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Coexpression of Neurocalcin With Other Calcium-Binding Proteins in the Rat Main Olfactory Bulb

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ABSTRACT

The distribution patterns of four calcium-binding proteins (CaBPs)—calbindin D-28k (CB), calretinin (CR), neurocalcin (NC), and parvalbumin (PV)—in the rat main olfactory bulb were compared, and the degrees of colocalization of NC with the other CaBPs were determined by using double immunocytochemical techniques.

All investigated CaBPs were detected in groups of periglomerular cells and Van Gehuchten cells, whereas other cell types expressed some of the investigated proteins but not all four. Double-labeling techniques demonstrated the colocalization of NC with CB, CR, or PV in periglomerular cells, whereas each neurochemical group constituted entirely segregated populations in the remaining neuronal types. This is evident in granule cells that demonstrated large but segregated populations immunoreactive to either NC or CR.

This study provides a further biochemical characterization of interneuronal types in the rat main olfactory bulb. On the basis of the distinct calcium-binding affinities, each neurochemically defined population may have different responses to calcium influx that would result in the existence of distinct functional subgroups within morphologically defined neuronal types. The expression of the investigated CaBPs in periglomerular cells with both single and colocalized patterns suggests that the local circuits in the glomerular layer are constituted by a complex network of elements with particular calcium requirements. J. Comp. Neurol. 407:404–414, 1999.

Indexing terms: calbindin D-28k; calretinin; olfactory system; parvalbumin; specific cell markers

The olfactory bulb is characterized by the laminar distribution of different classes of neurons whose properties and synaptic relationships are well known through morphological, electrophysiological, and biochemical analyses (among others, see Mori, 1987; Halász, 1990; Shepherd and Greer, 1990; Shipley et al., 1995). The detection of calcium-binding proteins (CaBPs) such as calbindin D-28k (CB), calretinin (CR), or parvalbumin (PV) in this brain region has allowed the identification of distinct biochemical subgroups within morphologically identical neuronal populations (Halász et al., 1985; Briñón et al., 1992; Résibois and Rogers, 1992; Rogers and Résibois, 1992; Kosaka et al., 1994). Each CaBP demonstrates a specific distribution in the olfactory bulb, although they are frequently expressed in cell groups belonging to the same neuronal type but in segregated populations, as has been demonstrated for CB and PV (Celio, 1990) and for CB and CR (Rogers and Résibois, 1992). This expression has been considered as evidence for the existence of hodological and/or physiological differences among morphologically and topologically similar neuronal populations (Celio, 1990).

Neurocalcin (NC) is a more recently identified CaBP (Nakano et al., 1992) and is also a member of the EF-hand family. Previous investigations have demonstrated that

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NC in the rat olfactory bulb is expressed in tufted cells (Bastianelli et al., 1993), a neuronal type that does not express CB, CR, or PV (Briñón et al., 1992; Résibois and Rogers, 1992; Kosaka et al., 1994). However, more recent studies have shown the presence of NC in additional cell types (Briñón et al., 1998), including periglomerular cells, granule cells, Van Gehuchten cells, and deep short-axon cells, similar to neuronal types that have been reported to be CB, CR, or PV immunopositive. Because the coexistence of NC with these other markers has not yet been investigated, we studied whether the NC-containing cells form a neuronal population separate from those expressing CB, CR, or PV, or, conversely, whether these groups overlap totally or partly. Their probable coexpression in specific neuronal populations would provide further characterization of neurochemically and morphologically identified neuronal groups. In addition, taking into account that NC belongs to the calcium-sensor group whose members have been proposed to be concerned with modulatory functions, whereas CB, CR, and PV are calcium-binding proteins involved in calcium buffering and transport (Ikura, 1996), the patterns of coexpression of NC with other known CaBPs could provide some insights into the functions of these proteins in individual neurons of the olfactory pathway and in other neuronal systems.

MATERIALS AND METHODS

Five adult male Wistar rats weighing 200–250 g were grown and kept under standard laboratory conditions. All experimental procedures conformed to NIH guidelines and were in accordance with the guidelines of the European Communities Council Directive (86/609/EEC) and current Spanish legislation for the use and care of laboratory animals (BOE 67/8509–12, 1988). After deep anesthesia (Ketolar, 50 mg/kg body weight), the animals were intracardially perfused with 100 ml Ringer's solution followed by 400 ml fixative solution made up of 4% paraformaldehyde and 2% picric acid in 0.1 M phosphate buffer, pH 7.3 (PB). The brains were then removed, and the olfactory bulbs were dissected and postfixed in the same fixative at 4°C for two additional hours. Four olfactory bulbs from different animals were dehydrated through an ethanol series and embedded in Durcupan (Sigma, St. Louis, MO). From each olfactory bulb, two series of adjacent 1-µm semithin sections were obtained in a Reichert Jung Ultramicrotome and mounted on slides. The six remaining olfactory bulbs were cryoprotected by immersion in 30% sucrose in PB at 4°C for 12 hours, and 25-µm-thick coronal sections were obtained by using a cryostat.

Calcium-binding protein immunohistochemistry

Series of free-floating cryostat sections from different animals were washed in PB and incubated for 30 minutes in either 5% normal goat serum or 5% normal horse serum in PB. After being washed in PB, one series from each animal was incubated for 48 hours at 4°C in primary antibody solution: 1:4,000 mouse anti-PV (Celio et al., 1988), 1:5,000 rabbit anti-PV (Swant antibodies, Bellinzona, Switzerland), 1:4,000 mouse anti-CB (Celio et al., 1990), 1:5,000 rabbit anti-CB (Swant), 1:15,000 rabbit anti-CR (Rogers, 1987, 1989), or 1:8,000 rabbit anti-NC (Nakano et al., 1992) diluted in either 2% normal goat serum or 2% normal horse serum and 0.1% Triton X-100 in PB. The sections were then processed according to the avidin-biotin immunoperoxidase method (Hsu et al., 1981). The sections were washed (3 × 10 minutes) in PB and incubated for 1 hour at room temperature in biotinylated anti-rabbit or anti-mouse immunogammaglobulin (Vectastain, Vector Laboratories, Burlingame, CA) diluted 1:250 in PB. After washing in PB (3 × 10 minutes), sections were transferred to Vectastain Elite ABC reagent (Vector Laboratories) diluted 1:200 in PB for two additional hours. Tissue-bound peroxidase was visualized by using 0.05% 3,3′-diaminobenzidine tetrahydrochloride (Sigma) and 0.003% hydrogen peroxide in 0.2 M Tris-HCl buffer, pH 7.6, for 10–15 minutes, until the desired staining intensity was obtained. Sections were then rinsed in PB, mounted on gelatin-coated slides, air dried, dehydrated through graded ethanol series, cleared in xylene, and coverslipped with Entellan (Merck, Darmstadt, Germany). The analysis was carried out using a Zeiss III microscope, and photographs were taken on a Zeiss Axiophot photomicroscope.

Analysis of coexistence of neurocalcin with other calcium-binding proteins

The analysis of coexistence of NC with PV, CB, or CR in the same neuron was carried out by using either the double-immunofluorescence technique or postembedding immunohistochemistry on adjacent semithin sections.

Double-immunofluorescence technique. Cryostat sections were processed for the immunofluorescence technique to analyze the coexistence of NC with either CB or PV. After preincubation, the sections were incubated for 48 hours at 4°C in a mixture of either 1:4,000 rabbit anti-NC and 1:2,000 mouse anti-CB or 1:4,000 rabbit anti-NC and 1:2,000 mouse anti-PV in 1% normal goat and horse serum and 0.1% Triton X-100 in PB. After washes in PB (3 × 10 minutes), the sections were incubated for two hours at room temperature in a mixture of horse anti-mouse fluorescein isothiocyanate-conjugated and goat anti-rabbit Texas red conjugated immunogammaglobulins (Vector Laboratories) diluted 1:75 in PB. After washing in PB, sections were mounted on gelatin-coated slides and coverslipped with an antifading-containing medium (0.42 g glycine, 0.021 g sodium hydroxide, 0.51 g sodium chloride, 0.03 g sodium azide, and 5 g N-propyl gallate in 100 ml of 70% glycerin). Double-labeled cells were identified and photographed in a Zeiss Axiophot photomicroscope equipped with epifluorescence and appropriate filter sets (Zeiss filter sets 487900 and 487909).

Postembedding immunohistochemistry on semithin sections. This procedure was used to determine the degree of coexistence of NC with CB, CR, or PV. Serial semithin sections were treated for 20 minutes with saturated sodium ethoxyde and then hydrated through an ethanol series until water. The immunohistochemical procedure was then carried out as follows: after incubation for 15 minutes in 5% normal goat serum in PB, the sections were incubated for 2 hours at room temperature in 1:4,000 rabbit anti-NC diluted in PB. The adjacent sections were incubated in 1:2,000 rabbit anti-CB, 1:8,000 rabbit anti-CR, or 1:2,000 rabbit anti-PV diluted in 2% normal goat serum and 0.1% Triton X-100 in PB. The sections were then processed according to the avidin-biotin immunoperoxidase technique as described above.

Immunoreactive perikarya and nearby blood vessels were drawn by using a camera lucida and a 100× oil-

TABLE 1. Density of Immunopositive Neurons for the Investigated Calcium-Binding Proteins in a 25-µm-Thick Section[1]

Cell type	Neurocalcin	Calbindin D-28k	Calretinin	Parvalbumin
Periglomerular cells	+++++	+++++	+++++	++
Superficial tufted cells	+++++	–	–	–
Superficial short-axon cells	–	++	+++	++++
Van Gehuchten cells	+	+	++	+++
Mitral cells	–	–	+++	–
Deep short-axon cells	+	+	+	++
Granule cells	++++	–	+++++	+

[1]Frequency: +++++, more than 500 cells per section; ++++, 250–500 cells per section; +++, 100–250 cells per section; ++, 25–100 cells per section; +, less than 25 cells per section.

immersion objective. The profiles of the same perikarya were located, by using capillaries as reference, on the adjacent section (incubated to detect the other antigen). Only those cells whose profiles were unequivocally present in both adjacent sections were included in the evaluation.

Controls

All primary antibodies have been fully characterized and they do not cross react with other brain proteins (Rogers, 1987, 1989; Celio et al., 1988, 1990; Nakano et al., 1992). Controls of the immunohistochemical procedure were performed by incubating sections in the same media but without the primary antibody, the biotinylated or fluorescent conjugated immunogammaglobulin, the avidin-peroxidase complex, or incubation of each individual primary antibody with noncorresponding secondary biotinylated or fluorescent conjugated immunogammaglobulin. In these cases, no residual reaction was observed. Incubation of each primary antibody with mixed secondary immunogammaglobulins did not demonstrate changes in immunostained elements as when incubating only antibody with its corresponding secondary immunogammaglobulin.

Counting procedures

Assessments of the density of immunoreactive neurons for each CaBP were performed by counting the stained neurons of each neuronal type in coronal sections at nine equidistant levels throughout the rostrocaudal axis of the olfactory bulb. The first chosen section was that showing all olfactory bulb layers; the remaining eight sections were selected at 300-µm intervals. These data are shown in Table 1.

The colocalization degree of NC with the other investigated CaBPs was assessed by counting immunostained cells in 12 paired semithin sections from four different animals at different rostrocaudal levels (48 paired sections per marker). Neurons included in the evaluation were those showing a clearly visible nucleus, which in most cases, allowed their identification with certainty. For the most numerous neuronal types, e.g., periglomerular cells and granule cells, cells counted were those appearing within a selected area. For periglomerular cells, four 200-µm-long strips of the glomerular layer were selected in each bulbar section at dorsal, ventral, medial, and lateral sides. For granule cells, neurons included in four nonoverlapped squares (100-µm side) per section were counted. For the remaining neuronal types, all cells appearing in the section were included in the evaluation. These data are shown in Tables 2–4.

RESULTS

After immunohistochemistry for all four CaBPs, positive elements showed a laminar appearance throughout the whole rostrocaudal axis of the olfactory bulb, with a constant staining intensity and distribution in all animals. The identification of positive elements was obtained from cryostat sections processed according to the peroxidase technique. Figure 1 shows schematically the distribution of cell types immunostained for each CaBP, and Table 1 shows the relative density. In addition, an overview of the olfactory bulb after immunostaining for each CaBP is shown in Figure 2.

The olfactory nerve layer did not demonstrate immunoreactivity for either NC or PV. Fibers coming from the olfactory receptors were always immunonegative for these markers, at least when entering the olfactory bulb (Fig. 2). A few nerve bundles were observed with a weak immunostaining for CB, whereas CR-immunolabeling showed many more positive fibers. The CR-positive fibers could be followed entering individual glomeruli, originating a moderately labeled glomerular neuropil. These stained glomeruli were intermingled among negative ones, and, although they appeared in different locations, they were most frequently observed at the lateral side of the olfactory bulb.

The olfactory glomeruli were surrounded by numerous cells, most of which were periglomerular cells. A large number of these cells was found in the sections immunostained for the detection of NC, CR, or CB (Fig. 2a–c; Table 1), whereas PV-immunoreactive periglomerular cells were much scarcer (Fig. 2d; Table 1). Despite the different biochemical characteristics of these cells, no differences in the somatic or dendritic morphological features were detected among periglomerular cells of each neurochemical group.

Colocalization studies demonstrated that NC-positive periglomerular cells partly overlapped those expressing the other analyzed CaBPs, although the degree of coexpression of NC with the other CaBPs investigated differed notably. Thus, as indicated in Table 2, coexpression of NC and CB in periglomerular cells was rare. In fact, most periglomerular cells expressing either NC or CB were entirely segregated (Fig. 3a,b), and only a small number (n = 10) of NC and CB double-labeled cells (Fig. 4a,b) were observed after analyzing a large sample of NC- and CB-stained periglomerular cells (n = 4,563). These double-stained cells represented 0.5% of those NC-positive and 0.4% of CB-positive periglomerular cells.

The periglomerular cells expressing PV were observed mainly at the medial and lateral glomeruli but were practically absent from dorsal and ventral ones. The few numbers of PV-immunoreactive periglomerular cells located at either dorsal or ventral glomeruli were almost entirely segregated from those expressing NC (Fig. 3e,f). Thus, most periglomerular cells coexpressing NC and PV (48 of 2,114) were found in medial and lateral glomeruli (Fig. 4c,d). Whereas the coexistence of these markers occurred in a small percentage of NC-positive periglomerular cells (2.6%), they represented a significant population (20%) of PV-immunopositive periglomerular cells (Table 3).

The largest number of double-stained cells was found after NC and CR immunolabelings (n = 955; Table 4). Periglomerular cells expressing both markers appeared with similar incidence regardless of the location of the

TABLE 2. Coexistence of Neurocalcin (NC) and Calbindin D-28k (CB)

Cell type	Number of NC cells	Number of CB cells	Number of NC–CB cells	%NC cells colocalizing CB	%CB cells colocalizing NC
Periglomerular cells	2,009	2,554	10	0.5	0.4
Superficial short-axon cells/Van Gehuchten cells	51	107	0	0	0
Deep short-axon cells	43	39	0	0	0
Granule cells	894	0	0	0	0

TABLE 3. Coexistence of Neurocalcin (NC) and Parvalbumin (PV)

Cell type	Number of NC cells	Number of PV cells	Number of NC–PV cells	%NC cells colocalizing PV	%PV cells colocalizing NC
Periglomerular cells	1,877	237	48	2.5	20.2
Superficial short-axon cells/Van Gehuchten cells	49	296	0	0	0
Deep short-axon cells	41	28	0	0	0
Granule cells	769	76	0	0	0

TABLE 4. Coexistence of Neurocalcin (NC) and Calretinin (CR)

Cell type	Number of NC cells	Number of CR cells	Number of NC–CR cells	%NC cells colocalizing CR	%CR cells colocalizing NC
Periglomerular cells	2,286	6,275	955	41.7	15.2
Superficial short-axon cells/Van Gehuchten cells	60	204	0	0	0
Deep short-axon cells	41	115	0	0	0
Granule cells	869	1,007	0	0	0

glomeruli (Fig. 5). Although NC- and CR-immunolabeled cells represented a moderate group with the large population of CR-immunopositive cells (15.2%), they constituted 41.8% of the total number of periglomerular cells expressing NC.

Intermingled among periglomerular cells were other neuronal populations immunoreactive to the analyzed CaBPs (Table 1). A large group of NC-immunopositive cells showed piriform cell bodies, larger than those of periglomerular cells, from which arose a single nonspiny dendrite branching inside the glomeruli. Some of these cells also showed secondary dendrites that coursed below the glomerular structures through the glomerular layer/external plexiform layer interface. By contrast, elements immunoreactive for CB, CR, or PV had fusiform cell bodies, with two or three main dendrites bearing occasional spines and frequent varicosities that extended in the glomerular layer/external plexiform layer border. These dendrites did not enter the olfactory glomeruli. On the basis of these morphological features, whereas NC-immunopositive neurons were identified as superficial tufted cells, neurons positive to the other investigated CaBPs were identified as superficial short-axon cells. In fact, investigation of the coexistence in the glomerular layer did not show any element, apart from periglomerular cells, demonstrating coexpression of NC with any of the other investigated CaBPs.

A moderate number of interneurons appeared in the external plexiform layer as immunoreactive to all investigated CaBPs. These interneuronal populations can be grouped into two morphologically different populations: Van Gehuchten cells and superficial short-axon cells. Van Gehuchten cells showed immunoreactivity to all CaBPs studied, being most frequently cells expressing PV and CR and those NC or CB immunoreactive much scarcer (Table 1). The populations positive to NC, CB, or CR occurred at the superficial half of the external plexiform layer, whereas those PV-immunolabeled were distributed throughout the whole extent of this layer. These biochemically identified subgroups exhibited particular morphological features according to their location regardless the marker they expressed. Thus, Van Gehuchten cells located at the superficial external plexiform layer demonstrated homogeneous somatic sizes and shapes and a similar branching pattern of their dendrites. Those located in the most internal region of the external plexiform layer, which were only observed after PV immunostaining, demonstrated more variable somatic morphology, and their dendrites were branched in a less dense and larger dendritic field.

Groups of superficial short-axon cells were immunoreactive for CB, PV, or CR. The PV-immunoreactive cells were the most numerous and morphologically variable and were observed throughout the whole extent of the external plexiform layer (Table 1). The CR-positive cells were located in the external and in the internal borders of the external plexiform layer; the central region of this layer was almost devoid of CR-immunopositive cells. Immunocytochemistry for NC did not show any neuronal population with morphologic characteristics of short-axon cells in either the superficial or the deep region of the external plexiform layer.

Analysis of the coexpression of NC with the other investigated CaBPs in these neuronal types located in the external plexiform layer was performed without distinguishing between these two morphologically different interneuronal types, given the difficulty to differentiate them in semithin sections. For this reason, data shown in Tables 2–4 include all interneuronal types in the external plexiform layer. According to our previous morphological analysis on cryostat sections, coexpression of NC with any other CaBP would be expected only in Van Gehuchten cells. However, after a detailed analysis, no cell expressing NC in the external plexiform layer colocalized with any other marker studied (Tables 2–4).

Mitral cells were clearly CB, NC, and PV negative but demonstrated CR immunolabeling. This staining was often weak and restricted to the cell body and the initial segment of the apical dendrite. This constituted the only neuronal type among principal neurons of the olfactory bulb that expressed a CaBP. Nevertheless, it is important

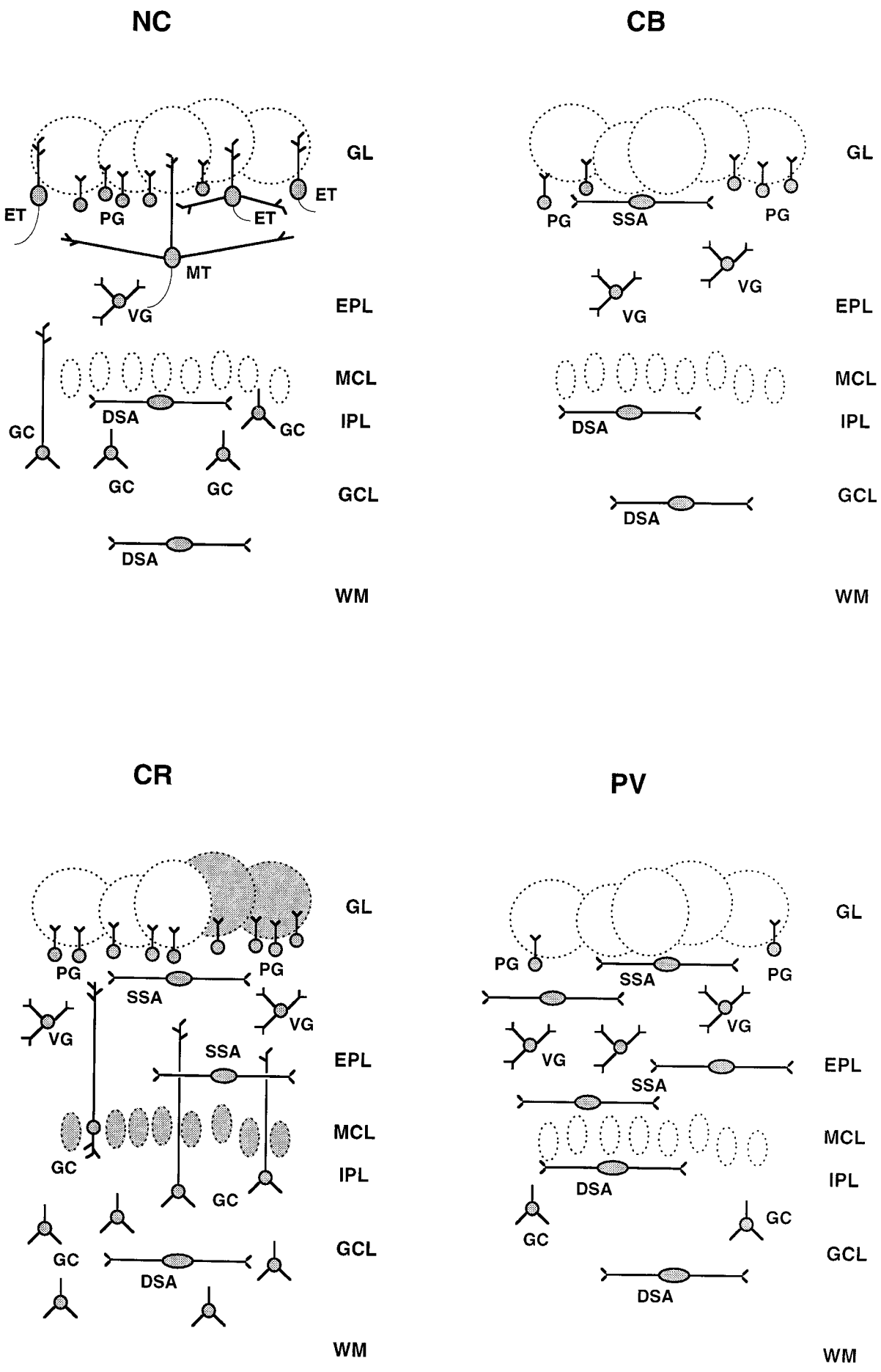
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Figure 1

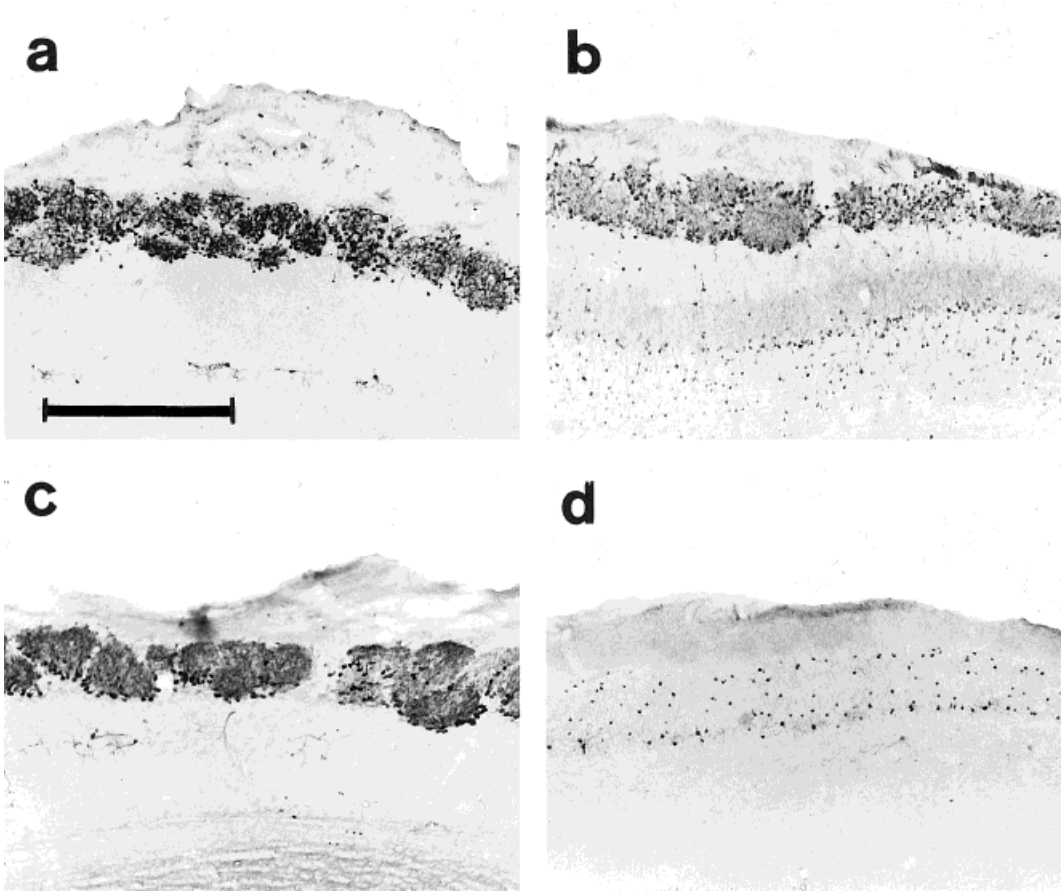

Fig. 2. Low power photomicrographs of coronal cryostat sections of the olfactory bulb immunostained for each calcium-binding protein showing the characteristic laminar pattern of immunoreactive elements. **a:** Calbindin D-28k. **b:** Calretinin. **c:** Neurocalcin. **d:** Parvalbumin. Scale bar = 500 µm.

to note that, despite the weak immunostaining in the mitral cell body, strongly CR-stained axons coursed in the lateral olfactory tract, which suggests a nonuniform intracellular distribution of this protein.

Inframitral layers demonstrated a moderate number of positive neurons for all studied CaBPs. On the basis of their location and morphologic features, they were identified as deep short-axon cells. Even though these different neurochemical groups demonstrated similar morphological and topological features, no neuron expressing NC was positive to any of the other investigated CaBPs (Fig. 3c,d).

Granule cells, the most abundant interneuronal type in the olfactory bulb, demonstrated immunoreactivity to NC, CR, and PV. Among these, NC- and CR-immunoreactive cells were numerous and PV-immunoreactive cells were much scarcer (Table 1). Regardless of the marker they expressed, they always showed small, round, or piriform cell bodies with a conspicuous apical dendrite coursing radially toward the external plexiform layer. Granule cells immunolabeled for CR showed stronger immunoprecipitate than did those that were either NC or PV immunoreactive. In addition, CR immunostaining demonstrated the apical dendrite in practically all its length, whereas the immunoprecipitate was restricted to the cell body and the initial region of the apical dendrite in NC- or PV-immunoreactive granule cells. CR-immunoreactive granule cells were distributed throughout the whole extent of the granule cell layer, whereas the NC-immunopositive ones were much more numerous in the superficial region of the granule cell layer, and the PV-immunoreactive ones were most frequently observed at the deep granule cell layer. Colocalization studies showed that the abundant population of NC-positive granule cells was entirely segregated from those expressing CR or PV (Tables 3, 4).

Fig. 1. Schematic drawing showing the distribution of neuronal elements expressing neurocalcin (NC), calbindin D-28k (CB), calretinin (CR), and parvalbumin (PV). DGC, displaced granule cells; DSA, deep short-axon cells; EPL, external plexiform layer; ET, external tufted cells; GC, granule cells; GCL, granule cell layer; GiC, giant cells; GL, glomerular layer; HZ, horizontal cells; IPL, internal plexiform layer; MCL, mitral cell layer; MT, middle tufted cells; ONL, olfactory nerve layer; PG, periglomerular cells; SSA, superficial short-axon cells; VC, vertical cells of Cajal; VG, Van Gehuchten cells; WM, white matter.

DISCUSSION

The main finding of the present report is the demonstration of the coexistence of NC with the other investigated CaBPs (CB, CR, and PV) in a specific population of interneurons of the rat olfactory bulb (periglomerular

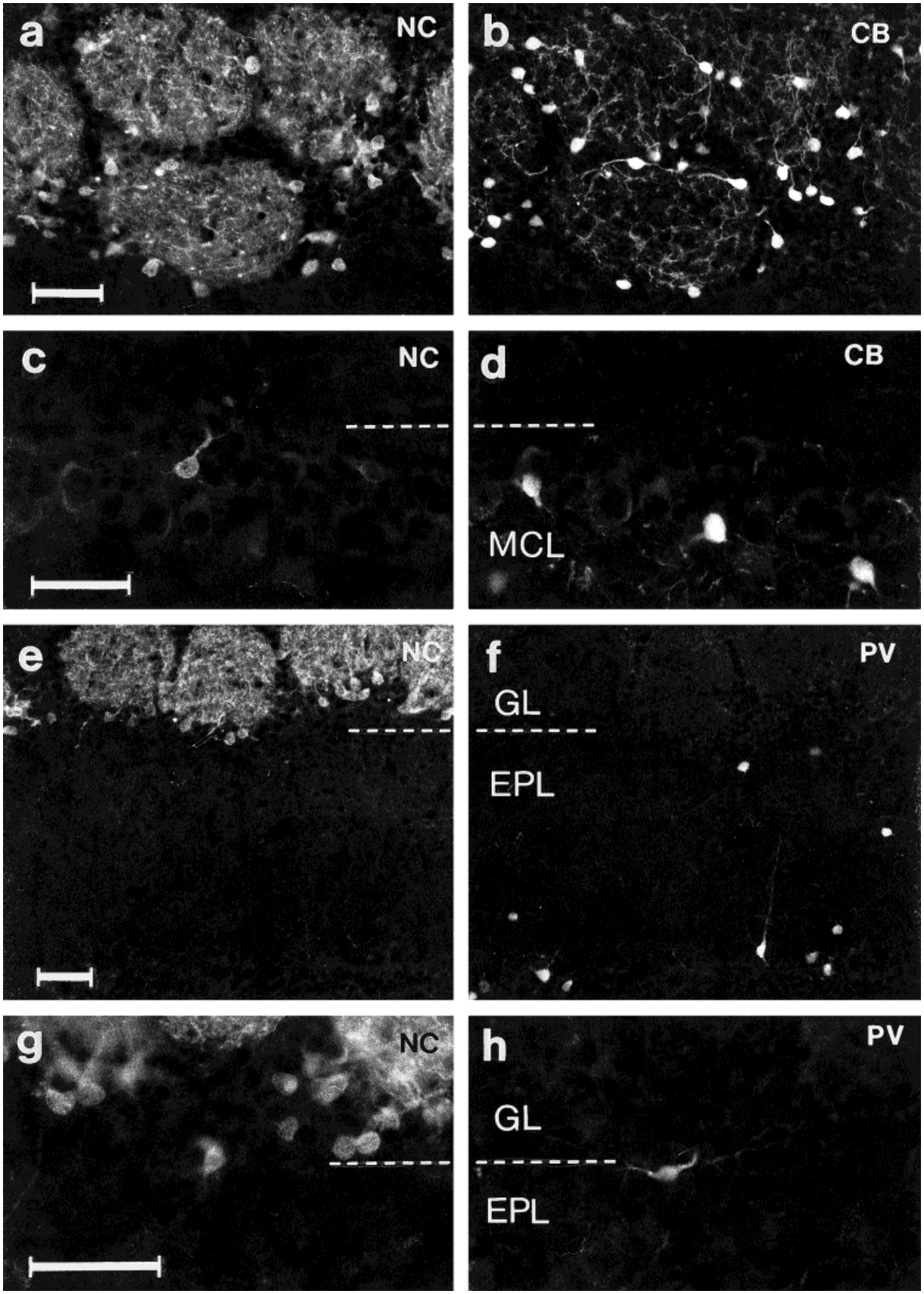

Fig. 3. Fluorescence photographic pairs of the same field from double-immunostained sections showing immunoreactivity for neurocalcin (NC) and calbindin D-28k (CB) and for NC and parvalbumin (PV). (**a**) NC- and (**b**) CB-immunoreactive neurons in this field of the glomerular layer appear as segregated populations. (**c**) NC-positive and (**d**) CB-positive deep short-axon cells in the mitral cell layer (MCL). Dashed lines indicate the boundary between the external plexiform layer (EPL) and the MCL. Partial view of the superficial layers of the rat main olfactory bulb shows differential distribution of NC-positive (**e**) and PV-positive (**f**) neurons. **g,h:** Border between the glomerular layer (GL) and the EPL, where segregated populations of NC-positive and PV-positive neurons are observed. Dashed lines in e–h indicate the boundary between the GL and the EPL. Scale bars = 50 μm.

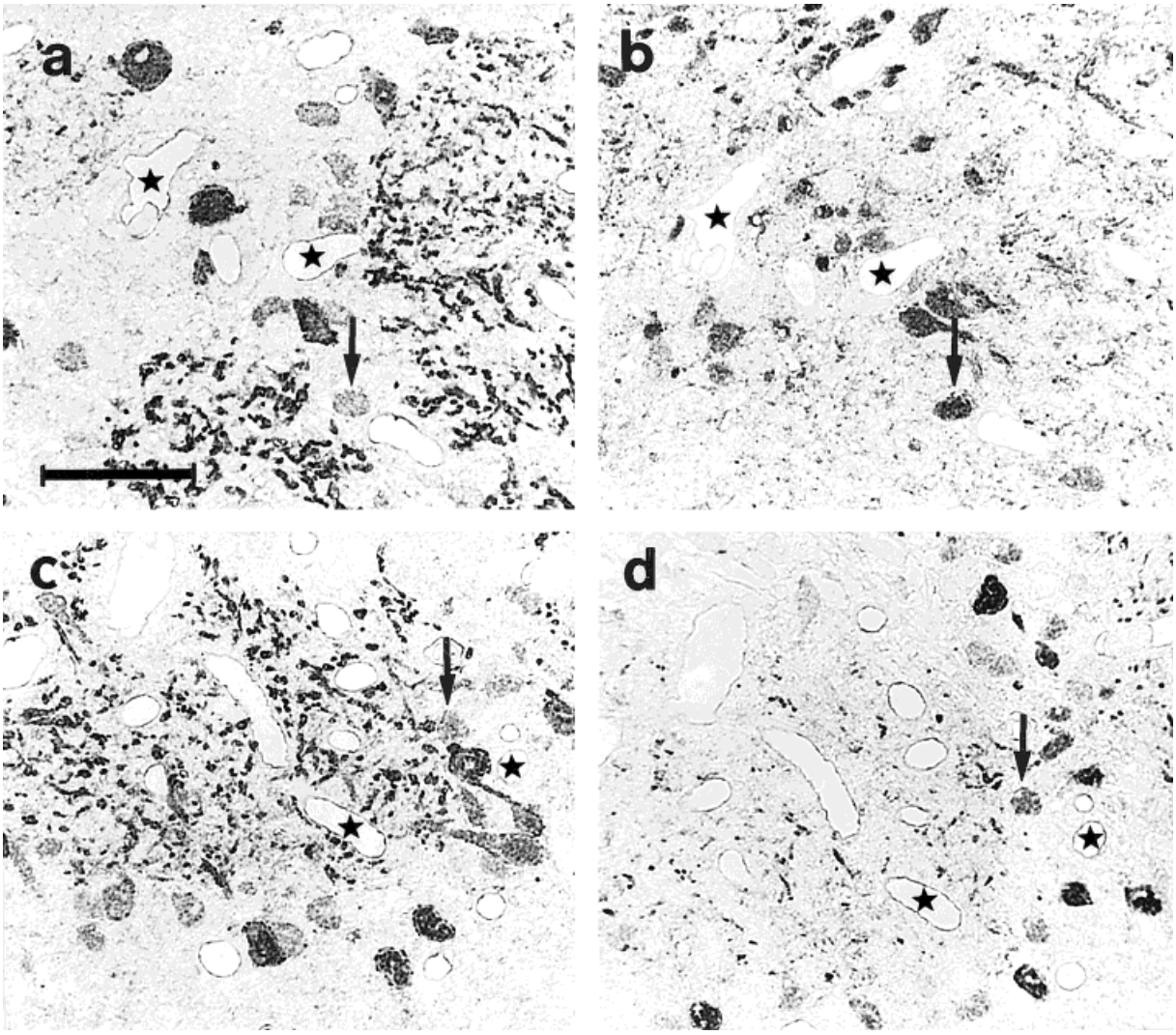

Fig. 4. Photomicrographs of adjacent semithin sections showing the glomerular layer after postembedding immunohistochemistry for detection of neurocalcin (NC; **a**) and calbindin D-28k (CB; **b**) or NC (**c**) and parvalbumin (PV; **d**). Blood vessels used as landmarks are marked with stars. NC (a) and CB (b) immunostaining in the glomerular layer shows a periglomerular cell immunoreactive for both calcium-binding proteins (arrows). Periglomerular cell (arrows) immunoreactive for NC (c) and PV (d). Other neuronal elements constitute entirely segregated populations. Scale bar = 25 μm.

cells), whereas the expression in the remaining neuronal types occurs in entirely segregated populations.

All investigated CaBPs were found to be associated primarily with interneuronal populations. The exception to this general rule are mitral cells, which demonstrated weak CR immunoreactivity in the cell bodies, although their dendrites remained unstained. This labeling has been described as highly variable in both animals and physiological conditions (Résibois and Rogers, 1992), although unequivocal immunostaining was detected in their axons (Wouterlood and Härtig, 1995). The other main group of projecting neurons, tufted cells, are clearly PV negative (Kosaka et al., 1994) and CR negative (Wouterlood and Härtig, 1995). Although a few tufted cells have been reported as CB immunopositive (Halász et al., 1985; Celio, 1990), most researchers have not found any CB-immunopositive neuron resembling the morphologic features of tufted cells (Jande et al., 1981; García-Segura et al., 1984; Briñón et al., 1992), as was the case in the present study. Despite the fact that previous (Briñón et al., 1998) and present observations have provided evidence that a large population of tufted cells exhibit NC immunoreactivity, the morphologic features and location suggest that they belong to groups that do not project to the olfactory tubercle and piriform cortex as other tufted cells and mitral cells do (Schoenfeld et al., 1985), and they cannot be strictly considered as relay neurons. In fact, only a few NC-immunoreactive fibers were observed in both the lateral olfactory tract and the olfactory tubercle. Therefore, with the exception of CR in mitral cells, the expression of the investigated CaBPs in the olfactory bulb occurs in interneuronal populations.

It is unknown why subpopulations of the same neuronal types express different CaBPs, and why the CaBPs expressed in a particular neuronal type are variable. Their most simple function could be protection against calcium-

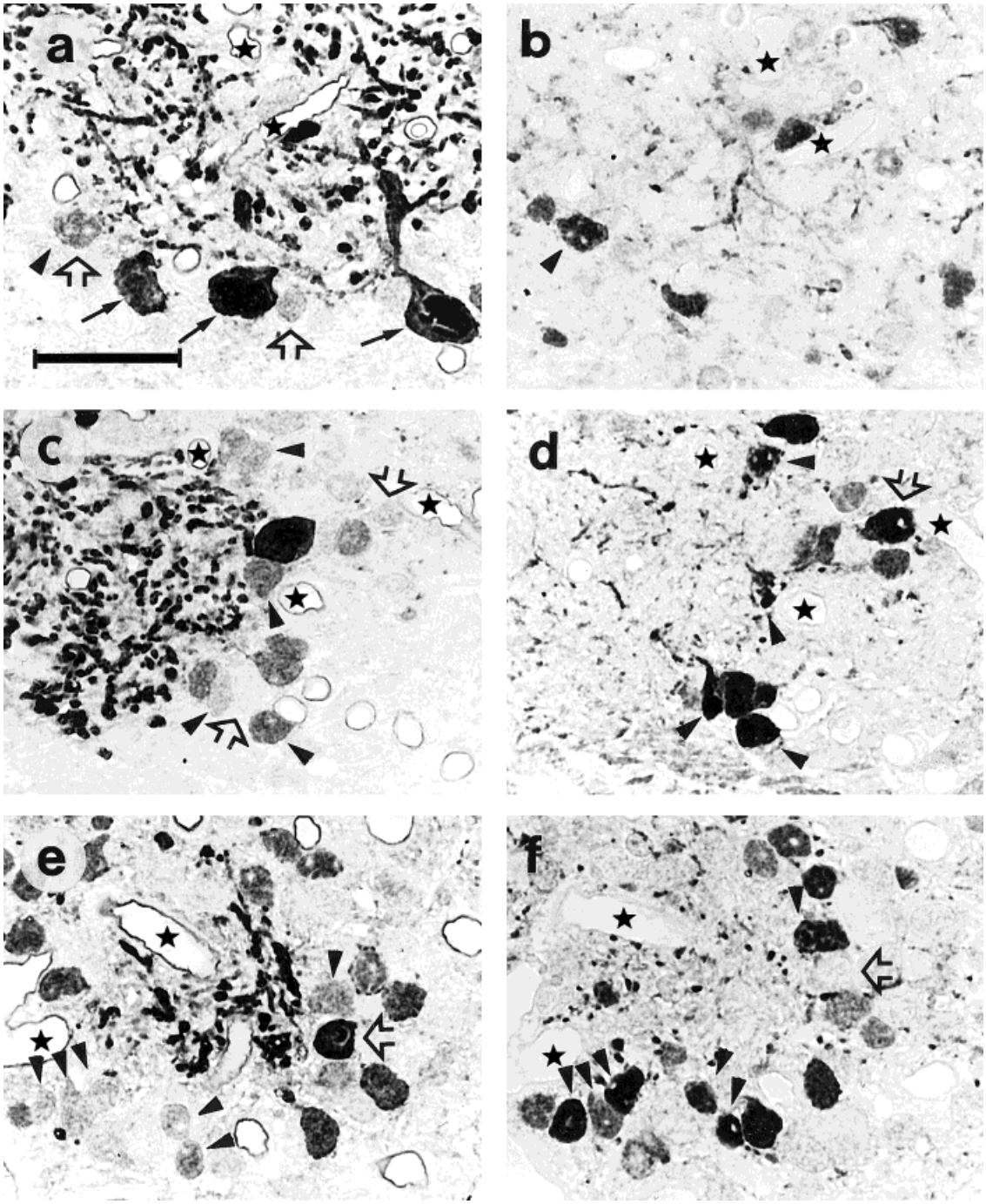

Fig. 5. Photomicrographs of consecutive semithin sections showing the glomerular layer after postembedding immunohistochemistry for detection of neurocalcin (NC; left) and calretinin (CR; right). Blood vessels used as landmarks are marked with stars. **a:** Partial view of a single glomerulus showing neuronal elements displaying NC immunoreactivity. Note the different size and staining intensity between immunoreactive tufted cells (arrows) and periglomerular cells (open arrows). **b:** Photomicrograph showing the same field as that shown in (a) after postembedding immunohistochemistry for CR. Tufted cells lack CR immunoreactivity, and only periglomerular cells are CR positive. Coexistence of NC and CR is found in one of these periglomerular cells (arrowhead). **c–f:** Coexistence NC and CR in a large population of periglomerular cells (arrowheads). Open arrows indicate groups of cells expressing CR and lacking NC (c–d) and others positive for NC and negative for CR (e–f). Scale bar = 25 µm.

mediated damage, but this notion alone does not explain why there are so many different CaBPs in sets of neurons involved in the same circuitry. In the olfactory bulb, there was a selective occurrence of the four investigated CaBPs among distinct layers and interneuronal types. Thus, CB, CR, and NC were expressed mainly in the glomerular

layer and the granule cell layer, whereas the PV-containing neurons were concentrated mostly in the external plexiform layer. These selective distributions support the notion that, if these CaBPs share some common properties, they may also be responsible for individual ones. This would be especially evident in periglomerular cells, where all investigated CaBPs are expressed with both single and colocalized patterns, and the existence of several functional subgroups has been hypothesized (Kosaka et al., 1997, 1998).

In periglomerular cells immunostained for a given CaBP, NC is present in only a few, and these cells are morphologically indistinguishable from other periglomerular cells labeled by this marker but lacking NC. However, Kosaka et al. (1998) described two major categories of periglomerular cells according to the characteristics of their intraglomerular dendritic arborization and their relationship to olfactory nerve terminals. These groups also are distinguishable by their neurochemical features. Thus, type 1 includes γ-aminobutyric acid (GABA)- and tyrosine hydroxylase-positive periglomerular cells. These cells have their dendrites closely apposed to olfactory axon terminals and preterminals. Type 2 cells have their dendrites rarely apposed to olfactory axon terminals or preterminals and include the CR- and CB-immunoreactive periglomerular cells. Therefore, periglomerular cells should be considered a heterogeneous population not only in their chemical nature but also in their hodological relationships. Our results indicate that the expression of NC is independent of these features. In fact, almost half of NC-immunopositive periglomerular cells colocalized CR or CB and therefore would share the characteristics defined for periglomerular cells of type 2, whereas the remaining group of NC-positive periglomerular cells, which would include those expressing NC alone (55.3%) and those coexpressing NC and PV (2.5%), would belong to type 1 or would constitute an additional group with their own specific features. Therefore, the expression of NC in periglomerular cells is not related to the GABAergic nature of cells, the CaBP expressed, the type of intraglomerular dendritic arborization, or the relationships with the olfactory nerve terminals.

In the remaining neuronal types of the rat olfactory bulb, NC does not colocalize with any of the investigated markers. As in the case of periglomerular cells, each distinct neurochemical group is morphologically indistinguishable. The most clear examples are granule cells, the most numerous interneuronal type of the olfactory bulb, which showed morphologically identical populations with segregated expression of NC and CR or PV. Several types of granule cells have been defined according to the zone of the external plexiform layer, where their apical dendrites extend, and the types of principal cell they contact (Mori et al., 1983; Orona et al., 1983; Halász, 1990). Although available evidence is still fragmentary, the distinct and nonoverlapped populations of granule cells may show distinct functional groups defined by the features of the principal neurons they are connected with.

It would be interesting to know why coexpression of NC and the other investigated CaBPs occurs only in periglomerular cells, whereas each neurochemical group constitutes segregated populations in the remaining neuronal types. Although it seems likely that the basic function of calcium-buffer proteins (CB, CR, and PV) is the regulation of intracellular calcium, NC was defined as a calcium-sensor protein (Ikura, 1996), which has been shown to inhibit rhodopsine phosphorylation in a calcium-dependent manner (Faurobert et al., 1996). Although the functional significance of the coexistence of different CaBPs is unknown, the variability in calcium-binding affinities and the structural and functional differences (Ikura, 1996) suggest that NC and the colocalized buffer-type CaBPs are most likely involved in different calcium-mediated events. Thus, NC may have a particular function and may play a role in association with or as a complement to other CaBPs in neuronal calcium-dependent events.

The abundance of different CaBPs in the olfactory system in both the olfactory epithelium (Bastianelli et al., 1995) and the olfactory bulb (present data) argues for their consideration as calcium mediators during olfactory signal transduction and modulation. Nevertheless, additional studies are required to ascertain their exact role in these processes. An interesting approach would be to investigate what experimental conditions alter their expression patterns. The olfactory bulb and in particular the periglomerular cells, where neuronal subpopulations express CB, CR, NC, or PV, with both exclusive and colocalized patterns, may be an excellent experimental region.

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