Heterogeneous targeting of centrifugal inputs to
the glomerular layer of the main olfactory bulb

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Abstract

The centrifugal systems innervating the olfactory bulb are important elements in the functional regulation of the olfactory pathway. In this study, the selective innervation of specific glomeruli by serotonergic, noradrenergic and cholinergic centrifugal axons was analyzed. Thus, the morphology, distribution and density of positive axons were studied in the glomerular layer of the main olfactory bulb of the rat, using serotonin-, serotonin transporter- and dopamine-β-hydroxylase-immunohistochemistry and acetylcholinesterase histochemistry in serial sections. Serotonin-, serotonin transporter-immunostaining and acetylcholinesterase-staining revealed a higher heterogeneity in the glomerular layer of the main olfactory bulb than previously reported. In this sense, four types of glomeruli could be identified according to their serotonergic innervation. The main distinctive feature of these four types of glomeruli was their serotonergic fibre density, although they also differed in their size, morphology and relative position throughout the rostro-caudal main olfactory bulb. In this sense, some specific regions of the glomerular layer were occupied by glomeruli with a particular morphology and a characteristic serotonergic innervation pattern that was consistent from animal to animal. Regarding the cholinergic system, we offer a new subclassification of glomeruli based on the distribution of cholinergic fibres in the glomerular structure. Finally, the serotonergic and cholinergic innervation patterns were compared in the glomerular layer. Sexual differences concerning the density of serotonergic fibres were observed in the atypical glomeruli (characterized by their strong cholinergic innervation). The present report provides new data on the heterogeneity of the centrifugal innervation of the glomerular layer that constitutes the morphological substrate supporting the existence of differential modulatory levels among the entire glomerular population.

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1. Introduction

Olfactory glomeruli are distinct structures in which the first synapse of the olfactory pathway occurs. An olfactory glomerulus constitutes an independent functional unit and is involved in the transmission and modulation of one or a few olfactory signals. Each olfactory receptor neuron (ORN) expresses one or a few molecule receptors (Buck and Axel, 1991) and the axons of the ORNs expressing the same molecular receptor converge in one or a few olfactory glomeruli (Ressler et al., 1994; Vassar et al., 1994; Mombaerts et al., 1996; Royal and Key, 1999). Olfactory glomeruli receiving the same type of olfactory axons have fixed positions in both main olfactory bulbs (MOBs) from the same animal and in MOBs from different animals. Therefore, any given specific glomerulus is localized in a precise topographic location (Schaefer et al., 2001) and hence specific odorants result in definite patterns of glomerular activity (Shepherd, 1994; Mori and Yoshihara, 1995). As regards this response to odours, the glomerular layer (GL) is heterogeneous, and indeed several groups of glomeruli have been identified according to their olfactory input. For example, the modified glomerular complex (MGC) is a small group of glomeruli that has been related to suckling behaviour, since it is specifically and selectively activated in pups during exposure to a lactation pheromone...
Additional glomerular groups have been defined using histochemical and immunohistochemical techniques, demonstrating specific subpopulations of ORNs that send their axons to glomeruli with fixed positions in the MOB. Examples of these groups are the glomeruli positive for human placental antigen X-P2 (hPAX-P2) (Shinoda et al., 1989), NADPH-diaphorase (Davis, 1991; Alonso et al., 1993, 1995, 1998; Hopkins et al., 1996) and NOC-3 (John and Key, 2001), among others. Another glomerular group has been defined as receiving axons from neurons that respond to odorants with a common straight-chain hydrocarbon structure (Johnson and Leon, 2000). Recently, using genetic approaches some works have identified glomeruli that respond to a specific odour (Schaefer et al., 2001; Bozza et al., 2002). Using this technology, Potter et al. (2001) performed three-dimensional reconstructions of specific olfactory glomeruli that respond to the same odorant, showing that mature glomeruli are not only heterogeneous neurochemically but that they also have a high degree of morphological variability.

Other evidence for glomerular heterogeneity is that related to the regulation of the transmission achieved in the glomeruli. For example, Zheng et al. (1987) demonstrated heterogeneity in the GL on the basis of the density of centrifugal cholinergic afferents to the olfactory glomeruli. Those authors described a glomerular population located in the ventrolateral and the dorsal borders of the caudal half of the MOB that is characterized by a high density of cholinergic afferents (atypical glomeruli), while most glomeruli contain a significantly lower density of acetylcholinesterase (AChE)- or choline acetyltransferase-positive fibres (typical glomeruli). Additional studies have shown that atypical glomeruli are not only different as regards their centrifugal innervation but also in terms of their olfactory afferents (Zheng and Jourdan, 1988; Juilfs et al., 1997) and their modulation by local interneurons (Crespo et al., 1996, 1997a,b). They receive axons from ORNs that have particular ultrastructural properties (Zheng and Jourdan, 1988) and use different second messengers for signal transduction (Juilfs et al., 1997). Atypical glomeruli have been related to the MGC and to hPAX-P2-positive glomeruli (necklace glomeruli) (Zheng et al., 1987; Shinoda et al., 1993), suggesting that they could be involved in the processing of specific olfactory cues, such as reproductive signals.

The modulation and/or processing of olfactory information occurring in the glomeruli is not only mediated by local circuits (interneurons); the centrifugal systems innervating the MOB also play a crucial role in these events. The cholinergic, serotonergic, and noradrenergic systems are involved in signal transmission in adult animals and during normal postnatal development. For example, noradrenaline converts weak olfactory nerve inputs that are unable to elicit a response into above-threshold ones (Jiang et al., 1996). It also appears to be necessary in early olfactory learning, in olfactory memory formation, and intraspecies olfactory recognition among individuals (Sullivan et al., 1989, 1992, 2000; Brennan et al., 1990). Serotonin (5-HT) has also been implicated in the normal transmission of olfactory information (Morizumi et al., 1994) and in different processes during the development of the nervous system, such as neurogenesis, neural differentiation (Whitaker-Azmitia et al., 1996), neural migration (Moiseiwitsch and Lauder, 1995), and synaptogenesis (Whitaker-Azmitia et al., 1996).

Acetylcholine (ACh) is involved in olfactory plasticity and is associated with olfactory learning (Kaba and Keverne, 1988). Additionally, ACh regulates the activity of the projection neurons of the olfactory bulb through an action on their modulatory interneurons, periglomerular cells, and granule cells (Nickell and Shipley, 1988; Elaagouby et al., 1991; Elaagouby and Gervais, 1992). All these data indicate that these centrifugal systems perform significant roles in signal transmission in the olfactory bulb.

Despite the importance of centrifugal systems in the physiology and development of olfactory structures, and whereas variations in the density of AChE fibres among different groups of olfactory glomeruli are known (Zheng et al., 1987), there are no data available about possible differences or segregated innervation patterns of the serotonergic and/or noradrenergic systems in the GL. Here we analyze the fibre morphology of these three extrinsic centrifugal systems as well as their distribution and the possible specific patterns within typical and atypical glomeruli in the MOB, using sequential labelling of the same glomeruli. The aim of this study was to determine whether each individual centrifugal system has a selective target innervation in the GL. Additionally, we compare; qualitatively and quantitatively, the serotonergic and cholinergic fibre densities in particular olfactory glomeruli, demonstrating a selective serotonergic innervation of the atypical glomeruli.

2. Materials and methods

2.1. Animals and tissue preparation

Ten adult (five male and five female) albino Wistar rats (180–230 g) were used in this study. In all cases, the experimental procedures conformed to NIH guidelines and were in accordance with the guidelines of the European Community Council Directive (86/609/EEC) and current Spanish legislation for the use and care of laboratory animals (BOE 67/8509-12, 1988). The animals were deeply anaesthetized with a mixture of ketamine (Ketolar, Parke-Davis, Barcelona, Spain) and tiletamine (Rompun, Bayer, Leverkusen, Germany), 10 µg/g body weight, and perfused intra-aortically with 100 ml of 0.9% saline followed by 300 ml of a fixative composed of 4% (w/v) paraformaldehyde and 0.2% (w/v) picric acid in 0.1 M phosphate buffer, pH 7.4 (PB). After fixation, the brains were removed and the MOBs dissected out and postfixed for 2 h in the same fixative. Then, tissue blocks were washed in PB, cryopro-
tected with 15% (w/v) sucrose over 12 h at 4 °C, and immersed in 30% (w/v) sucrose until they sank. After cryoprotection, 30 μm coronal sections were cut on a freezing-sliding microtome (Leica Frigomobil, Jung SM 2000, Nussloch, Germany). The sections were collected in six series in 0.05% sodium azide (w/v) in PB at 4 °C.

Serial free-floating sections were single-labelled using acetylcholinesterase (AChE) histochemistry, serotonin (5-HT)-immunohistochemistry or dopamine-β-hydroxylase (DBH)-immunohistochemistry. A one-in-three series was processed for AChE histochemistry, and immediately adjacent series were either 5-HT- or DBH-immunostained. This allowed us to perform comparisons between cholinergic, serotonergic or noradrenergic innervations of the same glomeruli. Additional series were processed for serotonin transporter (5-HTT) immunohistochemistry.

2.2. AChE histochemistry

The sections were first rinsed in 0.1 M sodium acetate buffer (pH 6.0) overnight. Then, they were immersed for 30 min at room temperature in an incubation medium made up of 1.7 mM acetylthiocholine iodide (Sigma, St. Louis, USA), 5 mM sodium citrate (Merck, Darmstadt, Germany), 3 mM cupric sulphate (Prolabo, Fontenay-sous-Bois, France), 0.2 mM potassium ferricyanide (Panreac, Barcelona, Spain) and 0.2 mM ethopropazine (Sigma) in 0.1 M sodium acetate buffer, pH 6.0. After incubation, AChE activity was visualized using 0.025% 3,3’-diaminobenzidine and 0.003% hydrogen peroxide in 0.2 M Tris–HCl buffer, pH 7.4. The course of the reaction was controlled under the microscope and it was stopped when the desired staining intensity had been reached. Two controls of the specificity of the histochemical staining were carried out: (1) omission of the substrate acetylcholine iodide, and (2) substitution of acetylthiocholine iodide by butyrylthiocholine iodide (Sigma) to check substrate specificity. No residual activity was detected in either case.

2.3. Immunohistochemistry

The immunohistochemical technique was carried out following the avidin–biotin-peroxidase method (Hsu et al., 1981). Sections were rinsed in PB (3 × 10 min) and sequentially incubated in: (1) 1:3000 rabbit anti-5-HT (Affinitini, SZ 1021, Exeter, UK), 1:10,000 rabbit anti-5-HTT (Zhou et al., 1996) or 1:2000 rabbit anti-DBH (Affiniti, DZ 1020) in PB with 0.2% Triton X-100 for 48 h at 4 °C; (2) 1:200 biotinylated goat anti-rabbit immunoglobulin (Vector, Burlingame, USA) in PB for 1 h at room temperature; and (3) 1:200 Vectastain Elite ABC reagent (Vector) in PB for 1 h at room temperature. Between each step, the sections were carefully rinsed in PB (3 × 10 min). The reaction product was visualized by incubating the sections in 0.025% 3,3’-diaminobenzidine and 0.003% hydrogen peroxide in PB.

2.4. Quantitative analysis

Eight rostrocaudal levels were defined, and each level was divided into eight sectors, as previously described (Wenuaga et al., 1999). Five randomly chosen glomeruli per sector were measured and only olfactory glomeruli larger than 100 μm in diameter were considered in the quantitative analyses.

In order to gain a better understanding of the present results, a semi-quantitative analysis of the glomerular morphology was performed. Images were taken with a photomicroscope (Olympus Provis AX70, Tokyo, Japan) interfaced with an Apogee Kx85 (Apogee Instruments, Tucson, USA) digital camera and a sequential trichromatic filter monitored by Capture software 2.0.7 (C.R.I. Cambridge Research & Instrumentation, Boston, USA). Using the NIH-Image software, the glomerular features (fibre density and glomerular area) were obtained. The digital images were treated to balance the signal-to-noise ratio in such a way that positive elements were clearly distinguishable from the background. Following this, they were manually transformed into binary images in which only immunostained elements appeared as white pixels. Then, the glomerular surface was delimited, using the original image as reference and fibre density was calculated as the white/black pixel ratio in the entire glomerular area.

The serotonergic and cholinergic innervations were compared to each other in caudal sections of the MOBs containing atypical glomeruli. Ten atypical glomeruli (five in the lateromedial area and five in the MGC area) were randomly selected from each MOB of each animal, as described previously (Crespo et al., 1996). For each of the 200 glomeruli selected in AChE-stained sections, its counterpart in the adjacent 5-HT-immunostained series was located and the features of the serotonergic innervation were analyzed.

3. Results

In this study, the distribution and morphological features of the serotonergic, noradrenergic and cholinergic centrifugal elements were analyzed. Our results point to a high degree of heterogeneity in some of these systems in the GL. Thus, several types of glomeruli were distinguished on the basis of the density and morphology of their centrifugal innervation. Also, a clear correlation in the fibre density could be established between the serotonergic and cholinergic systems in specific glomerular types.

3.1. Serotonergic centrifugal inputs

As in other cortical regions, the 5-HT-immunoreactive fibres in the MOB showed morphological heterogeneity, with differences in size, diameter and shape, allowing us to identify two types of fibres. There were thin axons (Fig. 1a),
very slim, bearing small pleomorphic varicosities separated by long intersegmental spaces. The other type, thick axons (Fig. 1b), had larger diameters and large spherical varicosities, which were usually connected to each other by short intervaricosal segments. The distribution of these two types of fibre was different among the bulbar layers. Infraglomerular layers were occupied by scarce 5-HT-immunoreactive thin fibres, while both types were observed in the GL. Moreover, most 5-HT-immunopositive axons were located in the GL, the density being two to three times higher than in the deeper layers (McLean and Shipley, 1987). These fibres branched in the olfactory glomeruli, giving rise to dense and intricate networks. The serotonergic fibre density in the remaining layers was clearly lower and the immunopositive fibres showed a patchy distribution, with large portions of the MOB devoid of immunopositive axons.

Within the GL, the innervation among glomeruli was not homogeneous, and based on the fibre density and distribution of the serotonergic inputs we were able to distinguish four types of glomeruli (Figs. 2 and 3). (i) Type I glomeruli (Fig. 2a and b) were those with the lowest serotonergic fibre density, with percentages (space occupied by 5-HT-immunoreactive fibres in relation to the glomerular area) lower than 0.1%. Most fibres in these glomeruli were thin, and although they extended throughout the glomerular volume they were mainly located in the outermost zone of the glomerulus. There was also a difference regarding their size since these glomeruli were the largest ones found in the GL, with cross-sectional areas of around 25000 μm² and 190 μm ± 21 maximum diameter. (ii) Most glomeruli of the bulb could be included in type II (Fig. 2c and d). In this group, the average density of 5-HT-immunostained fibres was 3%, although the values ranged from 2 to 4%. Both types of fibres were intermingled inside the glomeruli. These glomeruli displayed marked heterogeneity, with cross-sectional areas ranging from 10,000 to 20,000 μm² and the mean maximum diameter was 160 μm ± 18. They also showed variable shapes, ranging from spherical to flattened forms. (iii) Type III glomeruli (Fig. 2e and f) formed the group with the highest number of fibres. Quantitative analysis revealed that the average density was more than two-fold that of type II glomeruli (7%). Most axons were thick and they formed a very dense network, so the few thin fibres were partially hidden under the high number of varicosities. These glomeruli were small, with cross-sectional areas ranging from 5000 to 10,000 μm², and the mean maximum diameter was 115 μm ± 10. Their shapes were usually spherical although; rarely, a flattened shape could be observed. (iv) Type IV was a special group among all olfactory glomeruli because its main feature was the characteristic segregated distribution of serotonergic fibres within the glomerular volume. The 5-HT-immunoreactive axons did not occupy the whole glomerular volume but, instead, were distributed in specific zones of the glomerulus. This type was scarce and the glomeruli had fixed topological positions along the rostrocaudal axis of the MOB. Furthermore, they could be divided into two subtypes. Subtype IVa (Fig. 3a and b) had the 5-HT-immunopositive axons located at the periphery whereas their central domain was practically devoid of 5-HT-immunoreactive fibres. Most of these axons were thick and did not send collaterals to the inner glomerular region. These glomeruli were usually large, with a maximum diameter of 185 μm ± 30, and had flattened or rounded shapes. Subtype IVb (Fig. 3c and d) had fibres of both morphological types, but only in one portion of the glomerulus, whereas the remaining glomerular area was devoid of serotonergic inputs. They had variable sizes but were always elongated in shape.

Fig. 1. 5-HT-immunostained fibres in the olfactory bulb. Arrowheads point to varicosities (scale bar = 20 μm). Thin (a) and thick (b) 5-HT-immunolabelled axons.


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Fig. 2. 5-HT-immunostaining of serial sections in the glomerular layer, showing the three most common types of olfactory glomeruli according to the density and distribution of serotonergic fibres. Left panels show three different glomeruli (a, c and e) and right panels show the same glomeruli as in the left ones (b, d and f, respectively) but at different coronal sections (scale bar = 50 μm). (a) and (b) Type I, the scarce fibres are located mainly at the periphery; (c) and (d) Type II, increased fibre density occupying the whole glomerular volume; (e) and (f) Type III, are the smallest glomeruli with the highest fibre density.
3.2. Topographic distribution of the 5-HT glomeruli

The glomerular types described above were regionally located in the MOB, it being possible to describe a general pattern of distribution. In addition, there were some glomeruli that always belonged to the same type; these had unchanging morphological features and had similar distributions in all animals studied.

In order to define the distribution pattern of the glomerular types identified and to facilitate an accurate comparison with the other markers studied, the results are described following eight rostrocaudal levels representative of the structure of the MOB. These levels were readily identifiable in each MOB and among the animals since they were defined on the basis of anatomical landmarks (see Fig. 4 for details).

As a general pattern of distribution, the lateral region was occupied by type I 5-HT glomeruli; the medial region by type II; the dorsal region by type III, and the ventral region by types I and II. This general organization was constant along the rostrocaudal axis as long as the GL was continuous. In the caudal regions, where GL loses its continuity (lateral region of levels IV and V, dorsal region of level V and medial region of level VIII), the isolated glomeruli in these regions were predominantly of type III.

The scarce type IV glomeruli were intermingled with the predominant types I, II and III. Type IVa glomeruli could be seen along the whole of the rostrocaudal axis of the MOB, but type IVb glomeruli were only localized in the ventrolateral and medial regions of the caudal MOB.

Within this general distribution pattern, there were glomeruli with similar characteristics at similar regions of the MOB in all animals studied. In this sense, at level III the ventralmost glomerulus of the lateral region was a large flattened type IVa glomerulus with constant morphological features in all animals studied (Fig. 4c, arrow and Fig. 8a). This glomerulus was easily identifiable because, at this level, there is no continuity between the lateral and ventral regions of the GL. Thus, it is the ventralmost glomerulus appearing in the lateral region at this level. At level VI, two very large and morphologically constant glomeruli appeared (Fig. 4f, arrows). They were the dorsalmost and ventralmost glomeruli of the medial GL. Both of them were round; the dorsal one belonged to type III, while the ventral one was a type IVa glomerulus (data summarized in Fig. 4).

3.3. Serotonin transporter (5-HTT)

Immunohistochemical analysis revealed that the distribution of 5-HTT-positive-fibres matches the distribution...
of 5-HT-immunopositive-axons. Thus, the highest 5-HTT fibre density was observed in the GL, being lower in the remaining layers, the same distribution described above for 5-HT-immunostaining. Moreover, the different types of glomeruli identified according to 5-HT fibre density were also observed with 5-HTT immunohistochemistry and the distributions of the glomeruli with the highest and lowest 5-HTT fibre density corresponded also with those described after serotonin immunolabelling (Fig. 5). Glomeruli with the highest 5-HTT fibre density were mainly located in dorsal and medial regions (Fig. 5a and c), whereas those with the lowest number of 5-HTT positive-fibres were situated in lateral regions (Fig. 5b and d). Despite these similarities, the number of 5-HTT fibres in the external plexiform layer and the granule cell layer was always higher than those fibres labelled after 5-HT immunohistochemistry. In addition, the 5-HTT-immunolabelling did not reveal clearly the two types of serotonergic fibres identified according to their thickness, thin and thick (McLean and Shipley, 1987). Thus, no differences according to the morphology of 5-HTT-immunoreactive fibres could be observed between the GL and the remaining layers.
Fig. 5. 5-HTT-immunostaining in the glomerular layer. Note the regional differences according to the density of immunopositive fibres (scale bar = 200 µm (a and b) and 80 µm (c and d)). Olfactory glomeruli in the medial region (a and c) have a higher number of 5-HTT fibres than those located in the lateral region (b and d). Parts (c) and (d) show characteristic glomeruli from medial and lateral regions, respectively.
3.4. Noradrenergic centrifugal input

Noradrenergic fibres in the MOB showed a laminated pattern, with the highest density of DBH-positive axons in the internal plexiform layer and in the outermost region of the granule cell layer, where positive axons were oriented parallel to the bulbar lamination. The external plexiform layer also had a noteworthy amount of noradrenergic axons, although in this case the fibre density was lower than that observed in other layers and DBH-positive fibres were arranged perpendicular to the bulbar surface. The GL was almost devoid of noradrenergic innervation, olfactory glomeruli only being reached by a few isolated noradrenergic axons.

Noradrenergic fibres displayed two readily distinguishable morphological types. Thin fibres (Fig. 6a), very slim, which frequently had small spherical varicosities, and thick fibres (Fig. 6b), with larger diameters and varicosities. No specific distribution of these fibres among the bulbar layers was observed, with the exception of the granule cell layer, where the thick fibres were mainly located at its superficial region, while the deep granule cell layer was mainly innervated by thin fibres.

3.5. Cholinergic centrifugal input

AChE-positive fibres were widely distributed through all bulbar layers, the highest fibre density being observed in the internal plexiform layer and the GL-external plexiform layer interface.

Based on the density of cholinergic innervation, Zheng et al. (1987) reported the existence of two glomerular types. Most glomeruli, the typical glomeruli (Fig. 7a and b), exhibited a moderate degree of cholinergic innervation, while atypical glomeruli formed a subpopulation with a very dense cholinergic innervation (Fig. 7c). Atypical glomeruli

![Fig. 6. DBH-immunostained fibres in the olfactory bulb. Arrowheads point to varicosities (scale bar = 20 μm). Thin (a) and thick (b) DBH-immunolabelled axons.](image)

![Fig. 7. AChE-histochemical staining in the GL, showing the different types of glomeruli according to their cholinergic input (scale bar = 100 μm). (a) Typical type A glomerulus, with most fibres at the periphery. (b) Typical type B glomerulus. The fibres occupy the whole of the glomerular volume. (c) An atypical glomerulus.](image)
were located in the dorsal (MGC), medial and ventrolateral borders of the caudal half of the MOB. In our study, differences were found in the cholinergic innervation of typical glomeruli and this allowed us to recognize two subgroups. Thus, in most typical glomeruli the cholinergic fibres were preferentially located in the periglomerular region (typical A glomeruli) (Fig. 7a). The other group comprised glomeruli with fibres showing a homogeneous distribution both at the periphery and in the inner part of the glomeruli (Fig. 7b). This type of glomerulus (typical B) never had the high density of cholinergic fibres found in the atypical ones.

3.6. Serotonergic and cholinergic innervation in specific glomeruli

Comparisons between serotonergic and cholinergic innervations were performed in the GL. The results indicated that there is a correlation between the density of innervation by both systems in certain glomeruli, whose topographical locations were closely matched in all animals studied.

The large elongated ventrolateral glomerulus of level III (Fig. 8a) and the large glomerulus located in the ventralmost region of the MOB of level VI were characteristic because they were innervated by a large amount of both serotonergic and cholinergic fibres. According to their innervation pattern, they belonged to type IVa serotonergic glomeruli (Fig. 8a) and type A cholinergic typical glomeruli (Fig. 8b). In addition to their characteristic location in the MOB and their morphologic features, they were readily distinguished by having a higher fibre density than the adjacent ones. This feature was found in all animals studied. Another example of such glomeruli was the large round 5-HT-type III glomerulus located in the dorsal most region of level VI that was always a typical B cholinergic glomerulus.

Serotonergic fibre density was also studied in atypical glomeruli. It was found that atypical glomeruli display different types of serotonergic innervation, depending on their topological location. In the MGC region, most atypical glomeruli were 5-HT-type I (Table 1), whereas isolated atypical glomeruli of the lateral region of level V and the medial region of level VIII were 5-HT-type III glomeruli (Table 1 and Fig. 8c and d). This coincidence of atypical
cholinergic innervation and type III serotonergic innervation was observed in many glomeruli in all animals studied. However, it was different between males and females. In male rats, around half of the atypical glomeruli (54%; 27 of 50) were type III, whereas in females type III innervation was observed in 26% (13 of 50) and the most abundant serotonergic innervation of atypical glomeruli was classified as type I glomeruli (46%; 23 of 50) (Table 2).

Occasionally, zones with different densities of cholinergic inputs can be seen within the same glomerulus (Shinoda et al., 1993). Thus, a high number of cholinergic fibres innervated one region of the glomerulus, while the rest received a lower cholinergic input. In this glomerular type, mainly located in the lateral regions of level V and medial regions of levels VII and VIII, the serotonergic innervation pattern was usually complementary to that of cholinergic fibres (glomeruli IVb in Table 2). Thus, the region with the

Table 1
Correspondences in percentage between types of 5-HT innervation (I-IVb) and subsets of atypical glomeruli

<table>
<thead>
<tr>
<th></th>
<th>I (%)</th>
<th>II (%)</th>
<th>III (%)</th>
<th>IVa (%)</th>
<th>IVb (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lateromedial AtG</td>
<td>30</td>
<td>14</td>
<td>46</td>
<td>0</td>
<td>10</td>
</tr>
<tr>
<td>MGC AtG</td>
<td>58</td>
<td>10</td>
<td>30</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

According to the classification of Weruaga et al. (2001), based on the topological location of atypical glomeruli (AtG).

Table 2
Sex differences according to the percentage of type 5-HT glomeruli (I-IVb) that are atypical in the lateromedial region

<table>
<thead>
<tr>
<th></th>
<th>I (%)</th>
<th>II (%)</th>
<th>III (%)</th>
<th>IVa (%)</th>
<th>IVb (%)</th>
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</thead>
<tbody>
<tr>
<td>Male</td>
<td>18</td>
<td>16</td>
<td>54</td>
<td>0</td>
<td>12</td>
</tr>
<tr>
<td>Female</td>
<td>46</td>
<td>18</td>
<td>26</td>
<td>0</td>
<td>10</td>
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Fig. 9. 5-HT immunohistochemistry (left panels) and AChE histochemistry (right panels) in adjacent sections. Asterisks indicate blood vessels used as landmarks (scale bar = 100 μm). (a) 5-HT-immunostaining in a type IVb glomerulus, with fibres located on its right side (arrowhead). (b) Same glomerulus as in (a) in an adjacent section labelled after AChE histochemistry. The glomerular portion with the highest cholinergic innervation (arrow) corresponds to the lowest serotonergic fibre density region (arrow) and the zone with the lowest number of AChE-positive fibres is reached by a large amount of 5-HT-immunopositive fibres (arrowheads). (c) 5-HT-immunostaining in the MGC region. (d) Same region as in (c) after AChE histochemistry. The 5-HT type III glomerulus corresponds to the typical B glomerulus (black arrows), while the atypical glomerulus (arrowheads) is reached by few serotonergic fibres (5-HT type I-gglomerulus). White arrows point to a typical A cholinergic glomerulus that is 5-HT type I according to its serotonergic innervation.
highest cholinergic density showed a low serotonergic input, while the region with the lowest AChE labelling exhibited a high density of 5-HT-immunoreactive fibres (Fig. 9a and b). The glomeruli where this opposite serotonergic–cholinergic innervation pattern was observed were characterized by their large size and elongated shape.

The MGC glomeruli had a characteristic dense centrifugal innervation, both cholinergic and serotoninergic (Fig. 9c and d), but the fibres of these different systems did not coexist in the same glomeruli. In this region, atypical glomeruli had very little serotonergic labelling and were classified as 5-HT type I glomeruli (Fig. 9c and d, arrowheads), while 5-HT type III glomeruli were often cholinergic typical B (Fig. 9c and d, black arrows). Moreover, contrary to what was observed in the lateromedial area, no sex differences were found in the glomeruli of this region as regards the 5-HT-AChE innervation pattern. These data are summarized in Tables 3 and 4.

4. Discussion

The main finding of the present study is the demonstration of a greater degree of heterogeneity in the centrifugal innervation of the olfactory glomeruli than previously described. Four types of glomeruli were identified according to the density and distribution of the serotoninergic innervation and additional data are given about the heterogeneous pattern of the cholinergic centrifugal system. Moreover, comparisons between the new types of 5-HT glomeruli described in this report and those previously known on the basis of their cholinergic inputs have been carried out.

4.1. Methodological considerations

In this study, AChE-positive fibres were considered to be cholinergic since choline acetyltransferase immunoreactivity in the MOB closely matches the distribution of AChE-containing fibres (Godfrey et al., 1980; Ojima et al., 1988) and that of cholinergic receptors (Blaha et al., 1984). In addition, AChE-positive fibres disappear in the MOB after olfactory peduncle sectioning (Le Jeune and Jourdan, 1991).

4.2. 5-HT versus 5-HTT immunohistochemistry

The results found in this work showed that 5-HTT labelling fits relatively well with the 5-HT labelling, although two differences were found. The 5-HT immunostaining demonstrated two types of labelled fibres that after using 5-HTT antibody were not clearly distinguishable. Moreover, the 5-HTT fibre density observed in the GL was very similar to 5-HT labelling, but the density of 5-HTT-immunoreactive fibres was higher in the infraglomerular layers than that detected with the 5-HT antibody. A possible explanation is that these differences in the infraglomerular layers could be due to the staining of non-serotonergic fibres by the anti-5-HTT antibody. However, there are no previous evidences supporting this possibility and just a 5-HTT transient expression in non-serotonergic system has been reported during the early development (Lebrand et al., 1998; Zhou et al., 2000) but never in adult animals. On the other hand, the differences could be due to a different sensibility of both antibodies to demonstrate serotoninergic fibres, in such case, 5-HTT-immunolabelling would not reveal fibres with very low content of serotonin, while 5-HTT-immunostaining, less dependent of serotonin content, would. Several studies have demonstrated that 5-HTT is exclusively present in serotonin-producing neurons of the raphe and in their axonal arbors (Blakely et al., 1991; Hoffman et al., 1991). Moreover, a previous report using the same antibody has demonstrated that 5-HTT-immunolabelled fibres are serotoninergic in nature (Zhou et al., 1996). All these data seem to indicate that anti-5-HTT antibody is more sensible detecting serotoninergic fibres than 5-HT antibody and therefore, 5-HTT-immunopositive fibres observed in this report are likely serotoninergic axons arising from raphe nuclei.

4.3. Fibre morphology

Two morphological types of 5-HT-immunoreactive fibres were observed. The presence of these two types of fibres in the MOB of adult rats has already been reported (McLean and Shipley, 1987). These fibres differ not only in their morphological features but also in their origin, the source of thin fibres being the dorsal raphe, whereas the thick ones are originated in the medial raphe (Kosofsky and Molliver, 1987). According to their pharmacologic properties, thin fibres are susceptible to neurotoxic amphetamine derivatives while thick fibres are not (Battaglia et al., 1991; Mamounas et al., 1991), and it has been suggested that both serotoninergic types of fibres could carry out different functions (Köhler, 1982). In the GL, most serotoninergic fibres are thick (Mamounas et al., 1991), but our observations showed that there were some glomeruli where both types of fibres were present at the same proportion. Therefore, a “mixed” functional effect would be expected.
Concerning the morphology of the DBH-immunoreactive fibres, our results were also in agreement with those of McLean et al. (1989) and McLean and Shipley (1991), who described two types of fibres on the basis of varicosity diameters. These types could correspond to the thick and thin fibres identified in this study. Similar to what has been reported for the serotonergic system, two groups of noradrenergic axons have been identified on the basis of their pharmacological and morphological properties and their origin. A potent and highly selective neurotoxin (N-2-chloroethyl-N-ethyl-2-bromobenzylamine) induces the degeneration of a group of noradrenergic axons, although not all are destroyed by this drug (Fritschy and Grzanna, 1989). These two groups of noradrenergic axons are also different because noradrenergic axons susceptible to this drug contain the hyaluronan receptor for hyaluronic acid-mediated motility (an extracellular matrix receptor) while the others do not (Nagy et al., 1998). With respect to their origin, the noradrenergic axons susceptible to the drug originate from the locus coeruleus and those unsusceptible to it arise from extra-coerulear neurons (Fritschy and Grzanna, 1989). However, all noradrenergic fibres reaching the MOB appear to be originated in the locus coeruleus (McLean and Shipley, 1991). These data suggest that the two distinct types of DBH-containing fibres found in the MOB (thick and thin) would constitute two morphological subtypes within the group susceptible to the drug identified by Fritschy and Grzanna.

4.4. Segregated fibre distribution in type IV glomeruli

McLean and Shipley (1987) indicated that serotonergic fibres do not have a preferential distribution between the inner and outer parts of the glomeruli. This is true for most glomeruli, but not for those belonging to type IVa, where a specific intraglomerular fibre segregation is evident (see Fig. 3a and b). These glomeruli appeared with identical features in the same location in all animals, so this specific morphological image does not appear to be artificial. The location of these glomeruli in the MOB and the particular serotonergic fibre distribution identify them as a special group, in which a very selective and fine modulation by the serotonergic system probably takes place.

Each olfactory glomerulus is divided into two zones: the olfactory nerve-zone and the non-olfactory nerve-zone (Kosaka et al., 1997). According to the dendritic arborization pattern in these glomerular zones, periglomerular neurons have been differentiated into two categories (Kosaka et al., 1998). One type sends its dendrites into both the olfactory nerve and non-olfactory nerve-zone, while the other type sends its dendrites only into the olfactory nerve-zone. Since 5-HT-immunopositive fibres in the type IVa glomeruli occupy specific glomerular regions and the dendrite processes of these two types of periglomerular neuron are located in particular zones of the glomerulus, 5-HT could modulate the activity of only one of these two neuronal types. The interaction of 5-HT with its receptors may take place directly (through synaptic connections) (Aghajanian and McCall, 1980; Schaffar et al., 1984) or, as happens in most encephalic regions, indirectly (through extracellular diffusion) (Agnati et al., 1995; Aghajanian and Marek, 1997; Bunin and Wightman, 1999). It is thought that in the GL the interaction of 5-HT with its receptors takes place by means of extracellular diffusion, since serotonergic axon varicosities are found in the inner part of the glomeruli, while the most abundant serotonergic receptors in the MOB -5-HT2A (McLean et al., 1995; Hamada et al., 1998) are located at the interface between the GL and the external plexiform layer (Jansson et al., 2001). However, the position of 5-HT2A receptors in the glomerular periphery and the serotonergic fibre distribution in glomeruli IVa (fibres found only in the periglomerular region) suggest that in this glomerular type the interaction of 5-HT with its receptors could take place by direct synaptic connections, acting on particular juxtaglomerular groups. Taken together, all these aspects suggest that in this glomerular type serotonergic modulation may be more selective than in the others, and 5-HT could regulate the physiology of particular groups of local interneurons, carrying out a more specific regulation.

4.5. Centrifugal heterogeneity in the GL

A centrifugal heterogeneity has already described based on the density of cholinergic innervation. Thus, olfactory glomeruli have been classified into two types: typical and atypical glomeruli (Zheng et al., 1987). Shinoda et al. (1993) reported differences in AChE-staining intensity in atypical glomeruli and they divided this population into two subtypes. Our group (Wuruaga et al., 2001) subclassified atypical glomeruli in three groups according to the location, shape, and AChE labelling intensity. The findings reported here are in agreement with the above classifications and add further information about typical glomeruli.

Despite this known heterogeneity in the GL as regards its cholinergic innervation, no differences have been observed in the serotonergic centrifugal input among olfactory glomeruli. Serotonergic system elements have been detected in primary olfactory centres of the brain (Suzuki et al., 2000; Kinsey et al., 2001; Frontini et al., 2003), but the exact contribution of 5-HT to olfactory processing remains unclear. The glomerular heterogeneity demonstrated in this report supports the notion that there are different levels of serotonergic modulation in the GL. It has been reported that ORNs that express chemically related receptor genes project to neighbouring glomeruli (Tsuboi et al., 1999; Strotmann et al., 2000; Uchida et al., 2000; Belluscio and Katz, 2001). For example, different ORNs expressing receptors for aliphatic aldehydes of different carbon chain lengths systematically project along a rostral-caudal strip of the glomeruli in the dorsal region of the MOB (Belluscio and Katz, 2001). The present results revealed a regional
distribution of 5-HT-glomeruli in the GL. The glomeruli of the dorsal surface showed the most intense innervation by serotonergic axons, while the glomeruli of the lateral region received fewer serotonergic fibres. Taken together, these data would indicate that the distribution of serotonergic fibres in the GL depends on the olfactory signal transmitted by a specific glomerulus or related group of glomeruli. However, previous studies have reported that odour preference learning leads to alterations in the glomeruli that regulate the transmission of the learned odour (Woo et al., 1987). These changes include the number of juxtaglomerular cells (Woo and Leon, 1991), cFos expression, and 2-deoxyglucose uptake (Johnson et al., 1995), all of which are modulated, at least in part, by the centrifugal systems (Johnson and Leon, 1996). Since the serotonergic system is the main centrifugal system innervating the GL (McLean and Shipley, 1987), and since 5-HT is needed for olfactory learning, the preferential distribution of serotonergic fibres in a given glomerulus could be due to the involvement of the glomerulus in the processing of previously learned olfactory signals. These data suggest that serotonergic innervation in the GL could be adaptive and that serotonergic fibre density might depend on glomerular activity. This would be in contrast with the few glomeruli with a characteristic topographic location and a similar 5-HT fibre density identified in this report. Since there are no readily applicable criteria to identify the same glomeruli from animal to animal, it is not possible to know whether these glomeruli are the same in the different analyzed animals. However, their similar location, their equivalent morphology and their constant serotonergic innervation pattern do suggest that they would be involved in the same or similar olfactory signal pathway.

4.6. Serotonergic and cholinergic innervations of specific glomeruli

The strong similarity of the cholinergic and serotonergic fibre densities in specific glomeruli of the MOB indicates that 5-HT and ACh would work together in the regulation of olfactory transmission in these glomeruli. In several encephalic regions, an inverse relationship between 5-HT and ACh has been reported: serotonergic neurons perform a negative modulation on cholinergic systems and ACh release is inhibited by 5-HT (for a review, see Cassel and Jentsch, 1995). Robinson (1983) reported that an injection of 5,7-dihydroxtryptamine, a specific neurotoxin for serotonergic neurons, in the raphe nucleus increases the release and re-uptake rate of ACh in the hippocampus (Maura and Raiteri, 1986; Bolanos and Fillion, 1989). An inverse relationship has been also demonstrated. Thus, injury to the cholinergic projection by the selective toxin 192 IgG-saporin increases the 5-HT concentration in the frontoparietal cortex (Lehmann et al., 2000). According to our results, most atypical glomeruli also display an intense serotonergic innervation. Thus, 5-HT and ACh could modulate each other, leading to a fine tuning of olfactory modulation in this particular subset of glomeruli.

The number of atypical glomeruli in the lateromedial region receiving strong serotonergic input was higher in males than in females. This points to sexual dimorphism in the rodent MOB. In the accessory olfactory bulb, several sex differences have been described (Segovia and Guillamón, 1993). A recent study on two strains of mice by our group also showed a sex difference in the atypical glomeruli of the MGC (Weruaga et al., 2001). Both the accessory olfactory bulb and the MGC have been implicated in reproductive functions and in the transmission of pheromonal signals. Our results revealed sexual dimorphism in the MOB, providing additional evidence on sexual variability in the rodent olfactory system.
(Shinoda et al., 1993). Since the serotonergic innervation of the MGC does not match the distribution of the cholinergic innervation, and because these previous classifications were mainly based on the density of cholinergic fibres, our analysis provides additional information concerning the glomerular variability of the MGC. In neonatal animals, the MGC seems to be involved in suckling behaviour, whereas exposure of adult animals to the suckling pheromone does not elicit increased 2-deoxyglucose uptake in these glomeruli (Teicher et al., 1980). However, their function in adults is unknown. Considering the maintenance of the neurochemical characteristics of this region in adult animals and the different organization of their glomeruli at this stage, we hypothesize that the glomeruli of the MGC constitute a particular olfactory pathway, transmitting a restricted group of signals that require strong centrifugal modulation.

Finally, the present study shows that centrifugal systems to the MOB, in particular the serotonergic and cholinergic axons, have specific and segregated innervations of particular subsets of glomeruli, at least judging from the density of afferent axons and the distributions inside the glomeruli. However, olfactory glomeruli are almost not reached by noradrenergic fibres. This suggests that a heterogeneous central modulation takes place at glomerular level, receiving precise feed-back regulation from higher brain centers at the first synapse of the olfactory pathway.

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