



Atypical Olfactory Glomeruli Subset of the Rat: Quantitative Study and Organization of the Peripheral Afferents

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Abstract

The atypical glomeruli constitute a particular subset of olfactory glomeruli in the rat olfactory bulb which is mainly characterized by a strong centrifugal cholinergic innervation. In the present study, the topographical organization of the mucoso-bulbar projection of these glomeruli was analysed using small injections of WGA-HRP into the anterior nasal cavity of adult rats. The atypical olfactory glomeruli were visualized on adjacent bulbar sections using acetylcholinesterase histochemistry. A mean of 29 atypical glomeruli per bulb was observed in several areas of the posterior half of the olfactory bulb. Following the rostro-caudal axis of the olfactory bulb, the first atypical glomeruli were located in lateral positions, then in dorsal and ventral ones. The most posterior atypical glomeruli were located in the bulbar medial side. Concerning the projections from the periphery to the atypical glomeruli, various WGA-HRP patterns of labelling were observed. When the surface area of injection sites in the anterior part of the olfactory sheet was between 30 and 40 mm², half of the atypical population was labelled with the atypical glomeruli being heavily labelled. All sites of distribution previously described were represented. When the surface area of injection sites was inferior to 20 mm², only some positions distributed along the bulbar antero-posterior axis were represented. These atypical glomeruli were generally partially labelled. Taken together, these results suggest that, although atypical glomeruli are restricted in the posterior olfactory bulb, they receive peripheral projections diffusely organized along the antero-posterior axis of the olfactory mucosa. This profile was compared with that of other classical olfactory glomeruli. *Chem. Senses* 21: 303–312, 1996.

Introduction

In the main olfactory bulb of the rodents, the atypical olfactory glomeruli constitute an original subset of olfactory glomeruli whose functional implication in the processing of olfactory cues remains unknown (Zheng *et al.*, 1987). These glomeruli are mainly characterized by their intense centrifugal cholinergic innervation as revealed by acetylcholinesterase histochemistry or choline acetyltransferase immunocytochemistry (Zheng *et al.*, 1987; Ojima *et al.*,

1988; Le Jeune and Jourdan, 1991, 1993; Shinoda *et al.*, 1993). The atypical glomeruli are found on every aspect of the posterior part of the main olfactory bulb (Zheng *et al.*, 1987; Ojima *et al.*, 1988; Zheng and Jourdan, 1988; Le Jeune and Jourdan, 1991; Shinoda *et al.*, 1993). Interest has been focused on some atypical glomeruli located in the dorso-medial area at the medial border of the accessory olfactory bulb and being identified as the modified glomerular complex

(Teicher *et al.*, 1980; Greer *et al.*, 1982). Since the modified glomerular complex was identified in neonate rats using [14C]-2-deoxyglucose autoradiography and histological observation, it has been postulated that the entire atypical glomeruli subset may play a role in the processing of odor cues relevant to suckling behaviour (Le Jeune and Jourdan, 1991; Shinoda *et al.*, 1993).

Similar to 'classical' olfactory glomeruli, the atypical glomeruli receive afferents from the olfactory epithelium, since large instillation of wheat germ agglutinin conjugated with horseradish peroxidase (WGA-HRP) in the nasal cavity resulted in the labelling of both classical and atypical glomerular populations (Zheng and Jourdan, 1988). However, little is known on the olfactory neuroreceptors projecting to the atypical olfactory glomeruli. Previous light and electron microscopic studies of olfactory terminals in the atypical neuropil have shown that they have a homogeneous distribution and contain a significant quantity of dense-cored vesicles (Zheng *et al.*, 1987; Zheng and Jourdan, 1988; Le Jeune and Jourdan, 1993). These features strongly suggest the existence of a well-specified subset of neuroreceptors projecting to the atypical glomeruli (Zheng and Jourdan, 1988). Although several studies have extensively analysed the epithelio-bulbar projections using anterograde tract-tracing methods, no studies have reported the presence of labelled glomeruli in the posterior bulb corresponding to the atypical glomeruli (Land *et al.*, 1970; Land, 1973; Land and Shepherd, 1974; Stewart, 1985; Stewart and Pedersen, 1987). Since the injection sites were generally extended in these studies, the bulbar labelling might be too diffuse to allow the fine anatomical distinction of the atypical glomeruli.

The purpose of the present study was to further analyse the organization of peripheral afferents to atypical olfactory glomeruli in the adult rat using restricted injection sites in the main olfactory epithelium, the distribution of these glomeruli being revealed using acetylcholinesterase histochemistry.

Materials and methods

Experiments were performed on 11 adult Wistar SPR rats weighing 200–260 g (Iffa Credo, Les Oncins, France). Animals were anaesthetized with equithesin (0.3 ml/100 g body wt) and put on their back. Bilateral instillations of 0.5 µl of WGA-HRP (Sigma, 1% w/v in dimethylsulphoxide) were performed in the nasal cavity. WGA-HRP was injected through a thin catheter (0.3 mm diameter) connected to a 5 µl microsyringe. The end of the catheter was stretched

to reduce the flow rate. It was positioned at 5–7 mm deep to obtain injection sites in the anterior part of the olfactory sheet (eight animals) and at 1–2 cm deep to obtain injection sites in the posterior part (three animals).

Following a survival time of 24 h, animals were deeply anesthetized with sodium pentobarbital, perfused transcardially with a mixture of 1% glutaraldehyde and 1% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4), and then with a 10% sucrose solution in the same buffer. The nasal cavities and olfactory bulbs were dissected out. The olfactory bulbs were stored in 30% phosphate-buffered sucrose overnight at 4°C, while the nasal cavities were decalcified in 5% EDTA for 8–12 h prior to storing in the sucrose solution. Serial frontal sections of the nasal cavities (25 µm thick) were cut using a cryostat and thaw-mounted onto gelatin-coated slides. For the bulbs, sections were alternatively collected on two slides.

In order to study epithelial projections to the atypical glomeruli, WGA-HRP was detected by the tetramethylbenzidine method (Mesulam, 1978) on all sections of the nasal cavities and half of olfactory bulb sections. The distribution of the atypical olfactory glomeruli was identified on the alternative olfactory bulb slides using acetylcholinesterase histochemistry according to the procedure described by Tago *et al.* (1986). Briefly, after several rinses in maleate buffer (0.1 M, pH 6.0), the sections were incubated for 30 min at room temperature with acetylthiocholine iodide (25 µM, Serva Chem.) as substrate. Non-specific cholinesterases were selectively inhibited with ethopropazine (Sigma) added to the incubation medium at a concentration of 2×10^{-4} M. Sections were developed in Tris-HCl buffer (50 mM, pH 7.6) containing 0.04% 3–3' diaminobenzidine-4-HCl, 0.3% nickel ammonium sulphate and 0.003% H₂O₂. When ethopropazine was replaced by eserine (10^{-4} M), a total cholinesterase inhibitor, no staining was observed confirming the specificity of histochemical reaction. Finally, all sections were slightly counterstained with neutral red and mounted.

Data analysis was performed only on animals showing restricted WGA-HRP application. The bulb sections processed for AChE were enlarged and the labelled glomeruli plotted on these enlargements. The spatial distribution of acetylcholinesterase-labelled atypical glomeruli was analysed and their mean number evaluated for every bulbar location. Two glomeruli were counted when acetylcholinesterase labelling extended beyond 160 µm since the mean diameter of an olfactory glomerulus has been determined to be 130 (±30) µm in the adult rat (Royet

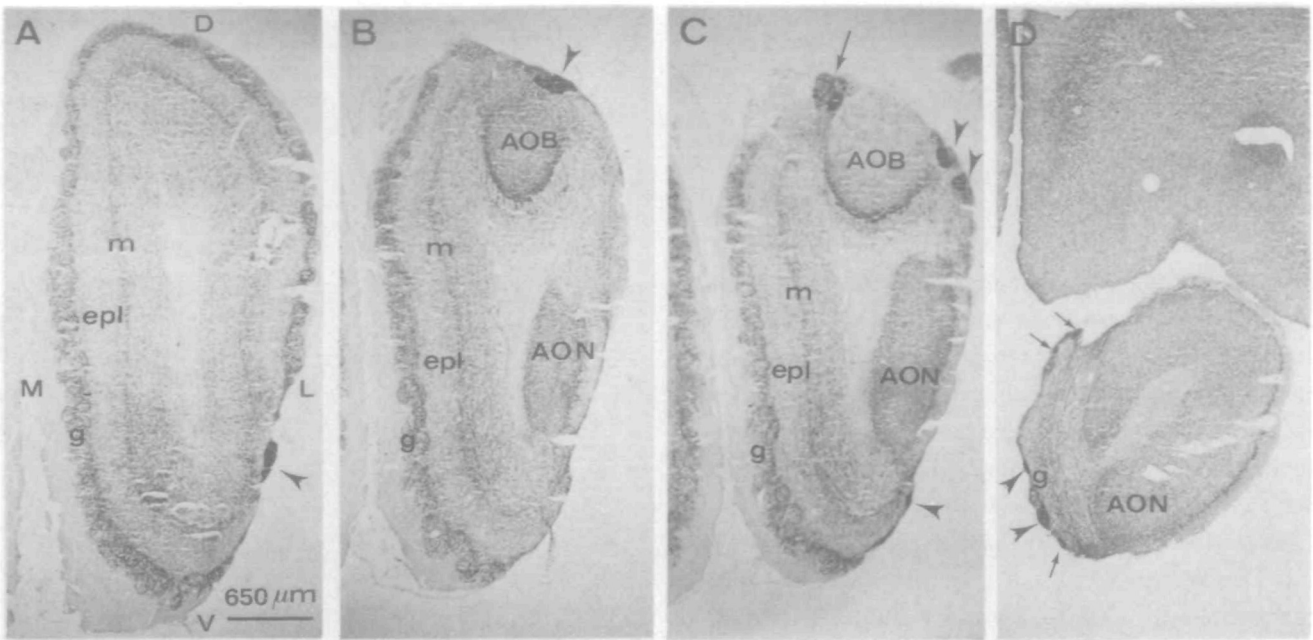


Figure 1 Photomicrographs of frontal sections of olfactory bulb reacted by using acetylcholinesterase histochemistry showing the antero-posterior distribution of atypical glomeruli **(A)** Anteriorly, these glomeruli were observed laterally (arrowhead). **(B)** Some atypical glomeruli were seen dorsally **(C)** More posteriorly, the atypical glomeruli were observed ventro-laterally, dorso-laterally (arrowheads) and dorso-medially, the group of acetylcholinesterase-reactive glomeruli (arrow) constitutes the MGC. **(D)** Posteriorly, atypical glomeruli are seen in a medial position (arrowheads). One can observe atypical glomeruli ending in dorso-medial and ventro-medial positions (arrows). AOB, accessory olfactory bulb; AON, anterior olfactory nucleus; epl, external plexiform layer, g, glomerular layer; m, mitral cell layer; L, lateral; D, dorsal; M, medial and V, ventral.

et al., 1989). The WGA-HRP-labelled epithelial projections to the glomeruli were then analysed. In order to determine the surface area of the injection site, serial sections of the nose were enlarged using an image analysis computer system (Imagenia, BIOCOM, France) and drawn to obtain a reconstruction of the rolled-out surface of injection sites following the antero-posterior axis of the mucosa. The bulb sections processed for WGA-HRP were also enlarged and the labelled glomeruli plotted on these enlargements as reported earlier (Giannetti *et al.*, 1992). The comparison of the obtained pattern with that of adjacent bulb sections processed with acetylcholinesterase histochemistry allowed the identification of the atypical glomeruli.

Results

Quantitative study of the atypical glomeruli along the antero-posterior axis of the adult rat olfactory bulb

The pattern of distribution of the strongly acetylcholinesterase-reactive atypical glomeruli is shown in Figures 1 and 2. These glomeruli were consistently identified in various areas in the posterior half of the olfactory bulb.

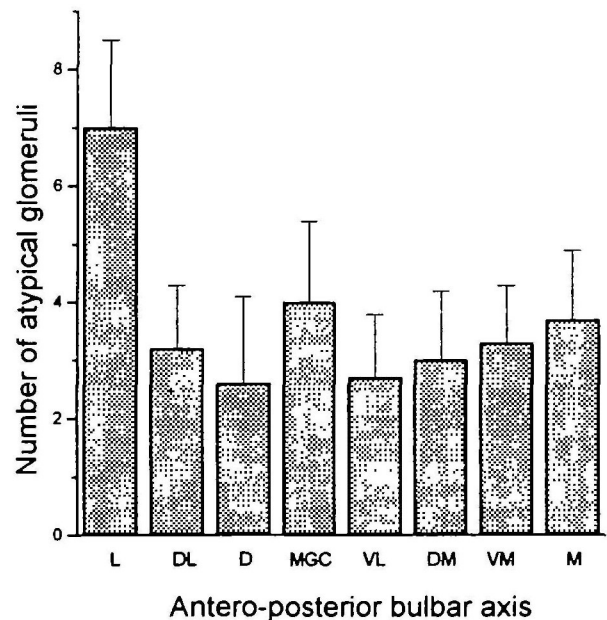


Figure 2 Mean number (\pm SD) of atypical glomeruli for each bulbar position along the antero-posterior bulbar axis. L, lateral; DL, dorso-lateral; D, dorsal; MGC, macroglomerular complex; VL, ventro-lateral; DM, dorso-medial; VM, ventro-medial; M, medial.

The most anterior atypical glomeruli were found in lateral position (Figures 1A and 2), where the lateral glomerular layer starts to disappear and the accessory olfactory bulb

begins to appear. More posteriorly, atypical glomeruli were observed outside the glomerular layer around the accessory olfactory bulb (Figure 1B, C). These glomeruli were either dorsal, dorso-medial or dorso-lateral (Figures 1B,C and 2). The dorso-medial atypical glomeruli located at the border between the main and accessory olfactory bulbs likely correspond to the modified glomerular complex (Figures 1C and 2). As the anterior olfactory nucleus develops, some atypical glomeruli were observed in a ventro-lateral position (Figures 1C and 2). Finally, atypical glomeruli were observed in medial positions with a second subgroup of dorso-medial glomeruli (Figure 1D), as well as medial and ventro-medial glomeruli in the more posterior bulbar part. At the caudal end of the bulb, where the glomerular layer persists only on the medial face, the last medial atypical glomeruli were found. The average (\pm SD) of atypical olfactory glomeruli was estimated to 29.5 (\pm 2.06) per bulb. As shown in Figure 2, each location corresponded to about three atypical glomeruli, except the lateral aspect where a mean of seven atypical glomeruli was counted.

Epithelial projections to the atypical olfactory glomeruli

Only eight injection sites were restricted enough to be analysed. They were all located in the anterior part of the olfactory sheet. According to their antero-posterior extent, two categories of injection sites were distinguished. The first one corresponded to four sites extending from the anterior dorsal recess to the anterior part of the endoturbinate II (Figures 3A and 4A,B). The surface area of these sites was estimated to be between 20 and 45 mm². The tracer was seen throughout the epithelium thickness, so that it could be assumed that all the neuroreceptors have taken up the tracer (Figure 3B). Only the posterior part of the injection site appeared less labelled (Figure 3C). The second category of injection sites consisted of sites restricted to the anterior dorsal recess (Figure 3D). In two sites of 5–6 mm², the tracer was apparently seen throughout the entire epithelial thickness. A representation is given in Figure 4C. In contrast, labelling of two other sites covering 12 and 14 mm² involved the whole epithelium only in the anterior half of the injection site and was sparse in the posterior half (Figure 4D).

At the level of the anterior olfactory bulb, the WGA-HRP labelled olfactory nerve terminals displayed a heterogeneous distribution pattern through many adjacent classical glomeruli (data not-shown). An antero-posterior fading was also noted. In the posterior olfactory bulb, the

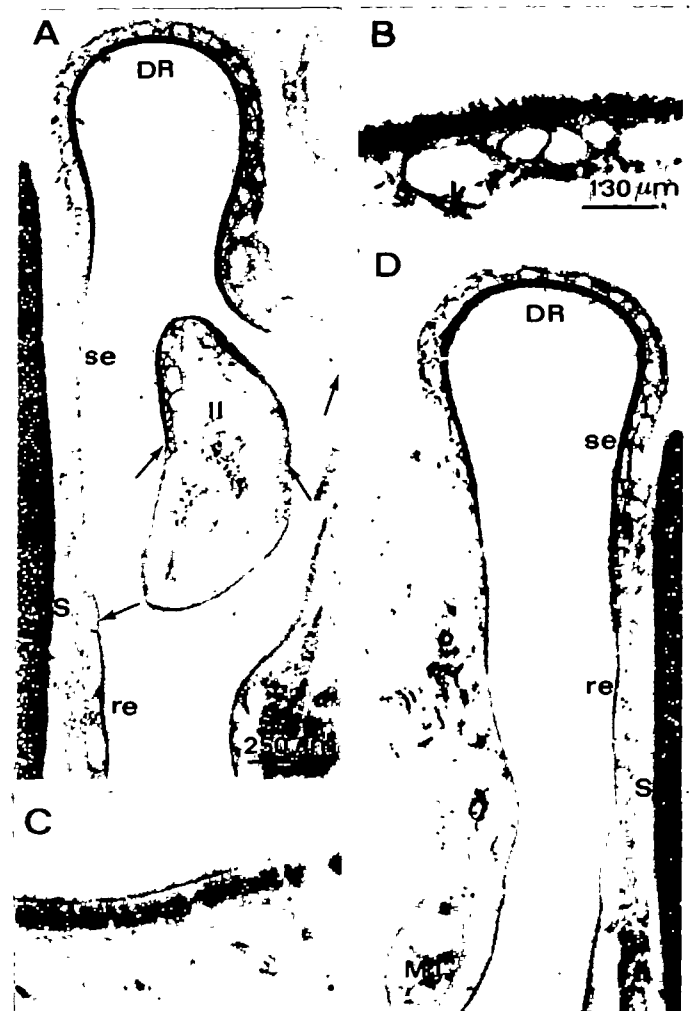


Figure 3 (A) Photomicrograph of a frontal section of a rat nasal cavity that shows the extent of the HRP-WGA injection site. The sensory epithelium (se) that lines the dorsal recess (DR) and the endoturbinate II (II) is delimited by arrows. The injection site extends on the dorsal part of the dorsal recess as well as the endoturbinate II. (B) At higher magnification, the labelling extends throughout the epithelial thickness. (C) In this example observed in the same animal but more posteriorly, the labelling was confined to the middle epithelial thickness and appeared not uniformly. (D) Photomicrograph of a frontal section of a rat nasal cavity in which the sensory epithelium (se) lining the dorsal recess (DR) was fully injected. MT, maxillary turbinate; re, respiratory epithelium; S, septum.

labelling was mostly expressed in the atypical glomeruli. Thus, with the more restricted injection sites, the labelled anterior classical glomeruli appeared isolated from the labelled posterior atypical ones. In contrast, the anterograde labelling of both of these glomerular populations overlapped for the larger injection sites. Regarding the glomerular WGA-HRP labelling in the posterior olfactory bulb, some olfactory glomeruli contained particularly intense staining (Figure 5). Their neuropil appeared massively built up by stained afferents (Figure 6A). The superposition of the

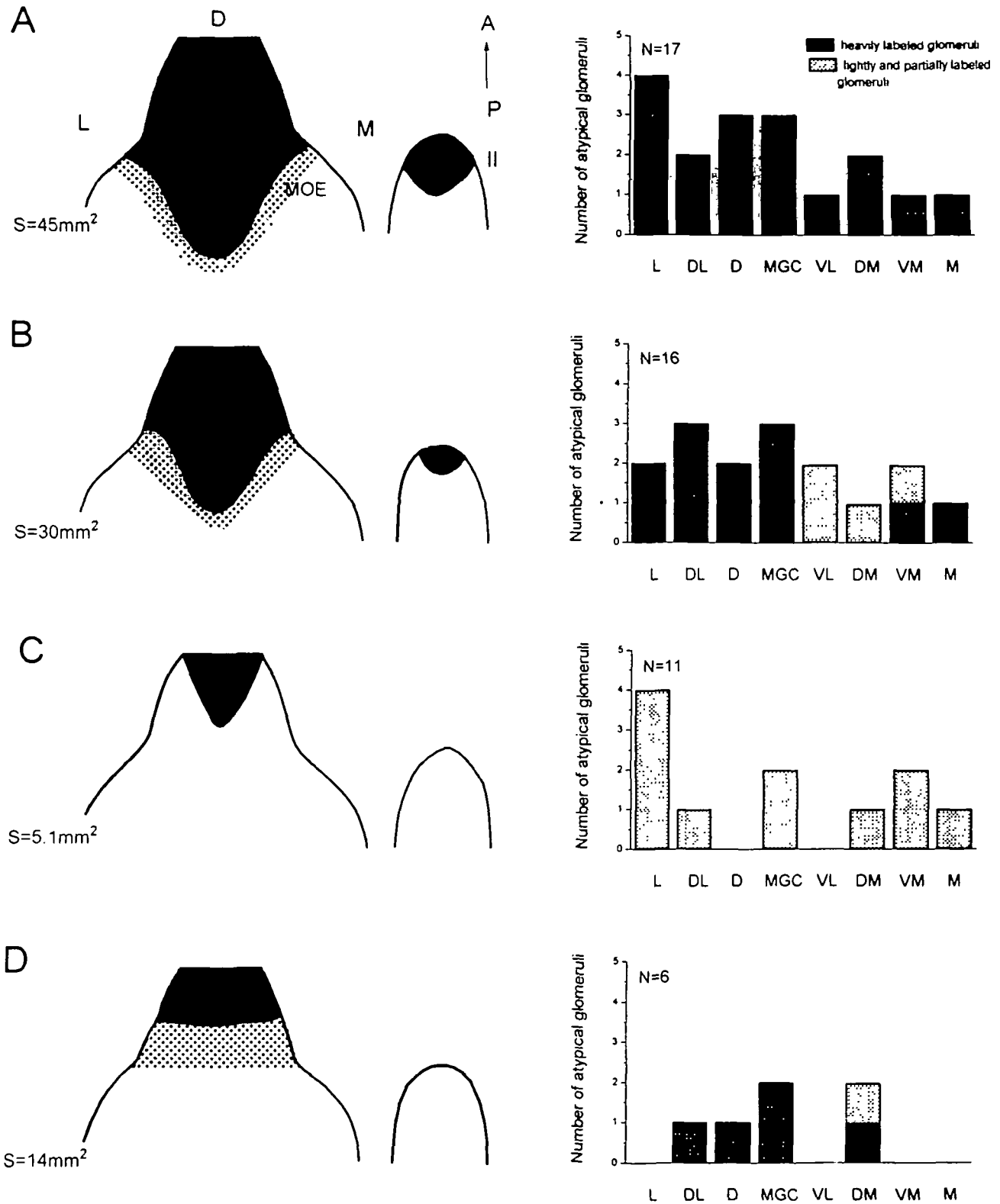


Figure 4 Four examples of injection sites (left part) and the corresponding distribution of the HRP-WGA-positive atypical glomeruli at the bulbar level (right part). On rolled-out reconstructions of the anterior part of the main olfactory epithelium (MOE) and the endoturbinate II (II), black areas represent regions fully injected, whereas dotted areas represent the regions partially injected. The total surface area of injection (S) was calculated. The distribution and the number (n) of atypical glomeruli presenting HRP-WGA labelling were reported. The histogram takes into account the heavily labelled glomeruli (dark grey), as well as the lightly and/or partially labelled ones (light grey). L, lateral; DL, dorso-lateral; D, dorsal; MGC, macroglomerular complex; VL, ventro-lateral; DM, dorso-medial; VM, ventro-medial; M, medial; A, anterior; P, posterior.

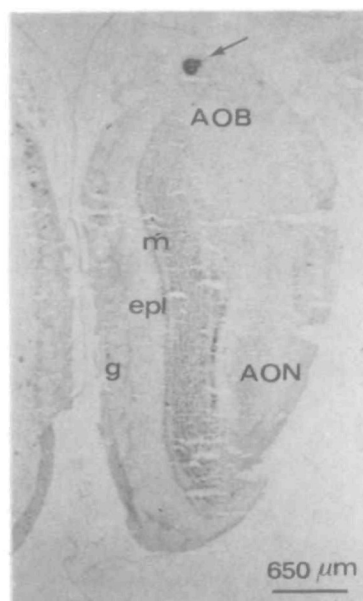


Figure 5 Photomicrograph of a frontal section of a rat olfactory bulb showing a heavily HRP-WGA labelled glomerulus in a dorso-medial position. The superposition of this section with the adjacent one showed in Figure 1C, allows us to say that this HRP-WGA-positive glomerulus (arrow) belongs to the MGC. AOB, accessory olfactory bulb, AON, anterior olfactory nucleus; epl, external plexiform layer; g, glomerular layer; m, mitral cell layer.

sections with adjacent sections processed by acetylcholinesterase histochemistry confirmed that they belong to the atypical glomeruli subset (comparison between Figures 5 and 1C). In other cases, the glomeruli were either partially labelled with only a part of the neuropil receiving massive WGA-HRP-positive afferents (Figure 6B) or weakly labelled with the whole neuropil displaying low density of WGA-HRP-reactive fibres (Figure 6C).

When the injection site was extended (20–45 mm²), the mean number of labelled atypical glomeruli was 15, representing half of their total number. In two cases, all glomeruli were heavily stained (Figure 4A), while one-third of them appeared either partially or lightly labelled in the two other cases (Figure 4B). In contrast, when the injection sites were more restricted (5–14 mm²), only some distributions were represented (Figure 4C–D). Most of the glomeruli (mean number 8) were lightly labelled, while a few of them still appeared strongly stained. Finally, in only one case, all atypical glomeruli receiving peripheral afferents were weakly stained (Figure 4C).

No topological relationship between the injection site and the distribution of WGA-HRP labelled atypical glomeruli was clearly observed. For example, at least two glomeruli of the modified glomerular complex were always labelled

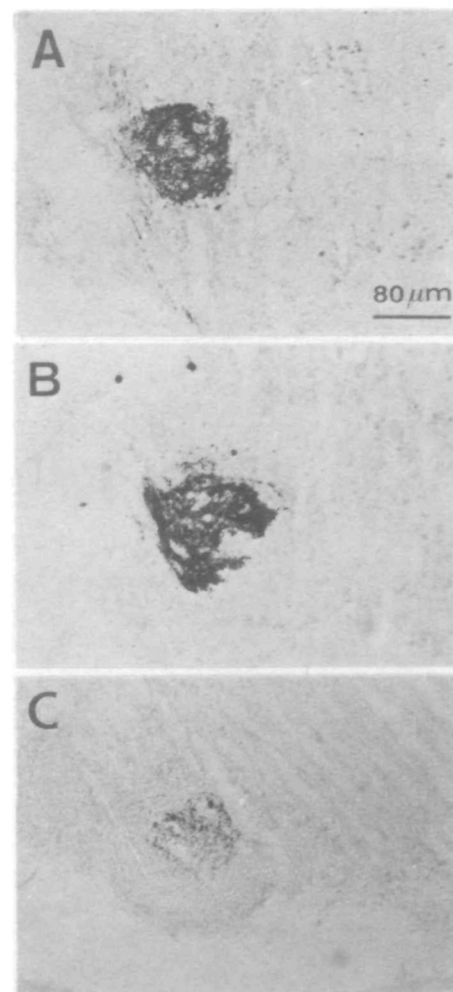


Figure 6 Photomicrographs of atypical glomeruli showing (A) heavily, (B) partially and (C) lightly HRP-WGA labelling

even for the smallest injection sites (Figure 4). Moreover, atypical glomeruli were generally seen in various positions distributed along the entire antero-posterior bulbar axis.

Discussion

In the present study, we have quantitatively analysed the distribution of the atypical olfactory glomeruli along the antero-posterior axis of the olfactory bulb. The results also revealed their original peripheral projections.

Results show that these glomeruli are exclusively located in the posterior half of the bulb, confirming previous studies using acetylcholinesterase histochemistry or choline acetyltransferase immunocytochemistry (Zheng *et al.*, 1987; Ojima *et al.*, 1988; Le Jeune and Jourdan, 1991; Shinoda *et al.*, 1993). Moreover, they consistently followed the same pattern of antero-posterior distribution. The more anterior atypical glomeruli were observed on the lateral face of the

olfactory bulb as reported previously (Zheng *et al.*, 1987; Shinoda *et al.*, 1993). Various atypical dorsal glomeruli were also seen surrounding the accessory olfactory bulb and among them, one could identify the modified glomerular complex in the dorso-medial position (Teicher *et al.*, 1980; Greer *et al.*, 1982). Finally, the most posterior atypical glomeruli were mainly located in ventral positions as previously demonstrated by Zheng *et al.* (1987), and also in a medial position as shown in the present study.

A mean of 29 atypical glomeruli were counted per bulb. This number was twice that reported by Ojima *et al.* (1988) using choline acetyltransferase immunocytochemistry. Differences in methodology such as the frequency of analysed sections (every 200 μm versus 40 μm for the present study) and the sensitivity of the immunocytochemical labelling could explain this discrepancy. For example, these authors reported two lateral atypical glomeruli, while seven were observed in the present study. We also demonstrated a second subgroup of dorso-medial atypical glomeruli distinct from the modified glomerular complex, while only two dorso-medial glomeruli were observed with choline acetyltransferase immunocytochemistry (Ojima *et al.*, 1988).

The more anterior subgroup of four dorso-medial atypical glomeruli well corresponds to the modified glomerular complex. This number does not seem very different from that observed in the young rats (Teicher *et al.*, 1980; Greer *et al.*, 1982), in spite of the important increase in the number of the classical olfactory glomeruli during the postnatal development (Meisami, 1979; Brunjes and Frazier, 1986). This suggests that the adult number of atypical glomeruli in the modified glomerular complex may be reached very early prior to those of other atypical and classical glomeruli. The ontogenic profile of cholinergic centrifugal innervation correlates with this assumption. Whereas most atypical glomeruli are innervated from the post-natal day 4 and classical glomeruli, only after post-natal day 6, the presence of cholinergic afferents is detected from postnatal day 1 in the dorso-medial area of the modified glomerular complex (Le Jeune and Jourdan, 1991). Taken together, these data support the involvement of this area from the first days after birth to adulthood.

WGA-HRP anterograde tract-tracing after intra-nasal injections is a well-established method to analyse the projections from restricted areas of the main olfactory epithelium to the bulb (Land *et al.*, 1970; Land, 1973; Land and Shepherd, 1974; Giannetti *et al.*, 1992). In the present study, the surface area of the injection sites (4–45 mm^2) was smaller than that of the most restricted injection sites

described elsewhere which involved several anterior sensory turbinates (Land *et al.*, 1970; Land, 1973; Land and Shepherd, 1974). The injection sites studied here corresponded to about 30% of the total epithelial surface estimated to represent approximately 120 mm^2 in the young adult rat (Meisami, 1989). Injection sites exclusively located in the more anterior part of the olfactory epithelium lining the dorsal recess, were analysed in the present study. Bulbar projections of the posterior injection sites were not analysed because WGA-HRP was generally taken up at the level of the dorsal recess while removing the injection catheter, which resulted in a fully injected mucosa.

In the glomerular layer of the anterior olfactory bulb, adjacent classical glomeruli presented considerable variations of the labelling density. Such a pattern suggests that the classical glomeruli are built up by afferents coming from various epithelial areas as it was already described for more extended peripheral injection or lesion sites (Le Gros Clark, 1951; Land, 1973; Land and Shepherd, 1974; Kauer, 1981; Stewart and Pedersen, 1987; Wellis and Scott, 1991) and by retrograde labelling of the main olfactory epithelium after restricted injections in the olfactory bulb (Astic and Saucier, 1986; Astic *et al.*, 1987; Schoenfeld *et al.*, 1994). The antero-posterior fading of the labelling also confirms that anterior epithelial areas mainly terminate on the anterior part of the bulb (Le Gros Clark, 1951; Land, 1973).

In contrast to the massive bulbar staining observed after large intra-nasal instillations (Stewart and Pedersen, 1987; Zheng and Jourdan, 1988), restricted injection sites resulted in a fine anterograde labelling of the posterior olfactory bulb. Following tracer application, labelling of the posterior olfactory bulb was mainly observed in atypical glomeruli. Two distinct types of glomerular labelling were observed. Concerning the fully stained atypical glomeruli, half of them received afferents from the anterior olfactory epithelium and it could be supposed that the atypical glomeruli are mainly, if not all, built up by afferents coming from this epithelial area. In contrast, the partially labelled atypical glomeruli may receive fascicles from various epithelial areas as already described in the classical glomerular layer (Le Gros Clark, 1951; Land, 1973; Land and Shepherd, 1974; Wellis and Scott, 1991). The non-labelled areas of these neuropils may correspond to fascicles of afferents from the turbinates or posterior dorsal recess as demonstrated for the MGC (Pedersen and Benson, 1986; Pedersen *et al.*, 1986).

Some glomerular subclasses have been identified using several methods. By immunohistochemical staining using monoclonal antibodies against glycoproteins or lactoseries

carbohydrates (Allen and Akeson, 1985a, b; Fujita *et al.*, 1985; Mori *et al.*, 1985; Schwob and Gottlieb, 1986), the glomerular subclasses corresponded to large glomerular regions extended on several bulbar faces. However, using simple tract tracing methods (Giannetti *et al.*, 1992) or examining immunohistochemical expression of the 70 kD heat shock protein (HSP70) family in the olfactory epithelium and bulb (Carr *et al.*, 1994), small subpopulations of olfactory neuroreceptors were labelled and observed projecting to small number of glomeruli (respectively 30 and 2). These glomeruli did not overlap at all the atypical glomerular subpopulation evidenced using an antibody against the human placental antigen (Shinoda *et al.*, 1989, 1993) or acetylcholinesterase histochemistry or choline acetyltransferase immunocytochemistry (Zheng *et al.*, 1987; Ojima *et al.*, 1988; Le Jeune and Jourdan, 1991) of which the glomeruli evidenced in the present paper belong to.

Interestingly, the analysis of the spatial distribution of labelled atypical glomeruli reveals the lack of a clear topographical organization of their mucoso-bulbar projection. Atypical glomeruli were observed in different antero-posterior positions, even with the most anterior and restricted injection sites. This observation strongly suggests that a small anterior part of the main olfactory epithelium may have diffuse projections to the atypical glomeruli distributed along the antero-posterior bulbar axis. In this, the sub-system of atypical olfactory glomeruli is similar to the main olfactory system in which the absence of transposition of the epithelial sheet to the rostro-caudal axis of the bulb has been shown (Le Gros Clark, 1951; Land, 1973; Saucier and Astic, 1986; Schoenfeld *et al.*, 1994). Such a dispersion of the neuroreceptors over the main olfactory epithelium projecting to some atypical olfactory glomeruli was previously reported using an antibody against the human placental

antigen (Shinoda *et al.*, 1989, 1993). Similarly, HRP injections in the modified glomerular complex area result in the labelling of neuroreceptors scattered on the olfactory sheet in spite of the large injection sites (Pedersen and Benson, 1986; Pedersen *et al.*, 1986). Finally, such diffuse distribution of epithelial projections has also been observed for the septal olfactory glomeruli which are observed in the ventro-medial bulbar aspect after total or partial injections of the septal organ (Giannetti *et al.*, 1992).

The difference between the atypical glomeruli projections and the classical projections may be related to the nature of their neuroreceptors. Ultrastructural features of the atypical glomeruli, in particular the organization and cytology of neuroreceptor axon terminals, might support the existence of a distinct subset of neuroreceptors ending here (Zheng and Jourdan, 1988). Olfactory axon terminals in the atypical glomeruli displayed at least two antigenic properties, since some atypical glomeruli constituting a necklace (Shinoda *et al.*, 1989), expressed immunoreactivity only to antibody raised against a human placental antigen (Shinoda *et al.*, 1993). To better understand the function of the atypical glomeruli, it would be interesting to know if specific olfactory cues bind on neuroreceptors located in the anterior part of the dorsal recess. Moreover, it has been suggested that these olfactory cues may be related to the reproduction function, since numerous luteinizing hormone-releasing hormone-containing fibres were observed in the areas of the atypical glomeruli (Zheng *et al.*, 1988). The establishment of a very precocious cholinergic innervation in atypical glomeruli is also in favour for their involvement in the suckling (Le Jeune and Jourdan, 1991). However, further studies are still required to precise the exact role of the atypical olfactory glomeruli in the adult and developing rat.

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