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Segregated distribution of nitric oxide synthase-positive cells in the periglomerular region of typical and atypical olfactory glomeruli

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Abstract

Using double-staining techniques, the distribution of NADPH-diaphorase (ND)- and nitric oxide synthase (NOS)-positive cells was compared in the periglomerular region of typical and atypical rat olfactory glomeruli. Dorsomedial and ventrolateral atypical glomeruli contained similar number of ND/NOS-positive periglomerular cells. The number of ND/NOS-stained periglomerular cells was much higher ($P < 0.001$) in typical than in atypical glomeruli. The present results indicate that, in addition to described neurochemical and ultrastructural differences between the neuropile of typical and atypical glomeruli, there are also marked differences in the phenotype of periglomerular interneurons of both glomerular subsets.

Keywords: Acetylcholinesterase; Glomerulus; NADPH-diaphorase; Nitric oxide; Olfactory bulb; Rat

The olfactory glomeruli are functional integrative units processing olfactory information. It is likely that some localized glomeruli of the main olfactory bulb (OB) are involved in the processing of specific olfactory signals [11,13]. By using cholinergic markers such as choline acetyltransferase and acetylcholinesterase (AChE), a subset of 'atypical' glomeruli has been described in the dorsomedial and ventrolateral areas of the posterior half of the rat OB [8,9,12,13]. Atypical glomeruli differ from 'classical' ones in their ultrastructure, the spatial distribution of primary olfactory axons within the glomeruli, and by a remarkably intense and early cholinergic innervation from centrifugal fibers [8,9,13]. This heterogeneity in the glomerular population suggests the presence of a local specificity which may have functional implications [13]. In this sense, atypical glomeruli have been implicated in the selective processing of olfactory cues related to suckling behavior [13].

Nitric oxide synthase (NOS) is present in specific neuronal populations in the OB [7]. Using antibodies against neuronal NOS and oligonucleotide probes for NOS mRNA in combination with NADPH-diaphorase (ND) histochemical staining, a population of OB interneurons has been identified. These neurons colocalize ND and NOS [7]. At the glomerular and periglomerular levels, ND/NOS is expressed by subpopulations of periglomerular cells and superficial short-axon cells [1,7]. Nitric oxide has been proposed as a retrograde messenger [3], and in the OB it may be involved in the intraglomerular synaptic integration of sensory inputs [4]. Whether the neurochemical characteristics of the interneurons surrounding the atypical glomeruli are distinct from the interneurons surrounding the classical glomeruli is unknown. The combination of immunocytochemical and histochemical techniques for the detection of ND/NOS-containing cells with the AChE histochemical technique allows us to compare the expression of ND/NOS in the periglomerular region of both typical and atypical glomeruli. The main goals of this study are (1) to analyze whether ND/NOS-positive neurons are distributed in the same manner in the periglomerular regions of both typical and atypical glomeruli, and (2) to establish whether the distribution of ND/NOS-positive cells is similar in the dorsomedial and ventrolateral populations of atypical glomeruli.

Five adult male and five adult female Wistar rats weighing 250–270 g were used. Animals were deeply anesthetized with Ketolar (50 mg/kg b.w.), and perfused with 4% paraformaldehyde and 15% saturated picric acid

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in 0.1 M phosphate buffer (PB) (pH 7.4). OBs were post-fixed for 4 h in the same fixative.

Free-floating 30 μm coronal and sagittal sections were cut on a cryostat and processed for NOS-AChE or ND-AChE double labeling. Both NOS immunocytochemistry and ND histochemistry were used for the demonstration of ND/NOS-positive cells. AChE histochemistry was used to discriminate between typical and atypical glomeruli. For NOS immunocytochemistry, sections were sequentially incubated in: (a) primary antibody (anti-neuronal NOS, a generous gift from Drs. Emson and Charles) diluted 1:16 000 in PB (4 h at 4°C); and (b) fluorescein-labeled anti-sheep serum (Vector) diluted 1:50 in PB (1 h at room temperature). The primary antibody used recognizes neuronal NOS specifically in immunocytochemistry and Western blots. ND histochemistry and AChE histochemistry were carried out as described [1,5]. Controls for the immunostaining and histochemical procedures were also carried out [1,2,5]. For the double labeling, sections previously processed for NOS immunocytochemistry or for ND histochemistry were stained for the detection of AChE activity.

The maximum diameters of ND/NOS-stained neurons, as well as the maximum diameters of 50 sections of atypical and typical glomeruli were calculated with a semiautomatic image analysis system (MOP-Videoplan Kontron). For the OB of each animal, the numbers of ND/NOS-positive cells were counted in the periglomerular region of twenty atypical glomeruli and twenty typical glomeruli of the dorsomedial and ventrolateral areas. The glomeruli in each region were selected at random. The results were statistically analyzed by ANOVA. Values of $P < 0.001$ for Fisher PLSD and Scheffé-F tests jointly were considered statistically significant.

Although all olfactory glomeruli were surrounded by AChE-positive fibers, atypical glomeruli were readily distinguished from the 'classical' ones by a very dense neuropile of AChE-containing fibers (Fig. 1c,e). Atypical glomeruli were located in the ventrolateral and dorsomedial areas of the caudalmost sections of the OB, and were larger (198.63 ± 6.52 μm in maximum diameter) than the adjacent typical ones (140.51 ± 5.56 μm). Although the olfactory fibers innervating a subpopulation of typical glomeruli in the dorsomedial region of the OB were ND-positive, no olfactory axons entering the atypical glomeruli were ND-reactive. No olfactory axons (neither the ND-positive nor the ND-negative olfactory axons) were NOS-immunoreactive.

In the periglomerular region of the rat OB, numerous cells demonstrated both ND and NOS staining. After ND/NOS histochemical/immunocytochemical labeling, the same neurons were stained (Fig. 1a,b). Most ND/NOS-stained neurons were typically small sized (7–9 μm) with the morphological characteristics of periglomerular cells (Fig. 1d). This neuronal type was found surrounding both typical and atypical glomeruli. The size and the morphological characteristics were similar in the periglomerular cells innervating typical glomeruli and in those found in the periglomerular region of the atypical ones. With a similar location to the periglomerular cells, another neuronal population was ND/NOS-positive. Their size, which was larger than periglomerular cells (15–18 μm), shape and dendritic pattern of these cells allowed us to identify them as superficial short-axon cells (Fig. 1a,b). Only a few ND/NOS-labeled superficial short-axon cells were found in the periglomerular region of typical glomeruli (one superficial short-axon cell per >50 glomeruli). After mapping 150 ND/NOS-positive superficial short-axon cells, all of them were found in the periglomerular region of typical glomeruli, and no ND/NOS-positive superficial short-axon cells were observed surrounding the atypical ones.

The quantitative analysis (Table 1) demonstrated that the number of ND/NOS-positive periglomerular cells innervating typical glomeruli was higher than the number of periglomerular cells innervating the atypical ones, the differences being statistically significant ($P < 0.001$). On the other hand, statistically significant differences were not found ($P > 0.001$) between the distribution of ND/NOS-labeled periglomerular cells surrounding dorsomedial or ventrolateral atypical glomeruli. Similarly, statistically significant differences were not detected ($P > 0.001$) between the distribution of ND/NOS-positive periglomerular cells in the periglomerular region of typical glomeruli from the dorsomedial and ventrolateral areas. These results indicate that typical and atypical glomeruli differ markedly in the number of ND/NOS-positive periglomerular cells, whereas both dorsomedial and ventrolateral atypical and typical glomeruli are homogeneous groups in this respect.

Neurochemical and ultrastructural differences in the glomerular neuropile in atypical glomeruli suggest distinct specificities in the integration of the olfactory information in these glomeruli. The high concentration of AChE-positive centrifugal fibers has been related to a strong modulatory influence from central structures on glomerular activity related to specific odorant cues [12]. On the other hand, the differences in the ultrastructure of olfactory axons in the atypical glomeruli suggest a spatial organization of the primary afferents and neuronal connections at the glomerular level [9,13]. The present results show that although a subpopulation of the olfactory fibers demonstrate ND-staining in the dorsomedial area of the rat OB, these fibers did not innervate any atypical glomeruli, confirming the presence of a defined spatial connectivity of olfactory axons at the glomerular level.

Periglomerular cells are the first-order interneurons modulating the integration of olfactory information in the glomeruli (see [6] for a review). A heterogeneous distribution of ND/NOS-positive periglomerular cells in the rat OB has not been previously described. A subpopulation of olfactory fibers is ND-positive and these fibers project

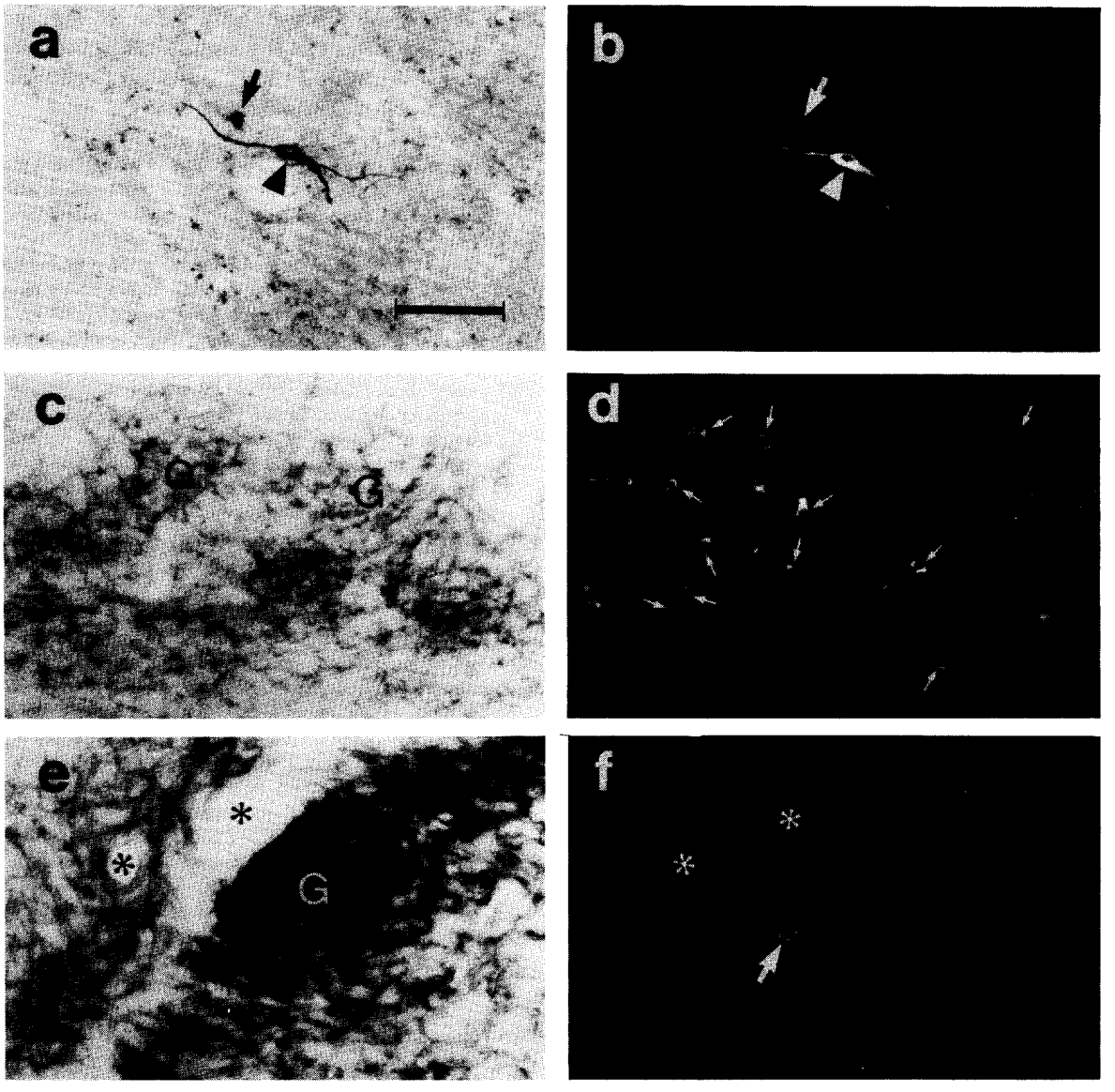

Fig. 1. Distribution of AChE staining, ND labeling, and NOS immunostaining in both typical and atypical glomeruli. Left panels show bright-field photomicrographs of ND- or AChE-stained glomeruli. Right panels show the same fields illuminated to reveal NOS immunofluorescence. Scale bar for all figures, 50 μm. (a) ND-stained periglomerular cell (arrow) and superficial short-axon cell (arrowhead) surrounding typical glomeruli. (b) Same field as in (a) showing that ND-positive cells are NOS-immunostained. (c) AChE-positive innervation of typical glomeruli (G). (d) Same field as in (c) showing NOS-immunopositive periglomerular cells (arrows). (e) Atypical glomerulus (G) showing the dense innervation of AChE-positive fibers. Two blood vessels are labeled for orientation (asterisks). (f) Same field as in (e) showing a NOS-immunopositive periglomerular cell (arrow).

exclusively to a subpopulation of olfactory glomeruli. The number of ND-positive periglomerular cells surrounding ND-positive and ND-negative glomeruli was similar [1]. Our current results confirm these previous observations. Nevertheless, the differential distribution of ND/NOS-stained periglomerular cells in relation to the typical and atypical glomeruli demonstrates that the spatial distribution of ND/NOS-positive periglomerular cells is not homogeneous. At the moment, atypical glomeruli are defined by the ultrastructure of their olfactory axons and the neurochemical characteristics of the centrifugal fibers [8,9,12,13]. Our data demonstrate clear differences

Table 1

Mean ± SEM of the number of ND/NOS-positive periglomerular cells per 30 μm-thick section of glomerulus

Typical glomeruli		Atypical glomeruli	
Dorsomedial	Ventrolateral	Dorsomedial	Ventrolateral
6.35 ± 0.18	6.50 ± 0.13	2.30 ± 0.15	1.78 ± 0.17

The number of ND/NOS-positive periglomerular cells was counted in 20 typical and in 20 atypical glomeruli of the dorsomedial and ventrolateral areas for the OB of each animal.

in the distribution of intrinsic elements in the periglomerular region of atypical glomeruli. This implies that atypical glomeruli differ from typical ones not only in the organization of primary afferents, but in the interneurons surrounding them.

The ultrastructural and neurochemical characteristics of both dorsomedial and ventrolateral atypical glomeruli are similar [8,9,12,13]. These similarities lead us to consider these atypical glomeruli as homologous. The identical distribution of ND/NOS-positive periglomerular cells surrounding both dorsomedial and ventrolateral atypical glomeruli supports this hypothesis.

A subset of dorsomedial glomeruli in the caudalmost rat OB has been defined as the 'modified glomerular complex' and this complex has been implicated in the processing of pheromonal cues relevant to suckling behavior in neonatal rats [11]. Zheng and Jourdan [13] have proposed that the atypical glomeruli might have morphofunctional homologies with the modified glomerular complex, and could be involved in similar functional processes. The spatial segregation of ND/NOS-positive periglomerular cells found in this report could be explained as directly or indirectly implicating nitric oxide in the function of these specific glomerular subsets. Breer and Shepherd [4] proposed that nitric oxide, by the induction of cGMP signals, could modulate at the glomerular level the neuronal response to odor stimulation in the olfactory mucosa. The low number of ND/NOS-positive periglomerular cells surrounding atypical glomeruli suggest a limited involvement of nitric oxide-producing neuronal circuits in the modulation of olfactory signals related to suckling behavior. The accessory OB in mammals is implicated in the processing of olfactory information concerned with pheromone-like molecules important in kin-recognition behavior [4]. The species-specificity of pheromones and the limited variety of these molecules could imply that the accessory OB glomeruli are less involved in the discrimination between many different odors than the glomeruli in the main olfactory pathway. Interestingly, the number of ND-positive periglomerular cells in the accessory OB is much lower than in the main OB [4,10]. Thus, glomeruli involved in the processing of specific signals rather than in the modulation of varied olfactory information have a lower number of ND/NOS-positive periglomerular cells in both the main and the accessory olfactory systems.

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