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Research report

Heterogeneities of size and sexual dimorphism between the subdomains of the lateral-innervated accessory olfactory bulb (AOB) of *Octodon degus* (Rodentia: Hystricognathi)

Rodrigo Suárez [a,b,*], Jorge Mpodozis [a]

[a] *Departamento de Biología, Facultad de Ciencias, Universidad de Chile, Chile*
[b] *Escuela de Postgrado, Facultad de Medicina, Universidad de Chile, Chile*

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ABSTRACT

The vomeronasal system (VNS) of rodents participates in the regulation of a variety of social and sexual behaviours related to semiochemical communication. All rodents studied so far possess two parallel pathways from the vomeronasal organ (VNO) to the accessory olfactory bulb (AOB). These segregated afferences express either Gi2 or Go protein α-subunits and innervate the rostral or caudal half of the AOB, respectively. In muroid rodents, such as rats and mice, both subdivisions of the AOB are of similar proportions; as there is no anatomical feature indicative of the segregation, histochemical detection has been required to portray its boundary.

We studied the AOB of *Octodon degus*, a diurnal caviomorph rodent endemic to central Chile, and found several distinctive traits not reported in a rodent before: (i) the vomeronasal nerve innervates the AOB from its lateral aspect, in opposition to the medial innervation described in rabbits and muroids, (ii) an indentation that spans all layers delimits the boundary between the rostral and caudal AOB subdivisions (rAOB and cAOB, respectively), (iii) the rAOB is twice the size of the cAOB and features more and larger glomeruli, and (iv) the rAOB, but not the cAOB, shows male-biased sexual dimorphisms in size and number of glomeruli, while the cAOB, but not the rAOB, shows a male-biased dimorphism in mitral cell density.

The heterogeneities we describe here within AOB subdomains suggest that these segregated regions may engage in distinct operationalities. We discuss our results in relation to conspecific semiochemical communication in *O. degus*, and present it as a new animal model for the study of VNS neurobiology and evolution.

1. Introduction

The vomeronasal system (VNS) of mammals is a senso-effector neuronal network that participates in the perception of semiochemicals, commonly called pheromones, and in the generation of bodily responses, such as oestrus induction or pregnancy block (for review see refs. [2,29]). Although both the main olfactory and vomeronasal systems participate in the neuroendocrine and behavioural functions, the sole perturbation of some of VNS components may severely disrupt many aspects of socio-sexual behaviours [4,7,16,35,59].

The sensory surface of the VNS is the vomeronasal organ (VNO), a tubular structure located bilaterally at the base of the nasal septum that sends primary axons to the accessory olfactory bulb (AOB). Fluids containing semiochemicals may reach the VNO of exploring animals by means of a vascular pumping mechanism [45,46], and physical contact with an odorous source is thought to be required to activate the system [36,48].

Two distinct populations of vomeronasal receptor neurons (VRN) are anatomically segregated in the neuroepithelium of the VNO and project to different subdomains of the AOB. VRNs with apically situated somata express a vomeronasal receptor protein of the V1R family, which is coupled to Gi2 α protein, and send projections to glomeruli of the rostral aspect of the AOB (rAOB); whereas basally located VRNs express receptors of the V2R family, are associated to Go α protein, and project to the caudal AOB (cAOB) [1,10,18,24,41,51]. The innervation of vomeronasal axons into the AOB is exclusively segregated in rostral and caudal regions, whose boundary has only been possible to define with the use of histochemistry or immunolabeling [8,17,31,49,60].

We studied the AOB of the new-world rodent *Octodon degus* (Rodentia; Hystricognathi; Octodontidae). The degu is a precocial, long-lived, diurnal and social rodent that makes active use of

* Corresponding author. Tel.: +56 2 978 7235; fax: +56 2 271 2983.
E-mail address: rsuarezsaa@gmail.com (R. Suárez).

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semiochemical signalling in social and sexual communication [5,11–15]. We found that the AOB of degus shows a bilobular organization and that these segregated regions differ in size and in the degree of sexual dimorphisms. We discuss several aspects of degus natural behaviour that may correlate with our findings.

2. Methods

2.1. Animals

A total of 24 adult individuals (195–286 g body weight) of *O. degus* were used in this study. F1 and F2 generations of a stock of animals captured near Santiago, Chile, were bred in captivity and maintained in an institutional animal facility under natural light and temperature conditions. They were kept in spacious cages (50 cm wide × 60 cm long × 40 cm high) with their siblings until adulthood. Water and food (rabbit pellets) were provided ad libitum. All the experimental procedures followed the "Principles of laboratory animal care" (NIH publication no. 86-23, revised 1985) and were approved by the faculty ethics committee (Comité de Ética de la Facultad de Ciencias, Universidad de Chile) according to Chilean legislation. The number of degus used was minimized, and every effort was made to reduce animal discomfort.

2.2. Tissue preparation

Sexually mature animals, i.e. older than 7 months, were deeply anesthetized with a mixture of ketamine and xylazine (2.4 and 0.4 ml/kg, respectively, i.p.) and perfused via the ascending aorta with a temperate solution of 0.1 M phosphate-buffered saline at pH 7.4 (PBS), followed by 4% paraformaldehyde in PBS. After a careful dissection of the brain and postfixation for at least 24 h in 4% PFA at 5 °C, we submerged the olfactory bulbs (OBs) in a 30% sucrose solution (w/v) in PBS until they sank (ca. 1–2 days). Then, we obtained sagittal slices 45 μm thick of the OBs using a freezing sliding microtome. Serial sections (from 10 males and 10 females) were mounted onto gelatin-coated slides, rinsed in dH_2O, dehydrated, stained with cresyl violet, cleared and coverslipped with Permount. All the slides were then coded.

2.3. Immunohistochemistry

Sagittal sections of the OBs were incubated free-floating in PBS with 0.05% Triton X-100 (PBST) and 0.3% H_2O_2 for 30 min, followed by 5% normal goat serum (NGS) in PBST for 1 h. The slices were then incubated with primary immunoglobulins raised against Gi2 α (1:200, cat no. sc-13534, Santa Cruz Biotechnology, Santa Cruz, CA) or Go α (1:200, cat no. sc-13532, Santa Cruz Biotechnology, Santa Cruz, CA) with 3% NGS in PBST for 16 h at 5° C. Subsequently, the sections were rinsed and incubated in biotinylated goat anti-mouse secondary antibody (1:200, cat no. sc-2039, Santa Cruz Biotechnology, Santa Cruz, CA) for 2 h and processed with the avidin-biotin complex (ABC Elite Kit; Vector Laboratories). The sections were reacted in PBS with 0.6 mg/ml of 3,3-diaminobenzidine (Sigma) and 0.003% H_2O_2 for 1–3 min.

2.4. Morphometrical measurements

Serial consecutive sections were examined under light microscopy and photographed using the SPOT camera and software (Spot Advanced, Diagnostic Instrument, Inc). For each section, we determined the surface area of both the complete AOB (from the vomeronasal nerve layer to the mitral cell layer in depth) and of the rostral subdivision of the AOB (rAOB). We estimated the area of the caudal AOB (cAOB) by subtracting rAOB from AOB. Then, we computed the volume of a section comprising eight consecutive 45 μm sections centered in the slice with maximum AOB area, thus spanning at least 80% of glomerular volume. The farther medial and lateral portions of the AOB were excluded from the analysis because its glomeruli were variable in number and because the rostro-caudal boundary was not evident. The investigator was blind to the identity of each series.

We counted and determined the diameter of glomeruli for each section at both rAOB and cAOB. We also estimated the density of projection neurons at both rAOB and cAOB by counting mitral cells in a restricted square area of 0.01 mm^2 at each subdivision.

2.5. Statistical analysis

Non-parametric statistics were performed to analyze the data, as they did not show a normal distribution; the Mann–Whitney *U*-test was chosen to compare between male and female values, and the Wilcoxon-matched pairs test was used to compare rostral and caudal regions of the AOB. All the statistical analyses were done using Statistica 6.0 (StatSoft Inc., Tulsa, OK). Data are presented as the mean ± one standard error.

2.6. Figure preparation

Photomicrographs were processed and assembled into figures by using Adobe PhotoShop CS3 (Adobe Systems, San Jose, CA). Images were cropped, resized, rotated and/or turned to grayscale for presentation purposes. Levels, contrast and brightness were adjusted when necessary.

3. Results

3.1. Bilobular segregation of the AOB of Octodon degus

The AOB of *O. degus* is a prominent structure embedded in the dorso-caudal extent of the main olfactory bulb (MOB). In relation with the MOB, the AOB of degus may perhaps be one of the largest amongst mammals (see Fig. 1). It is composed of five well-defined layers: the vomeronasal nerve (VNL), glomerular (GlL), external plexiform (EPL), mitral cell (ML), and granular cell (GrL) layers (Fig. 1A). Vomeronasal glomeruli are small and densely packed, as compared with glomeruli of the MOB, and are surrounded by abundant periglomerular cells, (Fig. 1B and C). Mitral cells are compactly distributed at the ML, although some mitral/tufted cells can be observed throughout the EPL. Granular cells are arranged in multiple clusters (5–8 sheets in depth) at the GrL, underneath the lateral olfactory tract (lot).

The rostral and caudal subdivisions of the AOB of *O. degus* are morphologically segregated by an indentation spanning all cellular layers, as depicted with arrowheads in Fig. 1A, thus defining two lobular subdomains. The segregation of the AOB coincides with the expression of G-protein α-subunits. Gi2 α showed immunoreactivity in the VNL and GlL of the rAOB only, whereas Go α was present in the VNL and GlL of the cAOB and in main olfactory glomeruli (see Fig. 2), as also reported in other species [56,62,64]. The expression of these proteins was exclusive to each subdomain of the AOB, and its boundary corresponded with the cellular indentation (arrowheads in Fig. 2D).

3.2. Size heterogeneity at the segregated AOB subdomains

The subdomains of the AOB of *O. degus* are highly disproportional. The volume of the rAOB was $0.289 \pm 0.009\ mm^3$, twice the size of the cAOB that measured $0.145 \pm 0.006\ mm^3$ ($Z = 6.96$, $p < 0.000001$; Fig. 3A). We compared the mean number and size of individual glomeruli at both subdomains, as a simple examination suggested differences. The mean number of glomeruli per section was 103 ± 2.5 for the rAOB, versus 62.7 ± 2.2 at the cAOB ($Z = 6.96$, $p < 0.000001$; Fig. 3B), and the mean diameter of rAOB glomeruli was 0.048 ± 0.001 mm, while glomeruli of the cAOB measured an average of 0.037 ± 0.001 mm ($Z = 5.91$, $p < 0.000001$; Fig. 3C).

3.3. Different degrees of sexual dimorphism at each AOB subdivision

The results regarding sex differences at degus AOB are summarized in Table 1. Volumetric estimations of the overall AOB showed significantly larger values for males than females ($Z = -2.15$, $p < 0.05$). However, when assessing sexual differences at each AOB subdivision we found that the rAOB, but not the cAOB, presented a larger mean volume in males than females ($Z = -2.15$, $p < 0.05$; Table 1), thus suggesting that the rAOB solely accounts for the dimorphic volumes observed in overall AOB.

The number and size of glomeruli also differed between sexes and subdivisions: males had more glomeruli than females, both at the rAOB ($Z = -3.01$, $p < 0.003$) and at the cAOB ($Z = -2.52$, $p < 0.02$). However, although males had larger glomeruli at the rAOB than females ($Z = -2.67$, $p < 0.008$), glomerular size at the cAOB showed no sex difference (see Table 1). However, the density of mitral cells showed an opposite pattern of male-biased dimorphism: although no sex differences in cell density were found at the rAOB ($p = 0.083$),

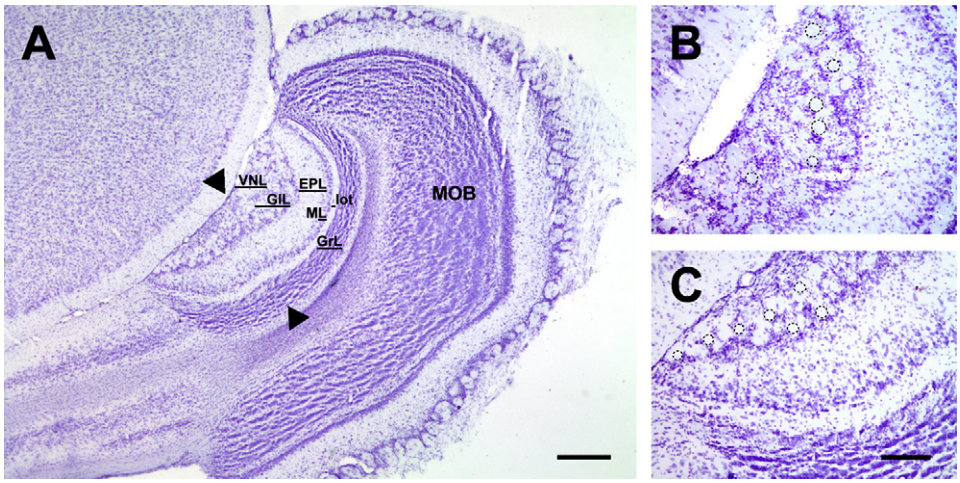

Fig. 1. Photomicrograph of a sagittal section through the olfactory bulb of *Octodon degus*. (A) The bilobular organization of the segregated accessory olfactory bulb (AOB) is defined by a cellular indentation, as depicted by arrowheads. Note the size of the AOB in relation to the MOB. (B) Enlarged view of the rostral, and caudal (C) AOB subdomains, showing individual glomeruli surrounded by periglomerular cells. VNL, vomeronasal nerve layer; GlL, glomerular layer; EPL, external plexiform layer; ML, mitral cell layer; lot, lateral olfactory tract; GrL, granular cell layer; MOB, main olfactory bulb. Lines underlying layers in (A) indicate the spatial extent of each. Scale bar: 500 μm (A), 200 μm (B and C).

the cAOB of males showed a higher cell density than that of females ($Z = -2.24$, $p < 0.026$).

Body mass of captive degus is larger in males than females (258.0 ± 6.37 g vs. 232.8 ± 8.45 g; $Z = -2.12$, $p < 0.05$). However, it has been reported that degus show no sex differences in overall brain dimensions [65]. In this study we scored three additional brain measurements: maximum cerebral width, maximum cerebral height, and ventral mesencephalon width, but found no sex difference ($Z = -1.14$, $p > 0.26$; $Z = -0.79$, $p > 0.44$; $Z = -1.84$, $p > 0.08$, respectively). We also assessed whether the season in which the animals were sacrificed affected AOB measurements, but found no effect (not shown).

3.4. Lateral innervation of the vomeronasal nerve into the AOB

Unexpectedly, and in opposition to what has been described in other species, we found that the vomeronasal nerve of degus arrives at the AOB from its lateral margin. A series of sagittal sections, presented from lateral to medial and immunolabelled against Gi2 α is shown on Fig. 4A. The vomeronasal nerve can be seen entering

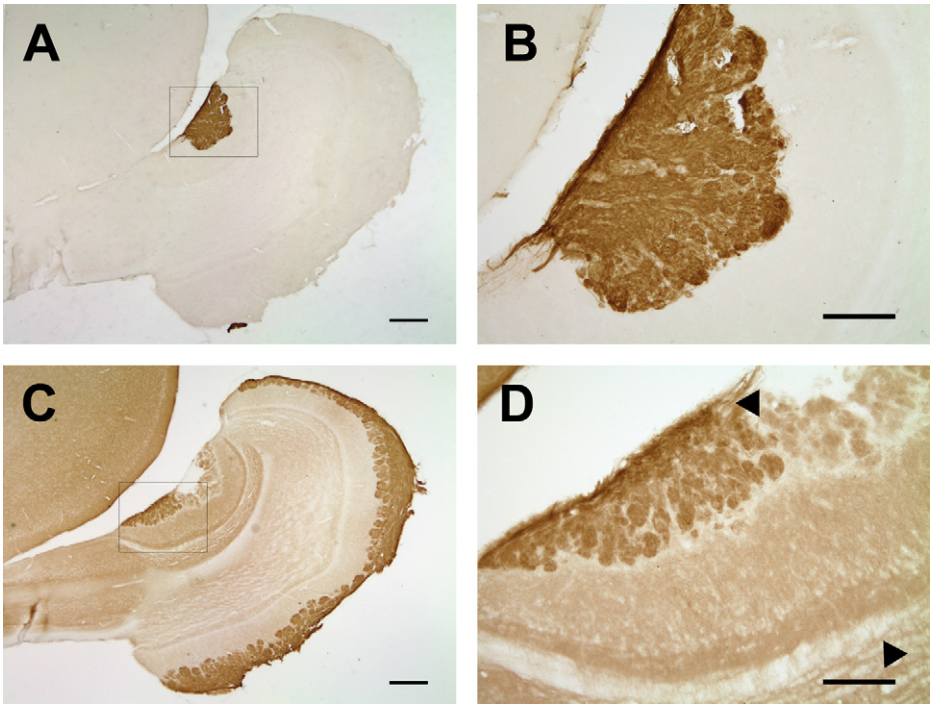

Fig. 2. G-protein α-subunit pattern of expression at the accessory olfactory bulb (AOB) of *Octodon degus*. (A) Gi2 α is expressed at the vomeronasal nerve and glomerular layer of the rostral AOB (rAOB) only. (B) Its margin of expression does not extend into the cAOB territory. (C) Go α expression is restricted to caudal AOB (cAOB) and main olfactory glomeruli. Although Go α expression can be observed to some extent throughout the olfactory bulb, the vomeronasal nerve layer of the rAOB completely lacks Go α expression. (D) The indentation across layers corresponds to the boundary between subdomains, as depicted by arrowheads. (B and D) correspond to an enlarged view of the area indicated in (A and C), respectively. Scale bar: 500 μm (A and C), 200 μm (B and D).

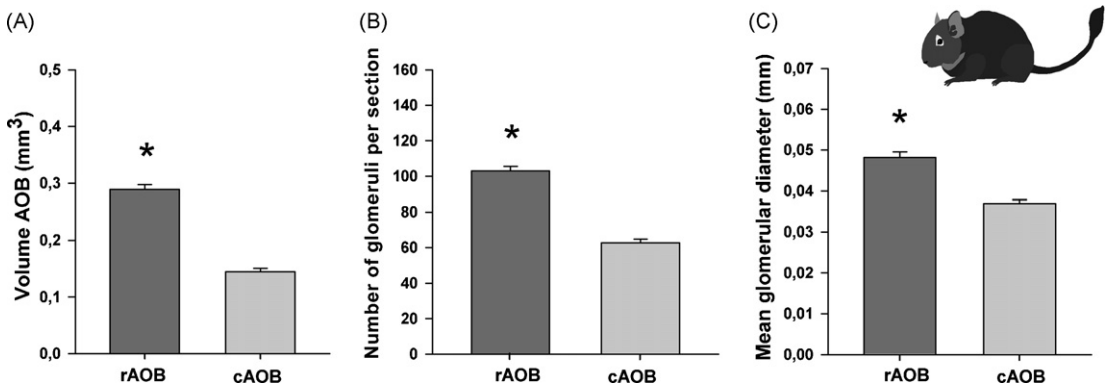

Fig. 3. The segregated subdomains of the accessory olfactory bulb (AOB) of *Octodon degus* are disproportional. (A) The rostral portion of the AOB (rAOB) is twice the volume of the caudal zone (cAOB). (B) Vomeronasal glomeruli are more numerous, and (C) larger in the rAOB than the cAOB. Data expressed as mean + S.E., $^{*}p < 0.000001$, Wilcoxon-matched pairs test.

into the AOB from its lateral margin to end in rostral glomeruli as advancing towards medial sections. After a maximum of area, the Gi2 α-positive staining becomes smaller to eventually disappear in medial-most sections. Fig. 4B shows a reconstruction of the left olfactory bulb, as if seen from above, after examining several sagittal and coronal series. The AOB lies just underneath the neocortex. Both the dorsal and ventral boundaries between the rAOB and cAOB are inclined towards the rostromedial aspect (continuous and dotted lines, respectively). The glomeruli of the rAOB are distributed rostrolaterally, while those of the cAOB are caudomedially distributed. The lateral innervation of the vomeronasal nerve into the AOB of degus contrasts with the medial innervation described in rabbits [19], mice [28,64], and rats [32], and whether it is a common feature of caviomorph rodents deserves further investigation.

4. Discussion

The AOB of *O. degus* shows several anatomical features that differ those of more studied old-world muroid rodents. While muroid AOB is lens-shaped with uniform-sized subdivisions, the AOB of degus is bilobular: an indentation spanning all cellular layers demarcates the rostral and caudal subdivisions. Moreover, the subdivisions of the AOB of degus present remarkable heterogeneities: the rAOB is twice as large as the cAOB and shows more accentuated sexual dimorphisms in terms of size and number of glomeruli. Furthermore, the lateral innervation of the vomeronasal nerve into the AOB, as opposed to the medial innervation described in rabbits and muroid rodents, leads to the supposition that this trait, together with those described above, might show variability across mammalian species.

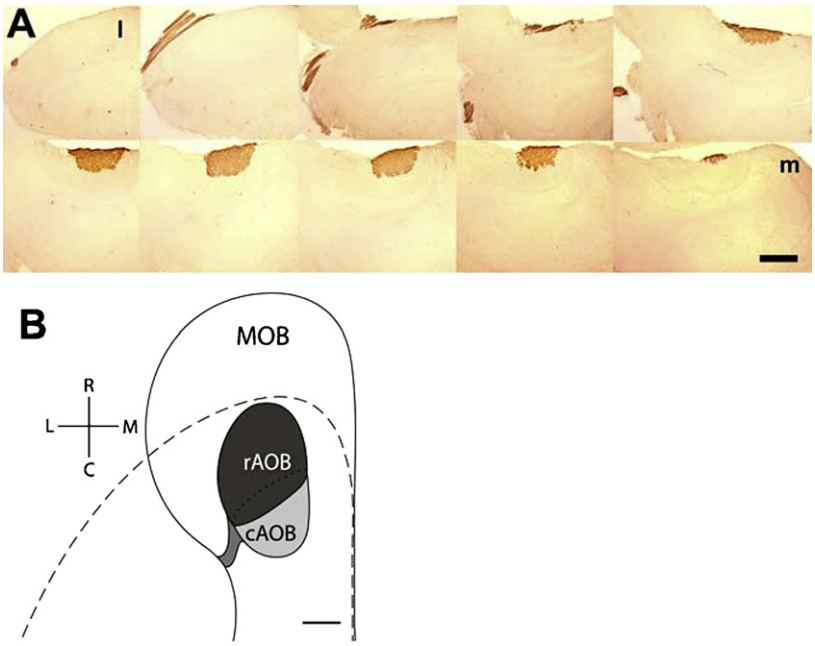

Fig. 4. Lateral innervation of the vomeronasal nerve into the accessory olfactory bulb (AOB) of *Octodon degus*. (A) A series of 40 μm sagittal sections, immunolabelled against Gi2 α and spaced at 160 μm intervals, is arranged from lateral (top left) to medial (bottom right). (B) Reconstruction of a left olfactory bulb of degus as if seen from the above. The boundary between both AOB subdivisions is tilted rostromedially. The ventral margin of the boundary is represented by a dotted line. The AOB lies just beneath the neocortex, which is represented by an interrupted line. These regions were reconstructed based on the observation of sagittal and coronal sections. MOB, main olfactory bulb; rAOB, rostral accessory olfactory bulb; cAOB, caudal accessory olfactory bulb; R, rostral; M, medial; C, caudal; L, lateral. Scale bar = 500 μm.

Table 1
Sexual dimorphisms in the accessory olfactory bulb (AOB) of *Octodon degus*.

	Morphometrical values (means ± S.E.)									
	Volume[a]			Mitral cell density[b]		Glomeruli number		Glomeruli diameter[c]		
	AOB	rAOB	cAOB	rAOB	cAOB	rAOB	cAOB	rAOB	cAOB	
Females ($n = 10$)	0.403 ± 0.02	0.267 ± 0.01	0.136 ± 0.01	17.3 ± 0.71	19.0 ± 0.81	96.23 ± 3.66	58.48 ± 3.51	0.045 ± 0.002	0.037 ± 0.002	
Males ($n = 10$)	0.456 ± 0.01*	0.304 ± 0.01*	0.151 ± 0.01	20.0 ± 1.18	22.4 ± 1.28*	110.90 ± 2.94**	67.57 ± 2.50*	0.052 ± 0.002**	0.037 ± 0.001	

AOB: accessory olfactory bulb, rostral (rAOB) and caudal (cAOB) subdivisions.
[a] Volume in mm^3.
[b] Number of neurons/0.01 mm^2.
[c] Diameter in mm.
* $p < 0.05$
** $p < 0.01$, Mann–Whitney *U*-test.

4.1. Functional significance of the segregated vomeronasal pathways

Vomeronasal neurons terminating in rAOB glomeruli express receptors of the V1R family that, like the main olfactory receptors, have a minute extracellular NH-terminal domain [10]. In this kind of receptors ligand binding is thought to occur at the membrane plane [18,41]. Accordingly, a diverse set of small lipophilic volatiles that elicit sex-related responses have shown to activate V1R neurons [30,33,49,61]. In contrast, V2R receptors have a large NH-terminal domain [18,41,51], and have shown to be responsive to large molecules such as protein fragments present in body secretions and urine [25,26,30,34].

It has been reported that volatile and non-volatile chemical stimulation elicit specific neuronal responses at the rAOB and cAOB, respectively [49,61]. Moreover, several studies have shown that the rAOB responds to semiochemicals in a sexual (male–female) context, while the cAOB would be involved in intrasexual (male–male) interactions [8,9,20–22,31,42,49].

As the AOB receives centrifugal projections from its recipient nuclei [40], as well as afferences from neuromodulatory nuclei such as the locus coeruleus [44], the behavioural milieu may affect neuronal activity at the AOB. Moreover, several nuclei in receipt of AOB afferences are known to be independently connected with hypothalamic nuclei involved in reproductive and defensive behaviours [3]. Under this scenario, a disproportionally large rAOB may be related either to a volatile form of chemical communication or to intersexual pheromonal appraisal, or both.

4.2. Social communication of Octodon degus and the disproportional AOB subdomains

O. degus is a diurnal group-living cursorial rodent that shows a structured social system [57,63]. While juvenile males disperse from their family group, females are phylopatric, i.e., they stay in a family burrow system. During the reproductive season (May–September), males defend and monopolize groups of 4–5 females [15] that engage in communal nursing of pups [13,23]. Non-aggressive male–male interactions, such as nose contact, can be observed in the field during the non-breeding season, but as the breeding season arrives all male–male interaction becomes aggressive [58]. *O. degus* scent-mark profusely their surroundings. They perform dustbathings near burrow entrances by rubbing rhythmically their flanks and ventrum against the soily substrate [15,27]. Dustbathing behaviour is affected by the sex and familiarity of past signallers [11,12], thus degus seem to engage in social communication by leaving chemical signatures on the soil.

As male–male body sniffing is seldom seen during the breeding season, we expect that non-contact social chemosignaling, like dustbathing, would be evolutionarily conserved as it minimizes aggression risk associated to male-male inspection. Indeed, male degus may use long-range chemosignaling, as captive males are able to discriminate urinary volatiles of virgin vs. sexually experienced males [57]. Thus, the disproportionally large rAOB may be related to a long-range volatile chemosignaling, as well as an intersexual, rather than male-male, pheromonal sampling with body contact, as might occur during copulation.

4.3. Sexual dimorphisms at the AOB and the mating system of degus

Sexual dimorphisms at the VNS have been described in rats [52,53], mice [55], voles [37], rabbits [54] and opossums [38], and have been traditionally associated with an epigenetic function of the endocrine system (for review see refs. [52,53]). However, only

a few studies have discussed how aspects of natural history, such as the mating system or parental care, could relate to sexual dimorphisms at the VNS [6,37]. In polygynous species, such as degus, the pheromonal stimulation associated with copulation is likely to be more frequent and more diverse in males than females. Consequently, sexual dimorphisms in sensory systems may emerge as historical differences in sensory stimulation between the sexes. Our findings that the rAOB of males had larger and more numerous glomeruli than females, and that overall sex dimorphisms were more accentuated in the rAOB than the cAOB, support the notion that the rAOB is involved in intersexual pheromonal appraisal. Moreover, the fact that glomerular development depends on sensory stimulation [67], suggests that epigenetic mechanisms might determine the dimorphic pattern seen at the AOB. The transgenerational conservation of a particular behavioural relationship between and within the sexes in *O. degus* may explain the anatomical traits described here for this species, as it has been proposed as a systemic mechanism of phenotypic conservation throughout lineages [43].

Similar patterns of vomeronasal heterogeneity and dimorphism have been described before: in the rabbit, the rAOB is also larger than the cAOB [19], and both rats and mice have more genes of the V1R than the V2R vomeronasal receptor families [66]. Likewise, in adult rats, there is a greater incidence of neurogenic cells at the rAOB than at the cAOB in males only, and only the rAOB showed more newly generated cells in males than females [50]. Finally, male-biased size dimorphisms have also been reported to be more accentuated at the rAOB than at the cAOB of the marsupial opossum (*Monodelphis domestica*) [38].

To our knowledge, no study has shown more pronounced dimorphisms occurring at the cAOB as compared to the rAOB. Here, we present evidence for a male-biased dimorphism in terms of mitral/tufted cell density occurring at the cAOB, but not at the rAOB. Although the size of cAOB glomeruli did not differ between sexes, their number was larger in males than females, so the possibility of differences in neuronal branching or connectivity of mitral/tufted cells remains open.

The evidence presented in this work strongly supports the notion of an operational independence between AOB subdomains. The possibility that the neuronal projections of the segregated AOB subdomains of degus conserve their segregation at their recipient nuclei, such as is the case of rats [47], and opossums [39], is supported by the disproportional nature of AOB subdomains and deserves further examination.

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