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The age-dependent change in Olfactory periglomerular neuronal populations is not affected by interrupting subventricular neuroblast migration in adult rats

Jatziri I. Contreras-García[b], Leticia Rodríguez-Castañeda[a], Gisela Gómez-Lira[a], Rogelio Ramírez-Hernández[a,b], Horacio Villafán[b], Leticia Granados-Rojas[a], Gabriel Gutiérrez-Ospina[b,*], Julieta G. Mendoza Torreblanca[a,*]

[a] *Laboratorio de Neuromorfometría, Instituto Nacional de Pediatría, Insurgentes Sur 3700-C, Col. Insurgentes Cuicuilco, Del. Coyoacán, 04530 México, D.F., Mexico*
[b] *Departamento de Biología Celular y Fisiología, Instituto de Investigaciones Biomédicas and Grupo de Investigación en Células Troncales IMPULSA 02, Universidad Nacional Autónoma de México, 04510 México, D.F., Mexico*

HIGHLIGHTS

- The number of the four main periglomerular cells change in an age-dependent manner.
- These changes do not correlate with the volume of the glomerular layer.
- The lack of new subventricular neurons does not affect this process.
- Barrier implanted rats showed a transient increase in the number calbindin cells.

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ABSTRACT

The olfactory bulb (OB) is rich in the number and variety of neurotransmitter and neuropeptide containing cells, in particular in the glomerular layer. Several reports suggest that numbers of some periglomerular phenotypes could change depending on age. However, it is unclear whether the different classes of periglomerular interneurons are modified or are maintained stable throughout life. Thus, our first objective was to obtain the absolute number of cells belonging to the different periglomerular phenotypes at adulthood. On the other hand, the olfactory bulb is continously supplied with newly generated periglomerular neurons produced by stem cells located in the subventricular zone (SVZ) and rostral migratory stream. Previously, we demonstrated that the implantation of a physical barrier completely prevents SVZ neuroblast migration towards the OB. Then, another objective of this study was to evaluate whether stopping the continuous supply of SVZ neuroblasts modified the different periglomerular populations throughout time. In summary, we estimated the total number of TH-IR, CalB-IR, CalR-IR and GAD-IR cells in the OB glomerular layer at several time points in control and barrier implanted adult rats. In addition, we estimated the volume of glomerular, granular and complete OB. Our main finding was that the number of the four main periglomerular populations is age-dependent, even after impairment of subventricular neuroblast migration. Furthermore, we established that these changes do not correlate with changes in the volume of glomerular layer.

1. Introduction

The olfactory bulb (OB) is rich in the number and variety of neurotransmitter and neuropeptide containing cells, in particular in the glomerular layer. Periglomerular cells contain at least four neuroactive substances and two calcium binding proteins [16]. These neurons can be divided into three groups defined by their principal chemical content: γ-amminobutyric acid (GABA) immunoreactive (IR) neurons, calretinin (CalR)-IR neurons and calbindin (CalB)-IR neurons; where tyrosine hydroxyalse (TH)-IR – presumably dopaminergic – neurons are considered a subpopulation of GABAergic cells [16,17].

The number of the different periglomerular cell types depend on the age of the animal; several studies have demonstrated that the number of cells expressing CalB-IR and CalR-IR increase at 6 months of age, compared to the first month; afterwards their

Abbreviations: OB, olfactory bulb; IR, immunoreactive; CalB, calbindin; CalR, calretinin; TH, tyrosine hydroxylase; RMS, rostral migratory stream; SVZ, subventricular zone; BI, barrier implanted; GAD, glutamate descarboxylase; PB, phosphate buffer.

* Corresponding author. Tel.: +52 55 1084 0900x1425; fax: +52 55 1084 3883.
E-mail address: julietamt14@hotmail.com (J.G. Mendoza Torreblanca).

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number remains constant [13,15]. In this sense, De Marchis et al. [6] showed that CalB-IR neurons are preferentially generated during the early neonatal period in rats, whereas CalR-IR and TH-IR neurons are mainly generated at later periods. These data suggest that some periglomerular phenotypes could change depending on the age. However, it is unclear whether the different classes of periglomerular interneurons are modified or are maintained stable throughout life. Thus, our first objective was to obtain the absolute number of cells belonging to the different periglomerular phenotypes during adulthood.

The nature of periglomerular neuronal populations could be maintained in part by the renewal capacity of the OB, since it is continuously supplied with newly generated neurons [20,28] produced by stem cells mainly located in the subventricular zone (SVZ) [3,7] and rostral migratory stream (RMS) [2,9,22]. Previously, we demonstrated that the implantation of a physical barrier completely prevents SVZ neuroblast migration to the OB [22]. Thus, another objective of this study was to evaluate whether stopping the continuous supply of new neurons from the SVZ modified the number of the different periglomerular cell types throughout time.

In summary, we estimated the total number of TH-IR, CalB-IR, CalR-IR and GAD-IR cells in the OB glomerular layer at several time points in control and barrier implanted (BI) adult rats. In addition, we estimated the glomerular layer volume to establish whether there was a relationship between this parameter and the number of periglomerular neurons.

2. Material and methods

2.1. Animals and experimental groups

Adult male Wistar rats were kept under controlled temperature and illumination (light on 7:00 a.m., light off 18:00 p.m.) with free access to food and water. At 12 weeks (300–350 g of body weight), the rats were divided into two groups: control rats ($n = 13$) and barrier implanted rats (BI, $n = 13$). BI animals underwent surgical implantation of a physical barrier (see below). The animals were sacrificed at different times (see below). This research was conducted with strict adherence to NIH Guide for the Care and Use of Experimental Animals and was approved by the local Animal Right's Committee.

2.2. Surgical procedures and barrier implantation

At week 12, barrier implanted rats were handled as previously reported [22]. Briefly, the rats were anesthetized with halothane and placed in a stereotaxic frame. The heads were then washed, shaved and a medial incision (2 cm long) was made on the skull. After retracting both the skin and the temporal muscle, a medium-lateral opening was drilled into the right side of the skull (AP: 2.5 mm from Bregma). A polypropylene physical barrier (4 mm × 6 mm; 60–80 μm thickness) was then inserted and pushed down to reach the ventral part of the brain. The procedure was performed unilaterally in the right olfactory bulb of each animal. The barrier entirely separated the distal segment of OB and RMS from the rest of the brain, thus isolating RMS from SVZ (Fig. 1). Afterwards, the wound was sutured and cleaned and an antibiotic (Baytril 5%; Bayer) was administered to avoid post-surgical infections.

2.3. Tissue sampling and processing

Both groups were sacrificed 12 days ($n = 4$), 3 ($n = 5$), and 9 ($n = 4$) months after barrier implantation (3, 6 and 12 months of age). The animals were anesthetized with pentobarbital (140 mg/kg) and perfused through the heart with saline solution followed by

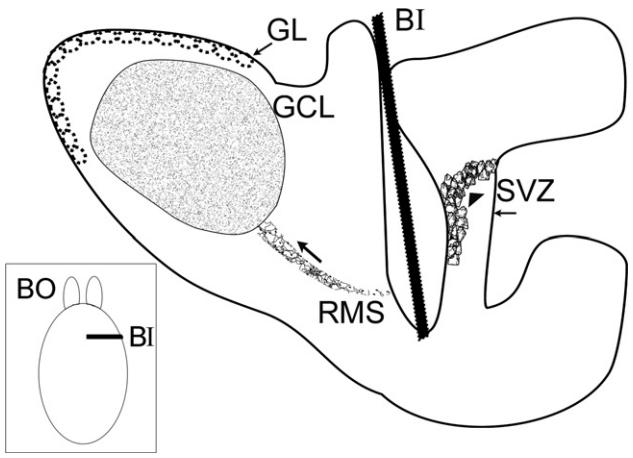

Fig. 1. Schematic representation of a sagittal section of an implanted rat. The black bar represents the physical barrier implant (BI). Notice the clear separation achieved by the BI and the thickening of caudal segment of the vertical limb of RMS and SVZ caused by the accumulation of migrating neuroblasts before the barrier (arrowhead). The insert shows a horizontal view of the site where the physical barrier was implanted in the right olfactory bulb (OB). GL, glomerular layer; GCL, granular layer; RMS, rostral migratory stream; and SVZ, subventricular zone.

buffered paraformaldehyde (4%). The brains were removed, post-fixed in the same fixative for 12 h, cryo-protected with sucrose (20% and then 30%), frozen in 2-methylbutane and stored at −74 °C. Coronal serial sections (50 μm thick) of the complete right OB (AP: 9.5–5.6 mm from Bregma) were cut and collected in phosphate buffer-saline. Afterwards, one of every tenth sections was systematically and randomly selected and were stained by immunocytochemistry for each marker tested (TH, CalB, CalR, GAD). A total of 10–12 sections per OB were considered for the stereological procedures.

2.4. Immunocytochemistry

Immunocytochemical procedures were carried out upon free-floating sections. The sections were always washed with phosphate buffer (PB) plus Triton X-100 (0.3%; PBT), except for those processed for GAD, which were washed only with PB. The sections were incubated with hydrogen peroxide (3%; Sigma) diluted in PBT or PB for 10 min at room temperature. After three washes (10 min each), the sections were incubated with a blocking solution (5% horse serum) for 60 min at room temperature and then incubated overnight at room temperature with the following primary antibodies (Millipore): goat anti-calretinin (1:7000), rabbit anti-GAD 65 (1:2000), rabbit anti-calbindin (1:5000), or rabbit anti-tyrosine hydroxylase (1:7000). Afterwards, the sections were washed three times (10 min each) and incubated either with anti-goat or anti-rabbit biotinylated secondary antibodies (1:5000) for 2 h at room temperature. Next, the sections were washed and incubated with the avidin–peroxidase complex for 1 h at room temperature and then washed and exposed to 3,3′-diaminobenzidine according to the manufacturer's recommendations (Vector Laboratories, Inc.). Sections were mounted and counterstained with methyl green (0.5%; Sigma). Finally, the slides were observed and digital photographs were taken using an Olympus BX5 microscope.

2.5. Stereology

A systematic random procedure termed as optical fractionator [27] was used to count the number of IR neurons of different phenotypes in the glomerular layer in the right OB. The size of the counting frame was 60 μm × 40 μm, its height was 15 μm and the guard zones were 1 μm; the size of the sampling grid was