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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)

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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.

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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

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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 ± 0.009 mm^3, twice the size of the cAOB that measured 0.145 ± 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 ($p = 0.083$),

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