits present substantial differences in their reproductive physiology and behavior. Female rats are spontaneous ovulators and female rabbits are reflex ovulators. Moreover, there are important differences between rats and rabbits in copulatory patterns. Male rats behave with multiple intromissions that end in one ejaculation and a postejaculatory period, while in rabbits ejaculation occurs in almost every intromission and the postejaculatory period is very short ([Dewsbury, 1972](#)). Females of both species, spontaneously or most commonly after the appropriate hormonal treatment, show male like copulatory patterns called pseudomale behavior ([Morali and Beyer, 1992](#); [Morali et al., 2003](#)). With respect to this behavior, rats are isomorphic while rabbits are dimorphic ([Morali and Beyer, 1992](#); [Morali et al., 2003](#)).

The participation of the VNS in the control of maternal behavior in rodents is well known. Most VNS structures exert a

tonic inhibition ([Del Cerro, 1998](#)) on the MPOA, a structure that receives VNS input and facilitates the expression of maternal behavior ([Del Cerro, 1998](#); [Numan, 1994](#)). With respect to rabbits, to our knowledge, there is only one study in the literature that performed by [Gonzalez-Mariscal et al. \(2004\)](#). These authors demonstrated a tonic inhibitory action of the AOB over the expression of maternal behavior in virgin rabbits and a stimulation of maternal responsiveness by ovarian hormones following AOB lesions.

Taking into account the differences in the reproductive physiology and behaviors above described between rats and rabbit species and in order to support the hypothesis that the VNS might be sexually dimorphic in mammals, this work studies the possible existence of sex differences in the AOB, BAOT, BST and C₃ in the rabbit. Moreover, the locus coeruleus (LC), which is sexually dimorphic in some strains of rats ([Garcia-Falgueras et al., 2005](#); [Guillamon et al., 1988b](#); [Luque et al., 1992](#); [Pinos et al., 2001](#)) and sends rich noradrenergic projections to the AOB ([Shipley et al., 1985](#)), is also studied.

2. Results

2.1. Accessory olfactory bulb

The AOB is displayed in [Fig. 1](#). The rabbit AOB is an ovoid structure, dorsocaudally embedded in the main olfactory bulb (MOB). It has five well differentiated layers: glomerular, external plexiform, mitral cells, internal plexiform and granular ([Fig. 1](#)). Four main characteristics can be detected: (a) glomerules are round shaped and well differentiated, (b) the mitral cell layer is not stratified, (c) bundles of fibers from the internal plexus invade the granular layer giving it an indented form and (d) light and dark granules can be distinguished in the granular layer.

There were sex differences in the overall volume ($t_8 = 2.52$, $P < 0.03$), since males had larger volume than female rabbits ([Table 1](#)). However, it may be considered as a technical artifact, since sex differences were not seen in the volume of the glomerular, external plexiform, mitral, internal plexiform and

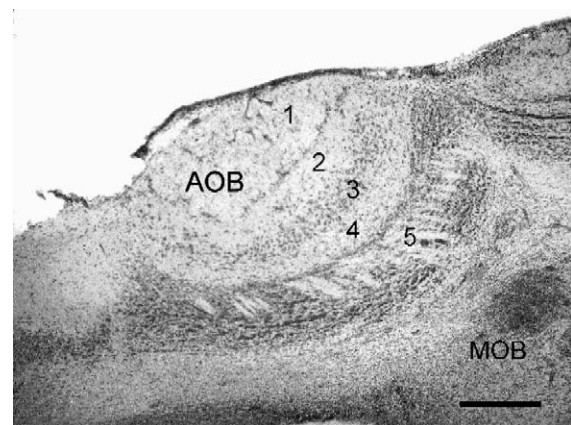


Fig. 1 – Photomicrograph illustrating the morphology of the rabbit (male) accessory olfactory bulb (AOB): (1) glomerular layer, (2) external plexus, (3) mitral cell layer, (4) internal plexus and (5) granular layer; 4×, scale bar = 300 μm; MOB: main olfactory bulb.

granular layers ($P > 0.05$, in all cases). Although neuron number did vary significantly by sex for mitral ($t_8 = 2.72$, $P < 0.02$), light granule ($z = 2.19$, $P < 0.03$) and dark granule cells ($t_8 = -2.62$, $P < 0.03$), females always showed more number of cells than male rabbits in all cases (Table 2).

Because no sex differences were found between the volumes of each of the AOB layers, a density study (number of neurons per volume unit) was performed for each layer. The light and dark granules were added together to calculate density in the granular layer. No sex differences in the density of cells were seen for the mitral ($z = 1.14$; $P > 0.31$) and for granule cells ($t_8 = 2.15$, $P > 0.71$).

2.2. Bed nucleus of the accessory olfactory tract

As can be seen in Fig. 2, the BAOT of the rabbit can be easily delimited in its ventral, lateral and medial edges, while the dorsal limits are not as clear as the others. However, the more scattered disposition of the cells and a higher affinity for cresyl violet in this nucleus make it possible to distinguish it from the dorsally located amygdaloid area. Fig. 2 shows that the BAOT is a round shaped structure, ventral to the anterior amygdaloid area and medial to the pyriform cortex (Fig. 2). The BAOT can also be distinguished by its medium-sized cells associated to the accessory olfactory tract. In medium and caudal portions, this nucleus is located lateral to the nucleus of the lateral olfactory tract and medial to the pyriform cortex. No sex differences were found for volume, number of neurons and density of neurons per unit of volume (Tables 1 and 2).

2.3. Medial amygdala

In the rabbit, the medial amygdala emerges as a nucleus where the nucleus of the lateral olfactory tract and the bed

nucleus of the accessory olfactory tract end. It first appears between the optic tract and the anterior cortical amygdaloid nucleus. Caudally, the Me accompanies the optic tract in its dorsal elongation and, as displayed in Fig. 3, the Me is delimited medially by the optic tract, laterally by the stria terminalis and ventromedially by the posteromedial basal amygdaloid nucleus (B3). The Me fades at the lateral ventricle when this cistern and the hippocampus appear. The dorsal and the ventral divisions of the rabbit Me can be distinguished by the fact that cells of the ventral Me appear more packaged.

Three separate statistical analyses were performed taking into account the whole Me and its ventral (Mev) and dorsal (Med) regions. The Me presents sex differences with respect to volume ($t_8 = 2.63$, $P < 0.03$) and number of neurons ($t_8 = 2.37$, $P < 0.04$), males showing greater morphological measures than females. The same pattern of sex differences was seen in the Mev (volume: $t_8 = 2.98$, $P < 0.01$; number of neurons: $t_8 = 3.52$, $P < 0.01$). However, sex differences in Med only affected volume ($t_8 = 2.30$, $P < 0.05$) (Figs. 3A and B; and Tables 1 and 2).

2.4. Posteromedial cortical amygdala (PMCo or C₃)

The amygdaline nucleus of the rabbit is shown in Fig. 4. The PMCo is a well-defined, large mass of cells situated in the ventromedial edge of the hemisphere. Rostrally, it begins as an ovoid-shaped structure situated ventral to the optic tract and that laterally borders the Me (Fig. 4A). Afterwards, it adopts a long form that then runs ventral to the posterolateral basal nucleus (B2) and the B3 and laterally borders the posterolateral cortical nucleus (C2) (Fig. 4B). After this point, the C₃ continues ventral to B3 and laterally borders the B2 until its end (Fig. 4C).

No sex differences were found with respect to the volume ($t_8 = 2.05$, $P = 0.074$, ns) and number of neurons ($t_8 = 0.56$, $P = 0.58$, ns), although female rabbits showed a significantly greater density of neurons per unit of volume than males ($t_8 = 2.54$, $P = 0.035$) (Tables 1 and 2). This neural population of the C₃ is composed, to a great extent, of oval or pyramidal neurons and occasionally fusiform neurons.

2.5. Bed nucleus of the stria terminalis

From a rostral-caudal view, the BST forms the floor of the lateral ventricle and is bounded rostrally by the nucleus accumbens, and ventrally merging with the preoptic area (Fig. 5A). The medial anterior (BSTMA) and lateral anterior (BSTLA) subdivisions of the BST are presented in Fig. 5A. The BSTMA of the rabbit (in rats also called anterodorsal part of the BST, Ju and Swanson, 1989) resembles a triangle, having one vertex on the end of the lateral ventricle and a leg lying on the anterior commissure. Rostromedially, it borders the BSTLA and the lateral septum (Fig. 5A).

The BSTLA (in rats also called dorsal part of the lateral bed nucleus by Ju and Swanson, 1989) can be distinguished by its elongated shape and its position, lateral to the BSTMA (Fig. 5A). This subdivision is bounded by the internal capsule, which separates it from the caudate-putamen nucleus, and by the juxtacapsular subdivision of the bed nucleus (BSTjx) (Fig. 5A). The BSTLA and BSTMA subdivisions run together caudally and become the lateral posterior (BSTLP) and medial posterior (BSTMP) subdivisions (Fig. 5B).

Table 1 – Sex differences in the rabbit vomeronasal nuclei: study of the volume

Structure		Male	Female
AOB	Overall	4.1374 ± 0.2196	3.506 ± 0.1201**
	Glomerular	1.2315 ± 0.1403	0.9114 ± 0.0721
	Ext. plexiform	0.4550 ± 0.0214	0.4536 ± 0.0652
	Mitra cell	0.6311 ± 0.0464	0.6185 ± 0.0427
	Internal plexus	0.4014 ± 0.0624	0.4649 ± 0.0736
	Granular	1.2341 ± 0.1294	1.2271 ± 0.1383
BAOT		0.0665 ± 0.0058	0.0505 ± 0.0053
Me	Me	2.8456 ± 0.1256	2.2932 ± 0.1686**
	Med	2.1369 ± 0.0940	1.7715 ± 0.1275*
	Mev	0.7086 ± 0.0408	0.5215 ± 0.0475***
C3		4.1264 ± 0.2533	3.5458 ± 0.1243
BST	BSTMA	0.4335 ± 0.0357	0.3959 ± 0.0182
	BSTLA	0.2309 ± 0.0133	0.2161 ± 0.0216
	BSTMP	0.3865 ± 0.0392	0.3282 ± 0.0323
LC		0.1304 ± 0.0078	0.1338 ± 0.0071

AOB: accessory olfactory bulb; BAOT: bed nucleus of the accessory olfactory tract; BST: bed nucleus of the stria terminalis; LA: lateral, MA: medial, MP: posteromedial subdivisions; LC: locus coeruleus; Me: medial amygdala, d: dorsal and v: ventral subdivisions; C₃: posteromedial cortical amygdala. Data show means ± SEM of the Student's t test * $P < 0.05$, ** $P < 0.03$, *** $P < 0.01$.

Table 2 – Sex differences in the rabbit vomeronasal nuclei: study of the number and density of neurons

Structure		Number of neurons		Density (neurons/mm ³)	
		Males	Females	Males	Females
AOB	Mitral cells	7122.6 ± 272.6	8473.6 ± 413.9**	11,451 ± 655.5	14,036.4 ± 1404
	Light granules	51,495.6 ± 1218.6	69,706.2 ± 6898+		
	Dark granules	106,361 ± 8563.3	147,296.4 ± 13,070.9**		
	Light + dark				
BAOT		3787.5 ± 487.5	2814 ± 399.3	57,394.3 ± 6496.9	54,989.3 ± 3731.9
Me	Me	52,218.2 ± 2956.4	42,318.2 ± 2955.5**	18,639 ± 1868.4	19,104.4 ± 2479.6
	Med	34,972 ± 3301.5	28,499 ± 3270.2	16,614 ± 2107.9	16,825 ± 2926.8
	Mev	17,245.6 ± 884.7	13,819.2 ± 408***	24,762 ± 2165.9	27,216 ± 2087.2
C3		154,423.6 ± 10,174.8	160,730.6 ± 4628.3	37,836.4 ± 2709.7	45,419 ± 1249.1**
BST	BSTMA	38,186.8 ± 3509.1	40,325.8 ± 4391	88,515.2 ± 4264.2	100,723.3 ± 7719.1
	BSTLA	15,786 ± 1652.3	15,271.3 ± 2007.2	67,834.7 ± 5032	69,950 ± 4590.8
	BSTMP	41,513.5 ± 5049.8	27,290 ± 2505.6*	110,259.5 ± 12,098.6	87,803.9 ± 11,182.2
LC		846 ± 82.1	815 ± 97.5	444.8 ± 520	6018 ± 428.2

AOB: accessory olfactory bulb; BAOT: bed nucleus of the accessory olfactory tract; BST: bed nucleus of the stria terminalis; LA: lateral, MA: medial, MP: posteromedial subdivisions; LC: locus coeruleus; Me: medial amygdala, d: dorsal and v: ventral subdivisions; C3: posteromedial cortical amygdala. Data show means ± SEM of the Student's *t* test **P* < 0.05, ***P* < 0.03, ****P* < 0.01 or Mann–Whitney test +*P* < 0.03.

The BSTMP is easily distinguished because of its heavy staining (Fig. 5C) and of its being bordered laterally by the internal capsule and medially by the fornix and the stria medullaris, and ventrally by the anterior hypothalamus. This subdivision becomes thinner caudally and disappears when fibers from fornix and the stria medullaris transected it.

With respect to sex differences, statistical analyses were done for volume, number of neurons and density in the BSTMA, BSTLA and BSTMP. Sex differences were only seen in relation to the number of neurons in the BSTMP, and males had more neurons than female rabbits ($t_{12} = 2.26$, $P < 0.04$) (see Fig. 6 and Tables 1 and 2).

2.6. Locus coeruleus

The rabbit locus coeruleus is situated in the ventrolateral edge of the IVth ventricle, medial to the mesencephalic nucleus of the trigeminal nerve (Me5) and dorsal to the motor trigeminal nerve

(Mo5) (Fig. 7A) The LC presents diffuse borders with the Me5 (Figs. 7A and B). This observation is consistent with tyrosine-hydroxylase (TH) or DBH-stained studies, which report difficulties in differentiating the limits of the LC (Schuenger and Balaban, 1999). As can be seen in Fig. 7B, the LC seems to be topographically organized into two subdivisions: one dorsal, with cells obliquely oriented from the dorsolateral to the ventromedial axis, and a ventral subdivision that is in a continuum with the subcoeruleus. Tables 1 and 2 display the volume, number of neurons and density of cells per unit of volume of the LC. No sex differences with respect to these parameters were found.

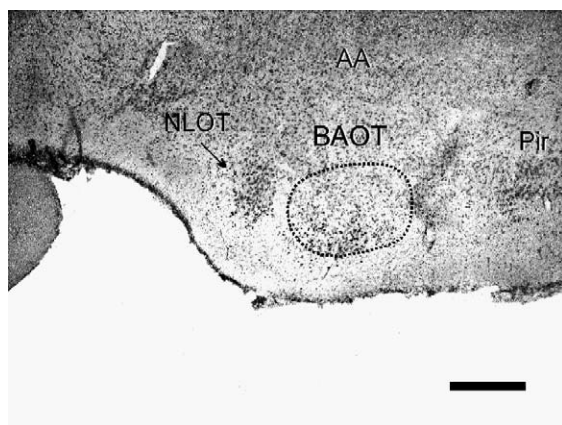


Fig. 2 – Microphotograph showing a coronal section of the rabbit (male) bed nucleus of the accessory olfactory tract (BAOT). AA: anterior amygdaloid area; BAOT: bed nucleus of the accessory olfactory tract; NLOT: nucleus of the lateral olfactory tract; Pir: piriform cortex; 4×, scale bar = 200 μm.

3. Discussion

The present study is the first morphological work that systematically describes brain sexual dimorphism in lagomorphs. The results show the existence of sex differences in most of the studied rabbit vomeronasal structures. The accessory olfactory bulb, medial amygdala, posteromedial cortical amygdala and the medial posterior region of the bed nucleus of the stria terminalis all present sexual dimorphism. However, the bed nucleus of the accessory olfactory tract seems to be isomorphic. The locus coeruleus, included in this study because it sends rich noradrenergic projections to the AOB and is sexually dimorphic in some rat strains (Garcia-Falgueras et al., 2005; Guillaumon et al., 1988b; Luque et al., 1992; Pinos et al., 2001), is isomorphic in the rabbit.

The rabbit AOB is quite similar to the rat AOB. As has also been reported by Meisami and Bhatnagar (1998), it is a very well-developed structure that contains all the structural components seen in the rat but in a highly differentiated form. With respect to the number of neurons, female rabbits show more mitral and granule (light and dark) cells than the male rabbits. The direction of sexual dimorphism in the number of neurons in the AOB of the rabbit is opposite to that reported by us in the rat (Segovia et al., 1986; Valencia et al., 1986).

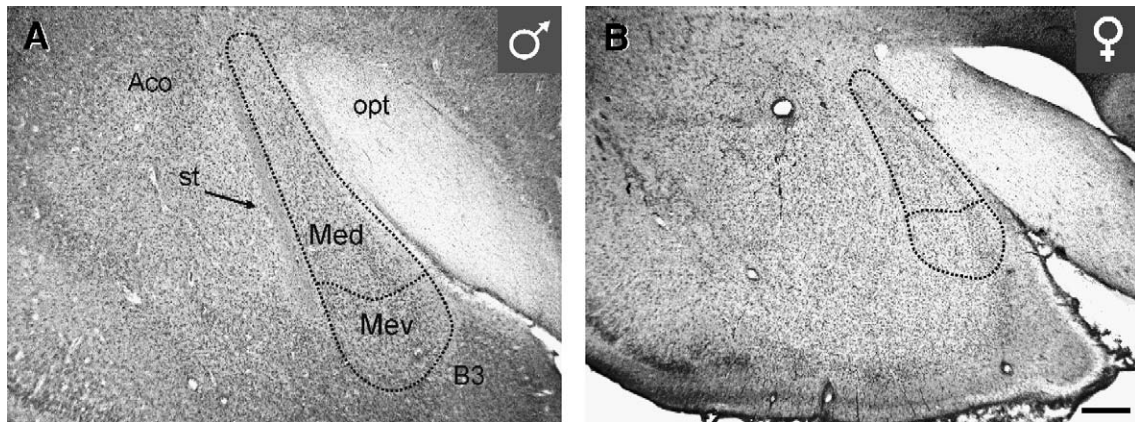


Fig. 3 – Microphotographs of coronal sections illustrating sex differences in the medial amygdala of (A) male and (B) female rabbits. Aco: anterior cortical amygdaloid nucleus; B₃: posteromedial basal amygdaloid nucleus; Med: medial amygdala, dorsal subdivision; Mev: medial amygdala, ventral subdivision; Opt: optic tract; st: stria terminalis; A and B: 2.5×, scale bar = 200 μm.

The rabbit BAOT, as was also observed in rats (Collado et al., 1990, 1993, 1998), can be distinguished by its medium-sized cells associated to the accessory olfactory tract. Contrarily to the data reported in the rat showing sexual dimorphism (Collado et al., 1990, 1993, 1998), the rabbit BAOT is an isomorphic structure.

In the rabbit, the Me emerges as a nucleus at the end of lateral olfactory tract and the BST. Analogously, as occurs in the rat (Shipley et al., 2004), a dorsal and a ventral subdivisions can be distinguished because the ventral Me cells are more densely packed. The Me of the male presents a greater volume and number of neurons than in the female rabbit. However,

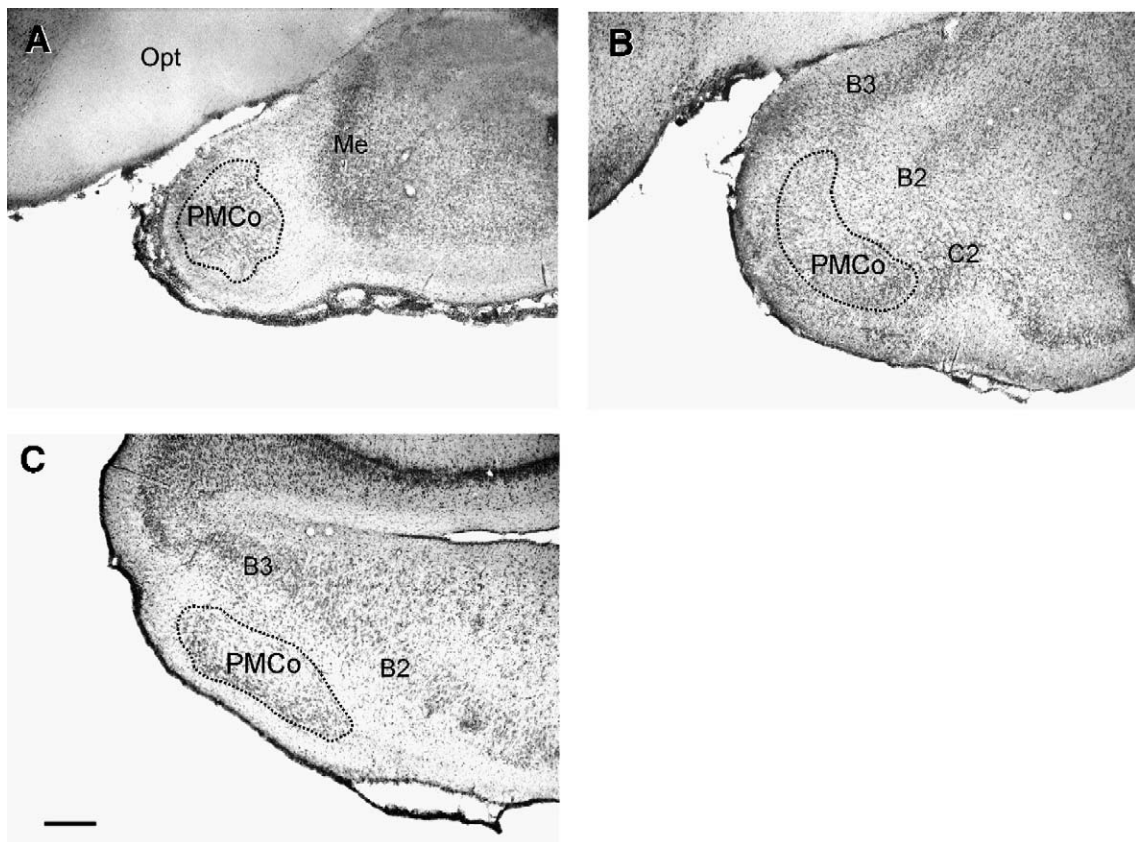


Fig. 4 – Photomicrographs showing the rostral (A), medial (B) and caudal (C) regions of the rabbit (male) posteromedial cortical amygdala (PMCo or C₃). B₂: posterolateral basal amygdaloid nucleus; B₃: posteromedial basal amygdaloid nucleus; C₂: posterolateral cortical amygdaloid nucleus; Opt: optic tract; 4×, scale bar = 300 μm.

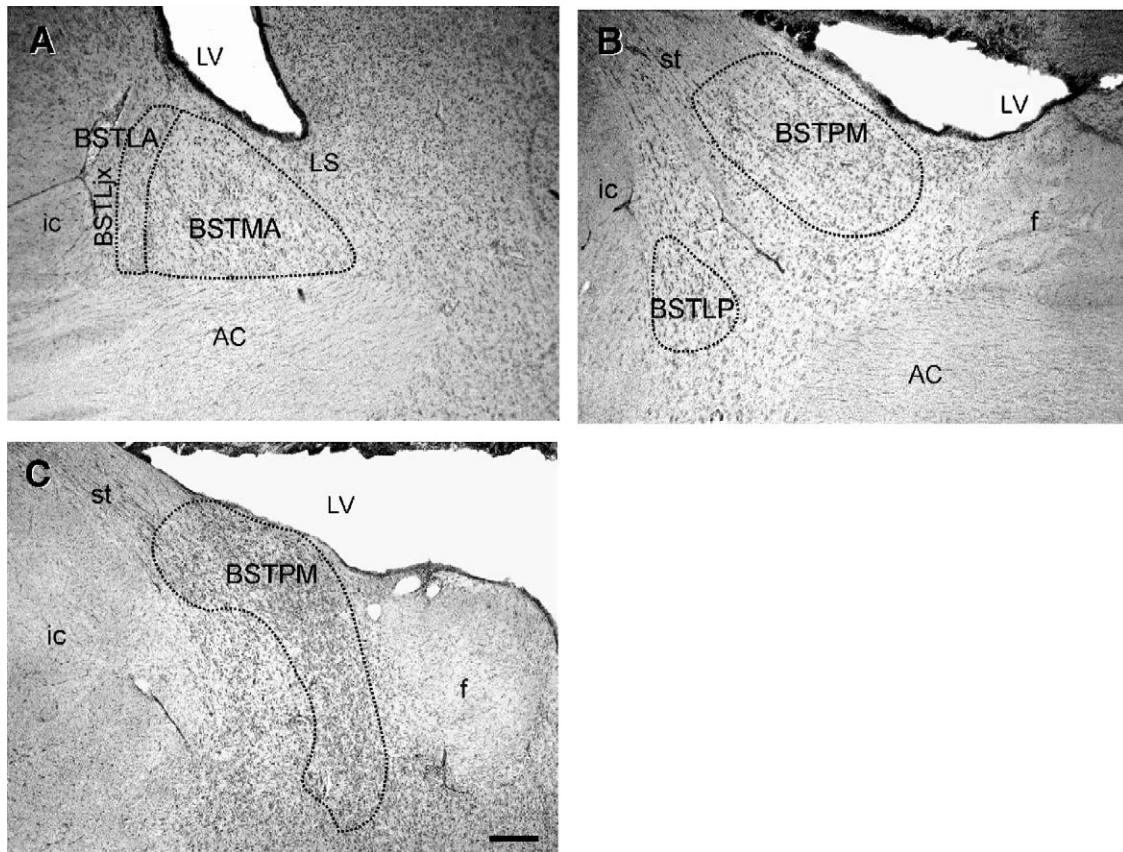


Fig. 5 – Coronal photomicrographs of a rabbit male showing (A) the lateral anterior (BSTLA), the medial anterior (BSTMA) and the juxtacapsular (BSTLjx) subdivisions of the bed nucleus of the stria terminalis (BST) and (B and C) the progression, from rostral to caudal, of the lateral posterior (BSTLP) and posteromedial subdivisions (BSTPM) of the BST. AC: anterior commissure; BSTLA: bed nucleus of the stria terminalis, lateral anterior subdivision; BSTLP: bed nucleus of the stria terminalis, lateral posterior subdivision; BSTLjx: bed nucleus of the stria terminalis, lateral juxtacapsular subdivision; BSTMA: bed nucleus of the stria terminalis, medial anterior subdivision; BSTPM: bed nucleus of the stria terminalis, posteromedial subdivision; f: fornix; ic: internal capsule; LS: lateral septum; LV: lateral ventricle; st: stria terminalis; 4 \times , scale bar = 300 μ m.

when the two Me subdivisions were analyzed separately, the Mev still presented sex differences in volume and number of neurons while only volume differences were observed in the

Med. In the rat, overall Me volume is larger in male than in female rats after weaning (Mizukami et al., 1983) and the synaptic organization of this nucleus is also sexually dimorphic,



Fig. 6 – Photomicrographs illustrating sexual dimorphism in the rabbit posteromedial region of the bed nucleus of the stria terminalis (BSTPM). As can be seen, males (A) show greater morphological measurements than females (B) (for details, see text); 4 \times , scale bar = 300 μ m.

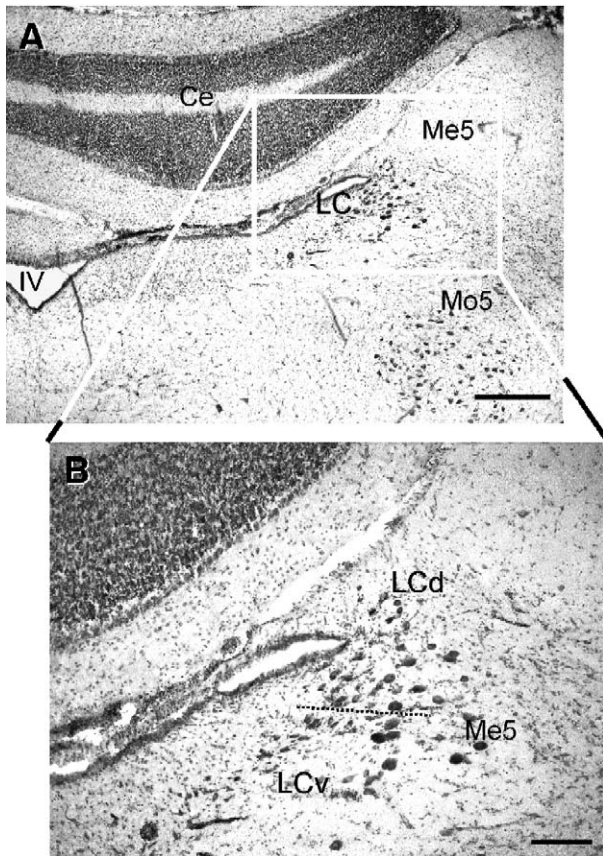


Fig. 7 – Microphotographs of coronal sections illustrating the Locus Coeruleus in a male rabbit (A). In panel B, the topographic organization of the LC is delineated in two subdivisions, the dorsal and the ventral parts and the absence of borders with respect to the Me5 can also be appreciated. Ce: cerebellum, LC: locus coeruleus, LCd: locus coeruleus, dorsal, LCv: locus coeruleus, ventral. Me5: mesencephalic nucleus of the trigeminal nerve, Mo5: motor trigeminal nerve, IV: fourth ventricle. A: 10 \times , scale bar = 300 μ m; B: 10 \times , scale bar = 100 μ m.

since males have more shaft synapses (Nishizuka and Arai, 1981, 1983).

The rabbit C_3 is a well-defined structure that is quite analogous to the rat (Shipley et al., 2004). With respect to sexual dimorphism, our results in the rabbit are different from those observed in the rat (Vinader-Caerols et al., 1998, 2000). The rat C_3 shows sex differences: males have a larger volume and a higher number of neurons than females. However, in the rabbit, no sex differences were found with respect to these two measurements, although the density of cells was different between sexes and the females showed a higher density of neurons than male rabbits.

The bounds of the rabbit BST and the borders of its subdivisions are similar to those described in the rat (Del Abril et al., 1987; Guillamon et al., 1988a). The rabbit BST presents sex differences only in the number of neurons of the BSTMP, where males have more neurons than females. These results are similar to those reported in the rat (Del Abril et al.,

1987; Guillamon et al., 1988a) With respect to other BST subdivisions, it should be taken remembered that the presence or absence of sex differences depends on the strain of rat studied. Sex differences, with females showing greater measurements than males, were reported for the BSTLA and the BSTMA in the Wistar strain (Del Abril et al., 1987; Guillamon et al., 1988a) but not in the Long-Evans rats (Garcia-Falgueras et al., 2005). In the rabbit, the BSTMA and the BSTLA are isomorphic. The male > female pattern of the rabbit BSTMP is also seen in other species of mammals like guinea pigs (Hines et al., 1985) and humans (Allen and Gorski, 1990), it seems that this pattern is so firmly established that it is preserved through species.

As in the rat, the LC of the rabbit is located at the ventrolateral edge of the fourth ventricle. However, there are two main differences between these two species: in the rabbit, the LC neurons are less packaged and the borders with the nucleus of the trigeminal nerve are more diffuse. In the rat LC, sex differences are associated to the strain. Females show greater volume and number of neurons than male Wistar rats but these differences are not replicated in the Sprague–Dawley and the Long-Evans strains (Garcia-Falgueras et al., 2005). At the morphological level, the LC is isomorphic in the rabbit. However, the noradrenergic function of the LC has been reported to be sexually dimorphic in rabbits (Yang et al., 1996).

Studies on sexual dimorphism in the rat have shown that sex differences in the central nervous system follow two opposite morphological patterns (Guillamon and Segovia, 1993, 1996, 1997; Segovia and Guillamon, 1986, 1993, 1996). In one pattern, males show greater morphological measurements (volume, number of neurons, density, etc.) than females (male > female pattern) while in others the opposite is true (female > male). In the rat, primary, secondary and tertiary VNS structures present the male > female sexual pattern. However, in the rabbit, we have found that some VNS structures, like the AOB and C_3 , present the female > male pattern, others like the BSTMP and the Mev show the male > female sexual pattern and another, the BAOT, is isomorphic. Thus, sexual dimorphism in the rabbit VNS is quite different from observations in the rat. We have no explanation for these species differences in the expression of sexual dimorphism, although we think they are probably related to differences in reproductive behavior and physiology between species. It should be remembered that female rabbits are reflex ovulators while female rats are spontaneous ovulators and that the sexual and maternal behaviors of these two species are also different (Dewsbury, 1972; Gonzalez-Mariscal, 2001; Morali and Beyer, 1992; Morali et al., 2003).

Gonadal steroids sexually differentiate neural networks related to reproduction (Guillamon and Segovia, 1993, 1996, 1997; Segovia and Guillamon, 1986, 1993, 1996; Segovia et al., 1999). In the rat, which shows the sexually dimorphic pattern male > female in all VNS structures, we have observed that early postnatal gonadectomy decreases the volume and the number of neurons of the male AOB, BAOT, C_3 and BSTMP while female androgenization at the same age increases these parameters (see for review Guillamon and Segovia, 1993, 1996, 1997; Segovia and Guillamon, 1986, 1993, 1996; Segovia et al., 1999). Furthermore, estradiol from testosterone aromatization might be responsible for VNS structures masculinization

(Collado et al., 1993; Perez-Laso et al., 1997; Vinader-Caerols et al., 2000). The findings of the present experiment and the fact that estrogen receptor- α have been found in the preoptic region, encapsulated BST, Me and C₃ of the female rabbit (Caba et al., 2003) suggest that, in rabbits, these structures might be sexually differentiated by gonadal steroids during periods of maximal susceptibility.

Rabbits are very interesting to research because they can bring us new insights for an evolutionary approach to sexual dimorphism and more experiments are needed to explain the findings obtained in the present work. Rabbits have species-specific reproductive behavioral responses and, as we have seen, their sexually dimorphic VNS pattern is also species-specific. Moreover, the findings of this experiment help to support the hypothesis that the VNS is a sexually dimorphic network in mammals. This hypothesis could be of great value in reaching a comprehensive approach to explain motivated behaviors like the reproductive behaviors.

4. Experimental procedures

4.1. Subjects

Twelve New Zealand adult rabbits (*Oryctolagus cuniculus* L.), six males and six females, weighting 3.5–4.5 kg (Centro de Investigación en Reproducción Animal, Tlaxcala, Mexico) were housed in standard cages in same-sex groups with free access to food and water and were maintained on a 12:12 h light/dark cycle and at a constant temperature of 22–25 °C. Animal care and handling throughout the experimental procedures were in accordance with the European Union Directive of 24 November 1986 (86/609/EEC).

4.2. Histology

When the subjects were about 290 days old, males and females (no estrous phase) were deeply anesthetized with an intraperitoneal injection (250 mg/kg) of tribromoethanol (Sigma Aldrich) and perfused intracardially with saline (0.9%) followed by 4% paraformaldehyde (Panreac) in PBS (Panreac). The brains were removed and stored in paraformaldehyde for 2 days followed by 3–5 days in 30% sucrose (Sigma Aldrich) at 4 °C. The brains were frozen and coronally sectioned at a thickness of 40 μm . All sections were stained with a 0.1% solution of cresyl violet (Merck) brought to pH 4 with glacial acetic acid (Panreac).

4.3. Quantitative analysis

Stereological methods were used to determine the volume of the AOB, BST subdivisions, BAOT, Me, C₃ and LC. It was not possible to apply stereological methods to estimate the total number of neurons in the LC due to the great dispersion of the cells, but the total number of neurons of the AOB, BST subdivisions, BAOT, Me and C₃ was estimated using the dissector and fractionator techniques (Gundersen et al., 1988; Sterio, 1984). These morphological parameters were measured with a Diaplan Leitz microscope (Olympus BX51 for the AOB) with a computer-controlled stage (MultiControl 2000; Mörzhäuser Wetzlar, Germany) allowing randomly chosen steps to be

generated on the x and y axes. This specially fitted rotating stage allows the slices to be shifted by 360°, independently of the x–y movements. The stereological software package (CAST2, version 0.9, Olympus, Denmark for the AOB; GRID; Interactivision, Denmark for the rest of the structures) makes it possible to superimpose the required grid patterns over the microscope image. Finally, an electronic microcator (Heidenhain, Germany) with a resolution of 0.5 μm was also attached to the microscope so the z axis measurements of the stage could be taken.

4.4. Volume measurement

The Cavalieri principle was used to estimate the AOB, BST subdivisions, BAOT, Me, C₃ and LC volume (Michael and Cruz-Orive, 1988). On each coronal section studied (only every other section for the BAOT, every fifth section for the BST, Me and LC and every fifteenth section for the C₃; the first section was randomly selected in all cases), a set of points (generated by the GRID system) was systematically placed and the points that coincided within the study area were counted. The total volume was obtained by multiplying the number of points by the area associated with each (AOB total volume: 154,195.6 μm^2 ; AOB layers volume 44,030.2 μm^2 ; BST: 18,468 μm^2 ; BAOT: 13,582 μm^2 ; Me: 32,928 μm^2 ; C₃: 36,936 μm^2 and LC: 3036 μm^2) and by the distance between the two sections being counted. This distance was obtained from the product of the cut thickness (40 μm) by the sampling interval in each structure.

4.5. Estimation of the number of neurons

The number of neurons in the AOB, BST subdivisions, BAOT, Me and C₃ was estimated using the optical fractionator, which combines the optical dissector and fractionator techniques (Gundersen et al., 1988; Sterio, 1984). The sections were cut with a cryostat to a thickness of 40 μm , but their actual width after using the microcator was approximately 20–25 μm . The optical disector was used as follows: at $\times 186$, magnification frames (dissectors) were generated over the area with a horizontal step of 120 μm and a vertical step of 180 μm for the AOB; 120 \times 120 for the BST; 90 \times 50 for the BAOT; 120 \times 160 for the Me; and 200 \times 150 for the C₃. All of the frames that included the nuclei surface were considered. The profiles (cell nuclei) that were completely enclosed in the test frame ($A(f) = 1445.7 \mu\text{m}^2$ for AOB mitral cells; $A(f) = 289 \mu\text{m}^2$ for AOB granular cells; $AA(f) = 842 \mu\text{m}^2$ for BST, BAOT, Me and LC; $A(f) = 631 \mu\text{m}^2$ for the C₃) and those intersected by the inclusion edges at an $\times 4323$ magnification were considered. The height of the dissector was 12 μm . Finally, the total number of neurons in all structures was obtained by applying the fractionator formula:

$$\sum Q = 1/ssf \times 1/asf \times 1/hsf$$

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. An observer, unaware of the group membership of each specimen, determined the volume and the number of neurons in each slide.

The estimation of the total number of neurons in the LC was performed with the following formula: $n_t = n_s \times P$ using a correction of split counting units following Abercrombie. In this

formula, n_t is the estimated total number of cells; n_s is the number of cells in each section counted and P is the frequency of seriation used. For the AOB, BAOT, BST, Me, C₃ and LC, Ling et al. (1973) criteria were used to discriminate between neurons and glial cells.

4.6. Statistical analysis

Volume and number of neurons within each region were submitted to a two-tailed *t* test. When there were no homogeneity of variance, the Mann–Whitney *U* test to compare group medians was used.

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