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Calcium-binding proteins in the periglomerular region of typical and atypical olfactory glomeruli

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Abstract

The distribution of chemically identified neuronal populations was studied in the glomerular layer of the rat olfactory bulb using calcium-binding protein immunocytochemistry combined with acetylcholinesterase histochemistry. Four calcium-binding proteins (calbindin D-28k, parvalbumin, calretinin, and neurocalcin) were analyzed in the periglomerular region of two different glomerular subsets: typical and atypical glomeruli. Atypical glomeruli were clearly distinguishable from typical ones by their dense network of acetylcholinesterase-positive centrifugal fibers. Each calcium-binding protein studied showed a specific distribution pattern in the rat olfactory bulb. Calbindin D-28k-, calretinin-, and neurocalcin-immunoreactive neurons were specially abundant in the glomerular layer. These three calcium-binding proteins had their main expression in neuronal subpopulations directly involved in the glomerular circuitries of the rat olfactory bulb. Specific populations of periglomerular cells were stained for calbindin D-28k, parvalbumin, calretinin, or neurocalcin, whereas external tufted cells were only immunoreactive to neurocalcin. Both neuronal types, periglomerular cells and external tufted cells, were found in the periglomerular region of both glomerular subsets. Nevertheless, a homogeneous distribution of calbindin D-28k- or calretinin-immunopositive periglomerular cells were found between typical and atypical glomeruli, whereas the neurocalcin-immunostained external tufted cells were statistically more abundant in typical glomeruli than in atypical ones ($P < 0.001$). These data suggest that some neuronal subpopulations are related with general properties of the glomerular physiology, and they have a homogeneous distribution in different subsets of glomeruli, whereas other chemically identified populations are related with a finer tuning of the olfactory processing, and they are segregately distributed in relation to particular glomerular subsets. In addition, this work adds new differences in the cellular composition of typical and atypical glomeruli.

Keywords: Calbindin D-28k; Calretinin; Neurocalcin; Parvalbumin; Olfactory system

1. Introduction

Olfactory glomeruli are complex structures where the first relay of the olfactory information takes place. In the olfactory glomeruli, dendrites from the projecting neurons of the olfactory bulb (OB) receive synapses from the axons of the olfactory receptor cells with an important local modulation [18]. Several lines of evidence demonstrate that olfactory glomeruli are not a homogeneous population [2,17,20,36,37]. Using both light and electron microscopy, a set of 'atypical' glomeruli has been described [37,38]. Atypical glomeruli differ from typical ones in their primary olfactory afferents as well as in their centrifugal innervation from higher brain centers [22,23,37,38]. Using acetylcholinesterase (AChE) histochemistry or choline acetyltransferase immunocytochemistry, both glomerular subsets can be easily differentiated because of the dense innervation by cholinergic centrifugal fibers of the atypical glomeruli [13,14,22–24,37,38]. In addition to these particularities in the extrinsic glomerular afferents, we have observed variations in the intrinsic OB interneurons surrounding either typical or atypical glomeruli. Thus, using NADPH-diaphorase (ND) histochemistry and nitric oxide synthase (NOS) immunocytochemistry, significant differences were observed in the number of ND/NOS-positive periglomerular cells located in both types of glomeruli [14]. This segregated distribution suggests differences in the neuronal circuitries between both glomerular subsets. It

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is presently unknown whether there are other neurochemical differences in the neuronal populations taking part in the circuitries of both typical and atypical glomeruli.

Several calcium-binding proteins (CaBPs) belonging to the 'EF-hand' homolog family have a wide distribution in specific neuronal populations of the central nervous system, including the OB [2,6,7,10,21,31]. Although it is assumed that cells containing one or more of these CaBPs may have a finer handling of calcium, the exact roles of these proteins remain to be elucidated [3,4,9,35]. In addition to their selective distributions, parvalbumin (PV), calbindin D-28k (CB), calretinin (CR), and neurocalcin (NC), four CaBPs of the 'EF-hand' family, share to be excellent neuroanatomical markers. They provide after immunostaining Golgi-like images including frequently the complete dendritic tree and lengthy axons [1,2,5,10,15,31]. Therefore, the characterization of the immunolabeled neuronal types can be easily carried out. Previous reports demonstrated the presence of these four CaBPs in different neuronal subpopulations of the rat OB, located in the glomerular layer and taking part in the glomerular circuitry [2,6,7,10,21,31].

Using Golgi impregnation and immunocytochemical techniques [18,32], three neuronal types could be identified in the glomerular layer: external tufted cells, periglomerular cells and superficial short-axon cells. External tufted cells are involved in rapid and topographically organized interbulbar and intrabulbar interactions [25,33,34]. Different neuroactive substances such as cholecystokinin, substance P, vasoactive intestinal polypeptide, and some CaBPs have been identified in this neuronal population [18]. Among CaBPs, NC stains external tufted cells in the periglomerular region of the rat olfactory bulb [6]. Periglomerular cells and superficial short-axon cells are first and second order interneurons respectively, and they also modulate olfactory information at the glomerular level. Periglomerular cells are a morphologically homogeneous but highly heterogeneous biochemically neuronal population [32]. Different subsets of periglomerular cells contain several neurotransmitters and neuroactive substances such as dopamine, γ-aminobutyric acid, somatostatin, NOS, cholecystokinin [18], and different CaBPs such as CB, CR, and PV [2,10,21,31]. Similarly, different subpopulations of superficial short-axon cells contain NOS, γ-aminobutyric acid, vasoactive intestinal polypeptide, dopamine [18], and different CaBPs such as CB, and PV [2,10,21,31].

Histochemical–immunocytochemical double-labeling allows to study the distribution of different neuronal subpopulations in typical and atypical glomeruli. Thus, using AChE-histochemistry combined with CaBP-immunocytochemistry, it is possible to study the neuronal subpopulations expressing CB, PV, CR, or NC surrounding both glomerular subsets. The aim of the present study is to analyze whether different chemically identified populations of juxtaglomerular neurons implied in the glomerular circuitries (periglomerular cells and external tufted cells) are segregately distributed around typical and atypical glomeruli.

2. Materials and methods

2.1. Animals and tissue preparation

Eight adult male Wistar rats (200–250 g) were used in this study. Animals were deeply anaesthetized with ketamine (Ketolar, 50 mg/kg body weight), and perfused intra-aortically with 100 ml of saline solution followed by a fixative solution composed by 4% (w/v) paraformaldehyde and 15% (v/v) saturated picric acid in 0.1 M phosphate buffer, pH 7.4 (PB). After perfusion, the brains were removed and the OB dissected out and postfixed for 4 h in the same fixative. Tissue was cryoprotected with 30% (w/v) sucrose. After cryoprotection, 20 μm coronal sections were cut on a cryostat and serially collected in cold (4°C) PB.

Serial free-floating sections were single- or double-labeled using AChE histochemistry and PV, CB, CR, or NC immunocytochemistry. Some sections were single-stained in a serial section method. In this method, consecutive sections were processed for each CaBP and AChE, respectively. This fact allows the identification of typical and atypical glomeruli in the CaBP single-labeled sections by comparison with the adjacent AChE-stained sections. In the double-labeled series, after AChE histochemistry a one-in-six series was processed for the demonstration of CB immunoreactivity, a second series was used for the detection of PV-positive elements, a third series was processed for the demonstration of CR immunoreactivity, and a fourth series was used for the detection of NC-immunopositive elements. A group of sections was stained in a reversal order, i.e., beginning with the calcium-binding proteins immunocytochemistry and afterwards with the AChE histochemistry. The fifth and sixth series were used for the single detection of CB, PV, CR, NC, or AChE, and for specificity controls.

2.2. AChE histochemistry

AChE histochemistry was carried out in the single- or double-labeled series for the discrimination between typical and atypical glomeruli as previously described [13,14]. The series were carefully rinsed in 0.1 M sodium acetate buffer, pH 6.0, and processed for the detection of AChE activity following a variant [19] of the Koelle method [16]. For AChE histochemistry, sections were incubated at room temperature for 15 min (series used for histochemical–immunocytochemical double-labeling) or for 30 min (sections used for AChE histochemical single staining) in an incubation solution made up of 1.7 mM acetylthiocholine iodide (Sigma #A5751), 0.49 mM sodium citrate (Sigma #S4641), 2.9 mM cupric sulphate (Sigma #C7631), 1.25

mM potassium ferricyanyde (Sigma #P8131) and 0.2 mM ethopropazine (Sigma #E2880) as inhibitor of non-specific esterases in 0.1 M sodium acetate buffer, pH 6.0. The AChE activity was visualized using 0.05% 3,3′-diaminobenzidine and 0.003% hydrogen peroxide in 0.2 M Tris-HCl buffer, pH 7.6. The course of the reaction was controlled under the microscope. The sections destined for double-staining were incubated for shorter periods (15 min) to visualize exclusively the dense fiber staining of the atypical glomeruli and to prevent the cell AChE staining which could be confused with the CaBP immunostaining. The reaction was therefore stopped when the neuropile of atypical glomeruli was stained and clearly distinguishable of the typical ones. The AChE histochemical reactions were stopped by rinsing the sections in 0.2 M Tris-HCl buffer, pH 7.6. For the specificity of the histochemical staining, three controls were carried out: (1) omission of the substrate acetylcholine iodide; (2) substitution of acetylthiocholine iodide by butyrylthiocholine iodide; and (3) addition of 10^{-5} M BW284C51 as inhibitor of AChE activity [8]. For all controls, no residual activity was detected.

2.3. Immunocytochemistry

After AChE histochemistry, the series destined for double-labeling were carefully rinsed in PB (3×10 min) and processed with the CaBPs single-stained series for the immunocytochemical staining. Primary polyclonal antisera against CR and NC, and primary monoclonal antibodies against PV and CB were used. The immunocytochemical techniques were carried out incubating the sections sequentially in: (a) primary antibody (anti-PV 1:1000; anti-CB 1:1000; anti-CR 1:20000; or anti-NC 1:8000; 48 h at 4°C); (b) biotinylated anti-mouse or anti-rabbit immunogammaglobulin (Vector Labs., Burlingame, CA, USA; 1:250 in PB; 2 h at room temperature); and (c) Vectastain Elite ABC reagent (Vector Labs.; 1:250 in PB; 2 h at room temperature). After each step, sections were carefully rinsed in PB (3×10 min). The reaction products were visualized incubating sections in 0.05% 3,3′-diaminobenzidine and 0.003% hydrogen peroxide in 0.2 M Tris-HCl buffer, pH 7.6. Some of the series were processed for an immunofluorescent labeling. In this procedure, sections were sequentially incubated in: (a) primary antibody (anti-PV 1:1000; anti-CB 1:1000; anti-CR 1:10000; or anti-NC 1:4000; 48 h at 4°C); and (b) fluorescein-labeled anti-mouse, or Texas Red-labeled anti-rabbit serum (Vector Labs., Burlingame, CA, USA; 1:50 in PB; 2 h at room temperature). The primary antibodies used in this report have been exhaustively characterized (CB [11]; CR [30]; NC [27]; PV [12]), and previously used in the rat nervous system (CB [1,2,10]; CR [29]; NC [6]; PV [10]). Additional controls of the immunohistochemical procedures were carried out omitting the first or second antibodies in each step, and incubating some sections exclusively in 0.05% 3,3′-diaminobenzidine and 0.003% hydrogen peroxide in 0.2 M Tris-HCl buffer, pH 7.6, to exclude the presence of endogenous peroxidases. No residual reactivity was observed.

2.4. Quantitative analysis

Using a digitizer tablet connected to a semiautomatic image analysis system (MOP-Videoplan Kontron), the maximum diameters of 100 PV-, CB-, CR- and NC-immunopositive cells were calculated. For the statistical analysis we have counted for each CaBP the number of immunopositive cells in the periglomerular region of 40 sections of typical glomeruli (20 in the dorsomedial area and 20 in the ventrolateral area) and 40 sections of atypical glomeruli (20 in the dorsomedial area and 20 in the ventrolateral area) in each OB of each animal (1280 sectioned glomeruli per each CaBP). The glomeruli were randomly selected as described previously [14]. Only sections of olfactory glomeruli larger than 50 μm were considered in the quantification. ANOVA was used for the statistical analysis of the results. Values of $P < 0.001$ for Fisher PLSD and Scheffé *F*-tests jointly were considered statistically significant.

3. Results

AChE showed a specific laminar distribution in the rat OB. When sections of the AChE single-stained series were incubated for long period (30 min), the AChE histochemistry demonstrated a high density of AChE-positive fibers and some AChE-stained neurons mainly located in the glomerular layer and internal plexiform layer. This histochemical procedure provided an excellent discrimination between typical and atypical glomeruli in the rat OB (Fig. 1a). Although all olfactory glomeruli were innervated by a network of AChE-positive fibers, atypical glomeruli placed in the dorsomedial and ventrolateral areas of the caudalmost OB were easily differentiated from typical ones by a remarkably intense innervation of AChE-positive fibers (Fig. 1b,c).

Calcium-binding protein immunocytochemistry provided a good visualization of the CB-, PV-, CR-, or NC-immunopositive elements (Fig. 2 and Fig. 3). The antibodies against the different CaBPs used in this work allowed a good morphological characterization of the labeled neurons. In this sense, dendritic trees, lengthy axons and dendritic spines were frequently stained in the immunopositive cells. This Golgi-like staining allowed an easy identification of the positive elements with the different neuronal types described in the periglomerular region of the rat OB. Identical results were observed using both the 3,3′-diaminobenzidine, and fluorescent immunostaining. Immunopositive cells were identified as periglomerular cells (Fig. 2a,c), superficial short-axon cells (Fig. 2d), or external tufted cells (Fig. 2b,e). Both

periglomerular cells and external tufted cells, whose dendrites take part in the glomerular neuropile, were included in the quantitative analysis. Since typical and atypical glomeruli are located adjacent, only the periglomerular cells and external tufted cells that showed one or more dendrites that could be followed innervating either a typical or an atypical glomerulus were quantified. Superficial short-axon cells showed a dendritic pattern with long dendrites coursing in the boundary between the glomerular layer and the external plexiform layer, but these dendrites did not enter within the olfactory glomeruli. This neuronal type was therefore not included in the quantitative analysis, since their dendrites could not be ascribed to a particular type of glomerulus.

In order to avoid the masking of immunopositive cells by the AChE-positive fibers, mainly in atypical glomeruli, short incubation periods for the AChE histochemical procedure (15 min) were used in series processed for double AChE histochemical–CaBPs immunocytochemical labeling. This short incubation period provided a weaker AChE staining where the neuropile of typical glomeruli and the intrinsic AChE-positive neurons were still unlabeled but it was strong enough to clearly differentiate typical and atypical glomeruli. The shortened incubations ruled out the possibility of confusion between AChE-stained and CaBP-immunopositive cells. In atypical glomeruli, it allowed a clear identification and an accurate quantification of the CB-, PV-, CR-, or NC-immunopositive cells surrounding and occasionally inside them.

The staining patterns for all four CaBPs as well as the distribution of AChE staining in double-labeled sections were similar to those observed in the CaBP or AChE single-labeled series. Identical results were found for both the AChE and CaBP staining, when the order of the

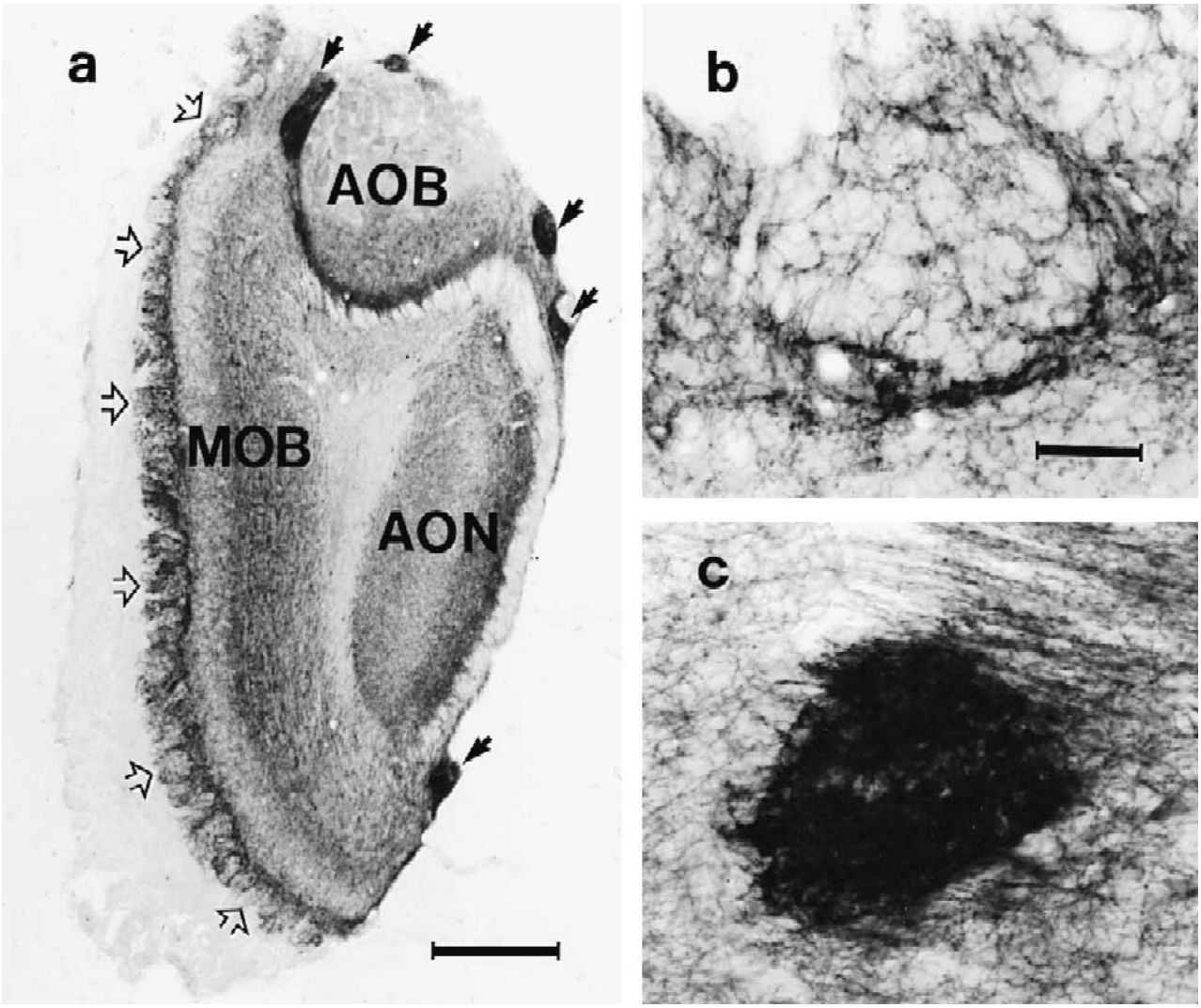

Fig. 1. AChE-positive staining in the rat olfactory bulb after standard incubation period (30 min). Scale bar = 500 μm (a) and 50 μm (b and c). a: overview of a coronal AChE-stained section of the rat OB showing the localization of both typical (open arrows) and atypical (solid arrows) glomeruli. AOB: accessory olfactory bulb; AON: anterior olfactory nucleus; MOB: main olfactory bulb. b: AChE-positive innervation of a typical glomerulus. c: AChE-positive innervation of an atypical glomerulus.

reactions in the double-stained sections was reversed, i.e., beginning with the immunohistochemical labeling. Nevertheless, the unspecific background staining was slightly higher and therefore the AChE histochemical technique was carried out first in most double-labeled sections.

3.1. Calbindin D-28k (CB)

For this CaBP, a laminar labeling was observed in the rat OB. Most CB-immunopositive neurons were located in the periglomerular region of the glomerular layer. In this layer, the CB-stained neurons were round or piriform, small (8.71 ± 0.82 μm of maximum diameter) cells with one or two dendrites innervating one or two adjacent glomeruli (Fig. 2a,c; Fig. 3a,b,c). These morphological characteristics made easy the typification of these cells as periglomerular cells. A second neuronal type demonstrated CB immunoreactivity in the glomerular layer/external plexiform layer boundary. Their larger size and the presence of two to five dendrites coursing through the boundary between both layers without entering the glomeruli allowed us to identify these neurons as superficial short-axon cells.

CB-immunopositive periglomerular cells were abundant surrounding both typical and atypical glomeruli (Fig. 3a,b,c; Table 1). The quantitative analysis showed a similar distribution of the CB-immunopositive periglomerular cells surrounding both glomerular subsets. Statistically significant differences were not detected ($P > 0.001$) between the number of CB-labeled periglomerular cells in typical and atypical glomeruli. Moreover, there were no statistically significant differences in the distribution of positive cells located in both groups (dorsomedial and ventrolateral) of either typical or atypical glomeruli (Table 1).

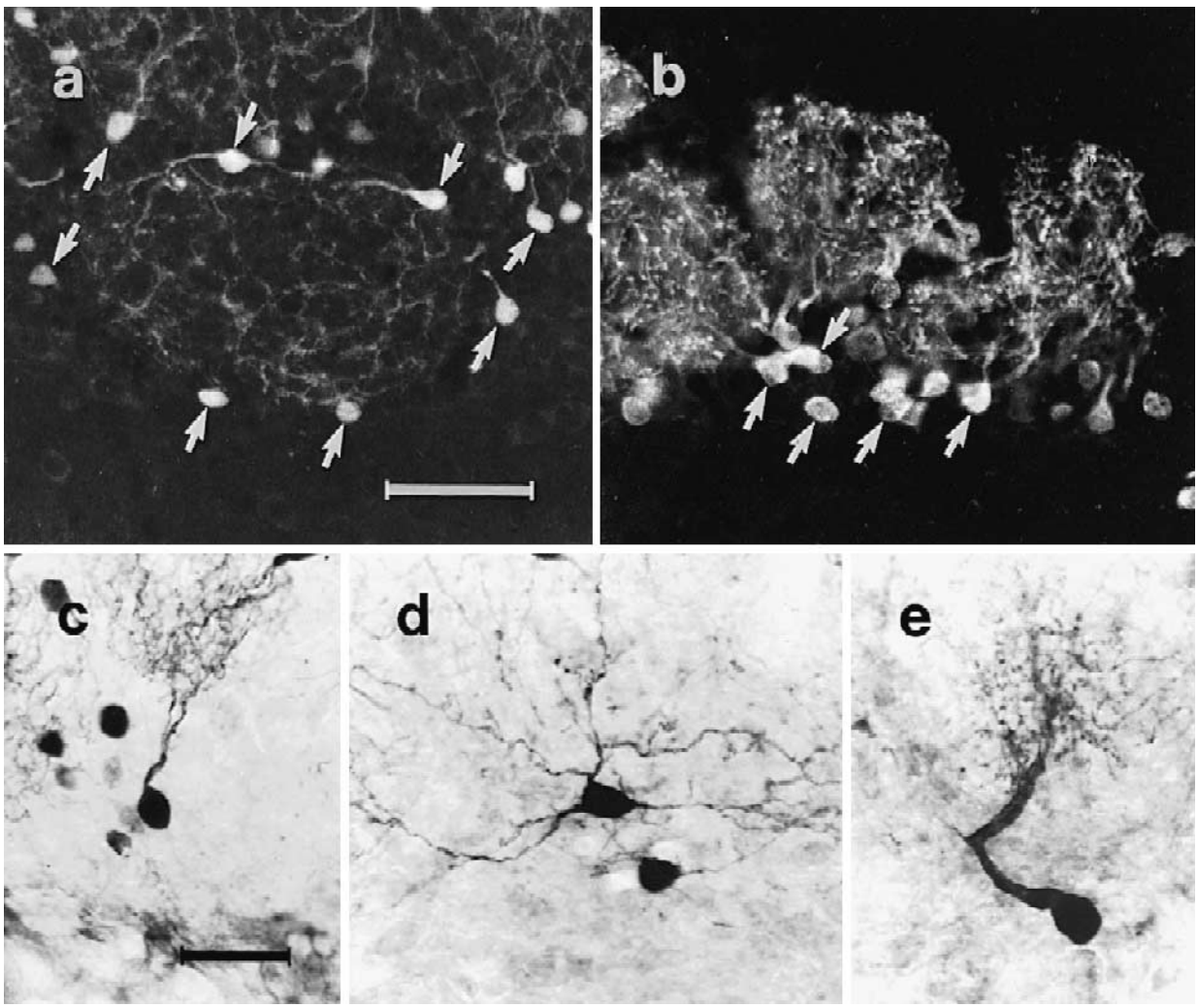

Fig. 2. CaBP immunostaining in the glomerular layer. Scale bar = 50 μm (a and b) and 25 μm (c–e). a: CB-immunofluorescent labeling in a typical glomerulus. CB-immunopositive periglomerular cells are observed (arrows). b: NC-immunofluorescent labeling in a typical glomerulus. NC-immunopositive external tufted cells can be observed (arrows). c: CB-immunostained periglomerular cell. d: PV-immunostained superficial short-axon cell. e: NC-immunostained external tufted cell.

3.2. Parvalbumin (PV)

The PV immunocytochemistry demonstrated a laminar distribution of this protein in the rat OB. The distribution pattern of PV was different to that observed for CB. Abundant PV-immunopositive cells were located in the external plexiform layer. In the glomerular layer, by contrast, only a few neurons were PV immunoreactive. These

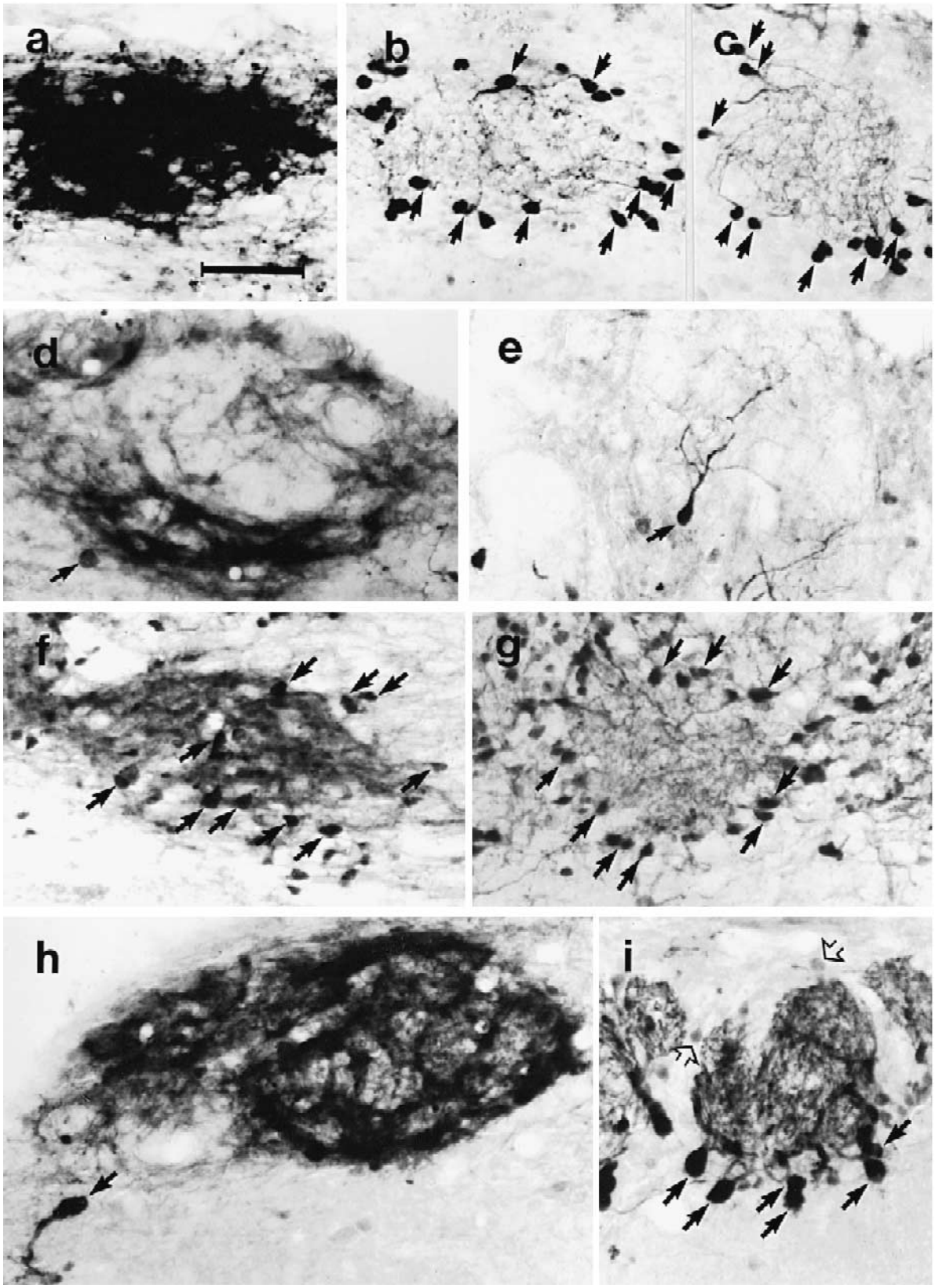

Table 1
Number of CaBP-immunopositive cells per sectioned olfactory glomerulus

	Typical glomeruli		Atypical glomeruli	
	Dorsomedial area	Ventrolateral area	Dorsomedial area	Ventrolateral area
CB-immunopositive periglomerular cells	10.37 ± 0.30	10.06 ± 0.27	9.52 ± 0.30	9.61 ± 0.31
CR-immunopositive periglomerular cells	33.53 ± 0.72	33.42 ± 0.72	32.55 ± 0.89	34.01 ± 0.90
NC-immunopositive external tufted cells	8.43 ± 0.25	9.51 ± 1.14	3.23 ± 0.20 *	3.32 ± 0.19 *

Mean ± S.E.M. of the number of CB-immunopositive periglomerular cells, CR-immunopositive periglomerular cells, and NC-immunopositive external tufted cells per 20 μm sectioned typical and atypical glomeruli of the dorsomedial and ventrolateral areas. Asterisks show statistically significant differences between the number of NC-immunopositive external tufted cells in the periglomerular region of typical and atypical glomeruli of the dorsomedial and ventrolateral areas. $n = 1280$ (number of quantified sections of glomeruli for each CaBP).

cells, mainly located in the internal area of the glomeruli, were identified as periglomerular cells (8.32 ± 0.67 μm of maximum diameter) (Fig. 3d,e) and superficial short-axon cells (16.21 ± 0.34 μm of maximum diameter) (Fig. 2d) with similar morphology that the same neuronal types observed after CB immunostaining. Both neuronal types were found surrounding typical and atypical glomeruli from the dorsomedial and ventrolateral areas. PV-immunopositive periglomerular cells were found innervating both glomerular subsets (Fig. 3d,e). Nevertheless, the very scarce number of PV-immunopositive periglomerular cells (less than one periglomerular cell per glomerulus) prevented us to carry out a similar statistical analysis of the distribution of these cells, as those carried out for CB, CR, and NC.

3.3. Calretinin (CR)

The highest number of immunoreactive structures of the four tested CaBPs in the rat OB was found for CR. Except the olfactory nerve layer, all OB layers displayed a high number of CR-immunopositive cells. In the glomerular layer, numerous (Table 1) periglomerular cells (8.96 ± 0.91 μm of maximum diameter), morphologically similar to the CB- and PV-immunopositive periglomerular cells, were CR-immunostained (Fig. 3f,g). In addition, a scarce number of larger cells (15.91 ± 0.52 μm of maximum diameter) identified by their morphological characteristics as superficial short-axon cells demonstrated CR immunoreactivity in the glomerular layer/external plexiform layer boundary.

A similar distribution of CR-immunopositive cells was observed in the periglomerular region of typical and atypical glomeruli (Fig. 3f,g and Table 1). The statistical treatment of the data demonstrated that there were no statistically significant differences ($P > 0.001$) between the distribution of CR-immunopositive periglomerular cells surrounding typical and atypical glomeruli in both the dorsomedial and ventrolateral areas (Table 1).

3.4. Neurocalcin (NC)

Like the other three CaBPs, NC showed a laminar distribution pattern in the rat OB. The highest number of NC-immunopositive cells in the rat OB was found in the glomerular layer, where two different neuronal types could be distinguished surrounding both typical and atypical glomeruli. The first neuronal population was formed by large piriform cells with one or two thick dendrites directed towards one of two different glomeruli. These dendrites branched profusely inside the glomeruli constituting an intraglomerular tuft. The size (12.91 ± 0.63 μm of maximum diameter) and morphology of these NC-immunopositive cells allowed their identification as external tufted cells (Fig. 2b,e and Fig. 3h,i). The second neuronal population was constituted by small weakly NC-stained neurons. The weak labeling in these cells allowed exclusively the visualization of the cell bodies and the proximal dendrites. According to their location in the periglomerular region of the glomerular layer and their small size (8.45 ± 0.83 μm of maximum diameter), these cells were typified as periglomerular cells (Fig. 3i). The NC-immunostained periglomerular cells could not be clearly ascribed to a typical or atypical glomeruli due to the lack of immuno-

Fig. 3. AChE histochemistry and CaBPs immunocytochemistry in adjacent sections, and AChE histochemical/CaBPs immunocytochemical double-labeling in typical and atypical glomeruli. Scale bar = 50 μm for all figures. a: AChE single-labeling in an atypical glomerulus. b: same atypical glomerulus as in 'a' in an adjacent section after CB immunocytochemistry. CB-immunopositive periglomerular cells are placed in its periglomerular region (arrows). c: CB-immunopositive periglomerular cells in a typical glomerulus (arrows). d: PV/AChE double-staining in an atypical glomerulus. A PV-positive periglomerular cell can be observed (arrow). e: PV-positive periglomerular cell innervating a typical glomerulus (arrow). f: CR-stained periglomerular cells innervating an atypical glomerulus (arrows). g: CR-labeled periglomerular cells in a typical glomerulus (arrows). h: NC/AChE double-staining in an atypical glomerulus. An NC-stained external tufted cells is close to the atypical glomerulus but does not innervate it (arrow). i: NC/AChE double-staining in a typical glomerulus showing NC-labeled external tufted cells innervating them (arrows). A few weakly NC-stained periglomerular cells also could be observed (open arrows).

staining in the distal dendrites, and it prevented us to include this neuronal population in the quantitative analysis.

The quantification of the NC-immunopositive external tufted cells demonstrated a segregation in their distribution between typical and atypical glomeruli. The number of NC-immunolabeled external tufted cells was lower in the periglomerular region of atypical glomeruli than in the periglomerular area of the typical ones (Table 1). These differences were statistically significant ($P < 0.001$). Comparing the distribution of NC-immunopositive external tufted cells in the atypical glomeruli of both the dorsomedial and the ventrolateral areas, statistically significant differences were not found ($P > 0.001$), showing a homogeneous distribution between both groups of atypical glomeruli. Similarly, statistically significant differences were not found between the dorsomedial and the ventrolateral typical glomeruli ($P > 0.001$).

4. Discussion

This work describes the distribution of four CaBPs in the periglomerular region of two different glomerular subsets: typical and atypical glomeruli. The AChE staining pattern and the CaBP-immunopositive neuronal types described in the present report in the periglomerular area of the glomerular layer are in close agreement with previous reports [2,6,7,10,21,26,29,31]. Only for NC, there are discrepancies between our results and previous descriptions [6]. These authors described external tufted cells as the only neuronal type NC-immunopositive in the rat OB, whereas we found small weakly labeled neurons surrounding glomeruli indicating that a subpopulation of periglomerular cells is also NC-immunopositive.

The main result of this report is that NC-immunopositive external tufted cells are segregately distributed in both glomerular subsets being statistically more abundant ($P < 0.001$) in the typical glomeruli, whereas, by contrast, CB-, and CR-immunopositive periglomerular cells were homogeneously distributed in both types of glomeruli. Other immunostained cells observed in the periglomerular region of typical and atypical glomeruli were not evaluated since either their very low frequency (PV-immunopositive periglomerular cells) or their weak labeling (NC-immunopositive periglomerular cells) did not allow a representative or a reliable quantification.

As demonstrated previously [2,6,29] and in the present results, three of the four analyzed CaBPs (CB, CR, and NC) have their main expression in neuronal subpopulations directly implied in the intraglomerular circuitries of the rat OB. It may suggest an important role of calcium regulation in the first relay of the olfactory information. In addition, our results suggest different and specific roles of these CaBPs in the glomerular activity. The identical distribution of CB- and CR-immunopositive periglomerular cells in typical and atypical glomeruli supports an implication of both CaBPs in general functions of the glomerular circuitries, instead of a topographically specific processing of the olfactory information in different sets of olfactory glomeruli. In the same way, a homogeneous distribution of the CB-stained periglomerular cells has been found in ND-positive and ND-negative glomerular subsets [2]. These results are in agreement with the homogeneity found in this report in relation to typical and atypical glomeruli. By contrast, the segregation of NC-immunopositive external tufted cells may indicate an involvement of NC in the specific functionality of different sets of glomeruli. NC immunoreactivity demonstrates also a characteristic segregation in the vomeronasal glomeruli of the rat accessory bulb [28]. Therefore, it may be hypothesized that some neuronal populations are related with general properties of the glomerular physiology and they are homogeneously distributed in different subsets of glomeruli (e.g., CB-immunopositive or CR-immunopositive periglomerular cells), whereas other chemically identified populations are related with a finer tuning, and they are more abundant in particular subsets of glomeruli (e.g., NC-immunostained external tufted cells).

Atypical glomeruli were firstly identified as a glomerular subset different to the typical ones on the basis of differences in their glomerular neuropile [37,38]. The presence of a high concentration of AChE- and choline acetyltransferase-positive centrifugal fibers in these glomeruli [22,38], and the particular ultrastructure of olfactory axons in their neuropile [23,37] suggest particularities in the integration and modulation of olfactory information in these glomerular subsets. A previous work of our group [14] demonstrated that atypical glomeruli differ from typical ones not only in the organization of primary and centrifugal afferents, but in the distribution of first-order interneurons surrounding them. In this sense, a three-folds higher number of ND/NOS-positive periglomerular cells was found in the periglomerular region of typical glomeruli than in the periglomerular area of the atypical ones [14]. Contrary to this segregation, the present report shows that other periglomerular cells (CR-, or CB-immunopositive periglomerular cells) have an identical distribution in both glomerular subsets. Although both CB and CR stain the same neuronal types in the glomerular layer of the rat OB, both neuronal markers did not colocalize in these neurons indicating that they are different neuronal subpopulations [31]. The present results indicate that not all periglomerular cells (e.g., CR- or CB-immunopositive periglomerular cells), but only specific periglomerular populations (e.g., ND/NOS-positive periglomerular cells) have a heterogeneous distribution related with typical and atypical glomeruli.

The segregation of NC-immunopositive external tufted cells found in this report adds new neuroanatomical particularities to the intrinsic neuronal circuitries of atypical glomeruli. External tufted cells project their axons to other

regions of the ipsilateral OB, or by the anterior olfactory nucleus to other regions of the contralateral OB [25,33,34]. Therefore, atypical glomeruli differ from typical ones in very different components of their circuitry, including primary afferents [23,37], centrifugal innervation [22,24,38], periglomerular cells [14], and external tufted cells (present data). These particularities have interesting functional implications. First, the particular olfactory connections suggest a specific organization of the olfactory information input in these glomeruli. Second, the atypical cholinergic innervation of this glomerular population from centrifugal fibers demonstrate a massive regulation of the olfactory information processing in this glomerular subset from superior centers. Third, this information is modulated by different chemically identified subpopulations of periglomerular cells. These neurons are similarly (e.g., CB or CR-immunopositive periglomerular cells) or down-represented (e.g., ND/NOS-positive periglomerular cells) in comparison with typical glomeruli. Finally, the particularities of the atypical glomerular circuitries may be related to the topographically organized interbulbar and intrabulbar connections as indicate the segregated distribution of NC-immunopositive external tufted cells in their periglomerular region.

Atypical glomeruli appear in the rat OB in two different locations. They are placed in the dorsomedial and the ventrolateral areas of the caudalmost OB. The neuroanatomical particularities found in this glomerular subset are identical for the dorsomedial and the ventrolateral atypical glomeruli. In this sense, the high concentration of AChE- and choline acetyltransferase-positive centrifugal fibers, the particularities of the olfactory axons, and the low number of ND/NOS periglomerular cells are similar in the atypical glomeruli of the dorsomedial and ventrolateral areas [14,22–24,37,38]. These similarities lead to consider these atypical glomeruli as homologous and implied in the same olfactory events [14,22–24,37,38]. The identical distribution of NC-positive external tufted cells in the periglomerular region of dorsomedial and ventrolateral atypical glomeruli supports this hypothesis.

The segregation of NC-immunopositive external tufted cells between typical and atypical glomeruli may suggest a more direct implication of these neurons in the complex organization of specific neuronal circuitries related with the processing of specific olfactory information at the glomerular level. In this sense, external tufted cells are likely to function in ways that tend to preserve and possibly enhance spatially distributed information across populations of neurons [25]. These authors reported that external tufted cells may play a key role in the processing of sensory information related to the topographically organization of the connections at the glomerular level. Zheng and Jourdan [37] reported that atypical glomeruli might have morpho-functional homologies with the modified glomerular complex described by Teicher et al. [36] and Greer et al. [17]. 2-deoxyglucose method demonstrates that this glomerular subset is involved in the processing of olfactory information concerned with pheromone-like molecules important in suckling behaviour in neonatal rats [17,22–24,36,37]. Thus, atypical glomeruli seem to be a crucial element for a particular olfactory pathway, transmitting a restricted group of signals, with a strong centrifugal control, and a limited local modulation in comparison with typical glomeruli.

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