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Calbindin D-28K and NADPH-Diaphorase Activity are Localized in Different Populations of Periglomerular Cells in the Rat Olfactory Bulb

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ABSTRACT

Calbindin D-28k (CaBP) immunocytochemistry and NADPH-diaphorase (ND) histochemistry have been combined in the rat olfactory bulb by successive incubations of the same sections. The outer strata showed a similar neuronal staining pattern for both markers with positive periglomerular neurons (although the CaBP-stained periglomerular cells were six-fold more abundant than the ND-active ones) and larger neurons scattered in the glomerular and external plexiform layers. Both populations of periglomerular cells were distinct but they did not show specific morphological characteristics nor a predominant distribution around ND-positive and negative glomeruli. The colocalization study demonstrates that the larger ND and CaBP-stained juxtaglomerular cells, identified according to their size, location and processes branching patterns as two types of short axon cells (superficial short-axon and Van Gehuchten Cells) were also independent populations.

KEY WORDS: Short-axon cell

INTRODUCTION

Calbindin D-28k (CaBP) is a cytosolic calcium-binding protein which is present in specific subpopulations of neurons throughout the brain. Different studies using immunocytochemistry and mRNA *in situ* hybridization have described the CaBP-positive neuronal types in the rat olfactory bulb (Baimbridge and Miller, 1982; Briñón *et al.*, 1992; Celio, 1990; Enderlin *et al.*, 1987; García-Segura *et al.*, 1984; Halász *et al.*, 1985; Jande *et al.*, 1981; Séquier *et al.*, 1988, 1990; Seroogy *et al.*, 1989). Although all authors agree about the immunostaining of some neuronal types (i.e. periglomerular cells), there are discrepancies on the identification of other positive elements closely located to these cells. Thus, some authors have identified CaBP-positive superficial short-axon cells (Briñón *et al.*, 1992; Seroogy *et al.*, 1989) and CaBP-positive external tufted cells in rats (Celio, 1990; Halász *et al.*, 1985) and in humans (Ohm *et al.*, 1991), whereas others (Briñón *et al.*, 1992; Garcia-Segura *et al.*, 1984; Jande *et al.*, 1981) have reported that this protein is absent from the tufted cells. Other authors (Baimbridge and Miller, 1982; Enderlin *et al.*, 1987; Séquier *et al.*, 1990) only indicated the presence of immunostaining in the periglomerular cells.

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NADPH-diaphorase (ND) activity is present in this region of the rat olfactory bulb in similar neuronal types. Thus, ND-staining has been identified in superficial short-axon cells and periglomerular cells (Davis, 1991; Scott *et al.*, 1987; Villalba *et al.*, 1989) and, with less certainty, in external tufted cells (Davis, 1991). Since superficial short-axon cells and external tufted cells share a common distribution and similar somal sizes and shapes, details of the dendritic or axonal branching patterns are required to distinguish them at the light microscopical level. The combined use of ND histochemistry and CaBP immunocytochemistry, both providing morphological details of the dendritic and axonal processes, may be a useful tool to characterize better these types of neurons. It is therefore possible to identify more clearly the discussed positive single-stained cells and, as a second objective, to analyse if both labellings are partially or totally colocalized by the same cells or, on the contrary, they are present in distinct subpopulations of local circuit cells (periglomerular and short-axon cells) and/or principal neurons (tufted cells).

MATERIALS AND METHODS

Five adult female Wistar rats (230–280 g body weight) were used. The animals were deeply anaesthetized with ketamine (Ketolar, 50 mg/kg body weight) and perfused through the ascending

aorta with 100 ml Ringer solution followed by a fixative containing 4% paraformaldehyde, and 15% saturated picric acid in 0.1 M-phosphate buffer (PB) pH 7.25. After 2 h, the olfactory bulbs were dissected out, postfixed at 4°C for a further 2 h in the same fixative, and cryoprotected with 30% sucrose (v/v). Thirty-micrometer frontal and sagittal sections were cut on a cryostat and collected in cold (4°C) PB.

Free-floating sections were processed for the demonstration of ND activity following the protocol described by Scherer-Singler *et al.* (1983). Briefly, the sections were incubated for 1–3 h at 37°C in a solution made up of 0.08% Triton X-100, 1 mM-reduced β-NADPH, 0·8 mM-nitroblue tetrazolium in 0.1 M-Tris–HCl buffer (pH 8). All reagents were obtained from Sigma. The course of the reaction was controlled under the microscope.

When the histochemical reaction was concluded, the sections were rinsed in Tris buffer and PB, and processed for immunocytochemistry as previously described (Alonso *et al.*, 1990, 1992; Briñón *et al.*, 1992). The sections were processed according to the avidin-biotin-immunoperoxidase method. They were sequentially incubated in: (a) primary antibody (McAB 300 anti-calbindin D-28k) diluted 1:2000 in PB containing 10% normal horse serum and 0.03% Triton X-100 for 48 h at 4°C; (b) biotinylated anti-mouse immuno-gammaglobulin (Vector Labs; 1:250 in PB; 3 h at 20°C); and (c) Vectastain ABC reagent (Vector Labs, 1:250 in PB, 90 min at 20°C). The tissue-bound peroxidase was revealed with 0.07% 3,3′-diaminobenzidine and 0.003% hydrogen peroxide in 0.1 M-Tris–HCl buffer (pH 7.6). After incubation, the sections were rinsed in PB, mounted on gelatin-coated slides, and air dried. The slides were dehydrated in a graded ethanol series and coverslipped with Entellan.

No ND activity was observed when the substrate (NADPH) was omitted in the incubation media. The staining obtained in the double-labelled sections was similar for each marker to that observed in single-stained sections. The primary antibody against CaBP has been fully characterized (Celio *et al.*, 1990). Controls of the specificity of the immunostaining procedure as described (Alonso *et al.*, 1990; Briñón *et al.*, 1992) were also carried out. No residual immunoreaction was observed.

RESULTS

The successive processing for ND-activity and CaBP-immunocytochemistry allowed us an easy way to discriminate between CaBP- and ND-positive neurons in the same section. ND-active elements were abundant in the outer strata of the olfactory bulb (Fig. 1a–e), whereas only scattered ND-stained elements were seen in the inner layers. In the olfactory nerve fibre layer, a subpopulation of ND-positive olfactory fibres was observed (Fig. 1a). These fibres coursed into the glomerular layer, where ND-stained (Fig. 1c) and ND-unstained (Fig. 1d) glomeruli, with a clearly segregated distribution, were found. The highest number of positive cells for both techniques was observed in this glomerular layer. CaBP- and ND-positive periglomerular cells—easily identified since both markers produced a 'Golgi-like' staining of the cell body and the whole dendritic arborization—were observed surrounding the ND-positive and negative glomeruli. No morphological difference was observed between the ND- and CaBP-labelled periglomerular cells. Both markers were evident not only in the somata but also in the intricate systems of fine varicose dendrites that arborized within the glomeruli.

We have counted ND- and CaBP-positive periglomerular cells surrounding 100 ND-positive and 100 ND-negative sectioned glomeruli, randomly selected. The results are shown in Table 1.

The distributions of both types of neurons around both types of glomeruli, after a 2-way analysis of variance test with interaction, did not show statistically significant differences (i.e. there was no predominant distribution of CaBP-positive or ND-positive periglomerular cells in the ND-positive and negative glomeruli). In more than 2000 ND-positive and 10 000 CaBP-positive periglomerular neurons, we have not observed a single cell that colocalizes with certainty both stainings. Thus, the combined use of both techniques separated two different subpopulations of periglomerular cells.

Apart from periglomerular cells, another group of ND-stained neurons was observed in the same region (Fig. 1b, e). These larger neurons were located at the boundary of the glomerular and external plexiform layers, and they showed their dendrites, which were completely labelled, coursing for relatively long distances parallel to this limit, mostly restricted to that former layer (Fig. 1b, e). These cells showed spineless medium-sized somata and two to six dendrites coursing around and between glomeruli; they never formed glomerular tufts similar to those of tufted and mitral cells. More rarely, we observed another group of neurons, CaBP-positive and ND-negative, with cell bodies larger than the periglomerular cells but smaller than those aforementioned ND-stained cells. These neurons were similarly located around the glomeruli and their dendritic trees, smaller and not so oriented as those of the ND-active neurons, remained in the periglomerular zone, not entering into the glomeruli.

On the other hand, we observed another population of interneurons, stained for CaBP and located in the external plexiform layer, although more internally (Fig. 1f). They were scarcer and their cell bodies smaller than those of the ND-active neurons. The quality of the staining allowed us to describe the characteristics of their axons and

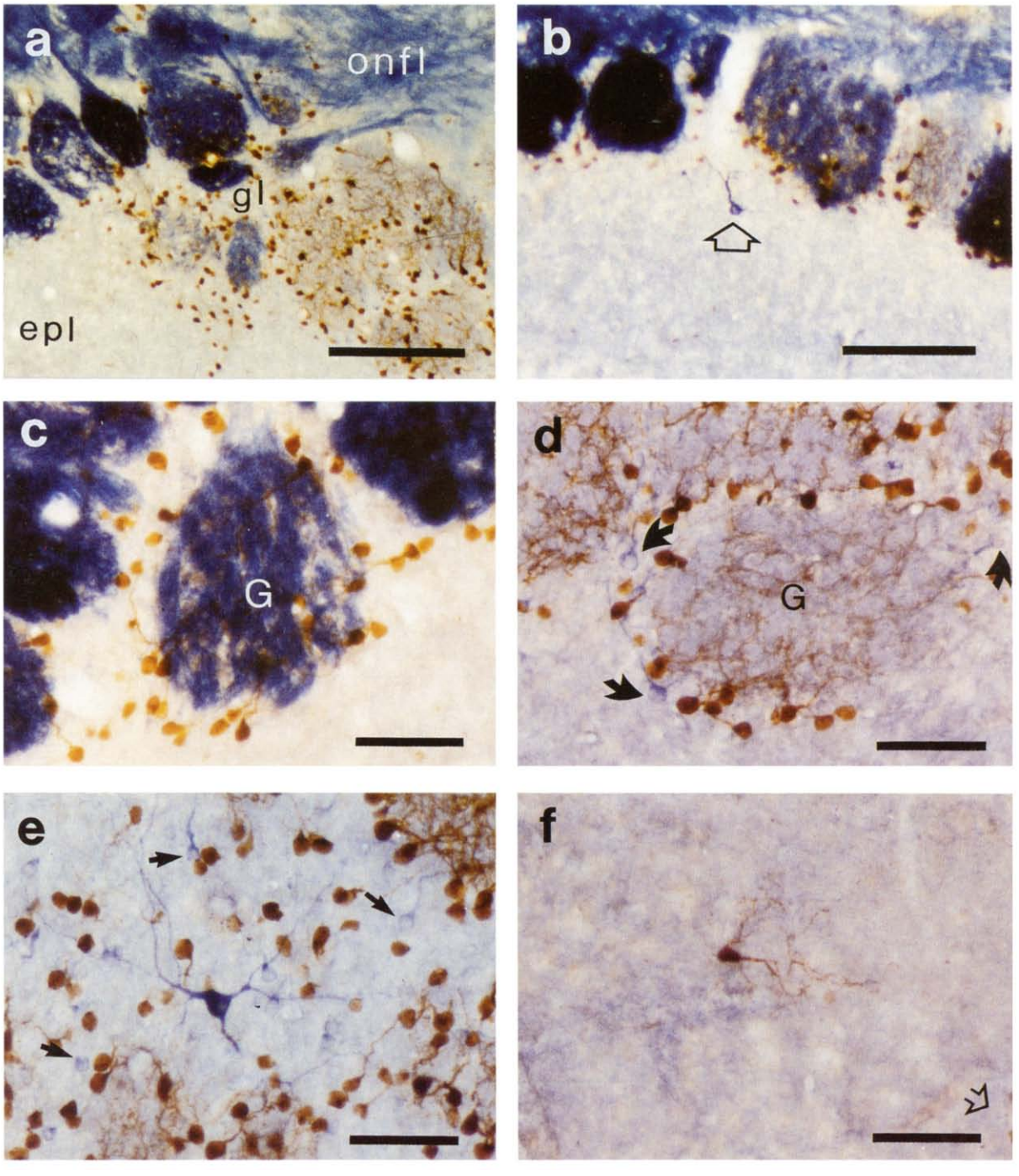

Fig. 1. (a) Overview of the outer strata of a sagittal section of the olfactory bulb showing ND-positive olfactory fibres and abundant CaBP-stained periglomerular cells. (epl: external plexiform layer; gl: glomerular layer; onfl: olfactory nerve fibre layer). Scale bar = 200 µm. (b) Transversal section of the olfactory bulb showing ND-positive glomeruli with CaBP-immunostained periglomerular cells and a ND-active superficial short-axon cell (arrow). Scale bar = 100 µm. (c) ND-positive glomerulus (G) surrounded by CaBP-positive periglomerular cells. Scale bar = 50 µm. (d) ND-negative glomerulus (G) surrounded by CaBP-positive and ND-active (arrows) periglomerular cells. Scale bar = 50 µm. (e) ND-active superficial short-axon cell in a sagittal section of the rat olfactory bulb. Note that most dendrites course in the same plane and the presence of more weakly stained periglomerular cells (arrows). Scale bars = 50 µm. (f) CaBP-positive Van Gehuchten cell in the external plexiform layer. Note the small dendritic field arborizing far from the periglomerular cells located in the lower right corner (arrow). Scale bar = 50 µm.

dendrites, which were also different to those of the ND-positive cells. Thus, the dendrites branched frequently, forming a dense and elaborate but small dendritic field, which was located relatively far from the periglomerular neurons. We did not observe a predominant orientation pattern of these dendrites

Table 1. Number (mean ± s.e.m.) of ND- and CaBP-positive periglomerular cells surrounding ND-positive and negative sectioned glomeruli

	Glomerular staining	
	ND(+)	ND(−)
ND(+) cells	2.06 ± 0.14	2.22 ± 0.19
CaBP(+) cells	13.02 ± 0.55	12.50 ± 0.49

with respect to the lamination of the bulb. Several CaBP-immunostained axonic collaterals arborizing close to the cell body were observed.

The differences in size and dendritic and axonic arborizations suggest that both markers stained separate, morphologically distinct neuronal populations. This was further verified by successive incubation of the same sections where no double-labelled neuron was observed.

DISCUSSION

This study demonstrates the CaBP and ND are localized in different populations of periglomerular cells and in different types of short-axon cells in the outer strata of the olfactory bulb.

The ND- and CaBP-positive periglomerular cells conformed to previous descriptions of the size, position and process arborization pattern of this neuronal type using conventional light and electron microscopy and Golgi impregnation (Pinching and Powell, 1971; Schneider and Macrides, 1978). Additionally, immunocytochemical studies have demonstrated that the periglomerular neurons constitute a much more heterogeneous population than was previously considered. Thus, subpopulations of periglomerular neurons, besides ND and CaBP, are positive to different neurotransmitters and neuroactive substances including parvalbumin, taurine, tyrosine hydroxylase, vasoactive intestinal polypeptide, cholecystokinin, methionine-enkephalin, somatostatin and gamma-aminobutyric acid (GABA) (Gall *et al.*, 1987; Halász *et al.*, 1985; Halász, 1990; López-Mascaraque *et al.*, 1989; Mugnaini *et al.*, 1984; Sakai *et al.*, 1987; Seroogy *et al.*, 1985). Davis (1991) indicated that the coexistence of any combination of these substances is possible and, for example, partial colocalization of tyrosine hydroxylase and GABA has been reported (Gall *et al.*, 1987). On the contrary, we demonstrated that CaBP and ND, as is the case for CaBP and tyrosine hydroxylase (Halász *et al.*, 1985) were present in completely segregated populations of periglomerular cells. Since ND and CaBP are reliable markers, our data provide a further characterization of the periglomerular cells.

The typification of the larger positive neurons was less evident. On the basis of perikaryal size and position, they can correspond to external tufted cells or to two types of short-axon neurons: Van Gehuchten cells and superficial short-axon cells (Davis, 1991; Schneider and Macrides, 1978). However, we have never observed in the ND-positive neurons nor in the CaBP-immunostained cells the presence of the tuft, a broad, smooth dendritic process which enters and arborizes within a glomerulus, typical of tufted and mitral cells. In good agreement with this absence of ND- or CaBP-positive external tufted cells in our sections (although Davis (1991) suggested that the medium-sized ND-active neurons should correspond to this neuronal type) is the fact that no ND-positive axon is observed in the superficial granule cell layer (Davis, 1991), a position used by the output axons destined for the lateral olfactory tract, and the output nature of the external tufted cells has been confirmed, at least in the rat, with orthodromic stimulation and horseradish peroxidase iontophoresis (Halász, 1990).

The ND-active neurons showed morphological characteristics of superficial short-axon cells, as previously described by Schneider and Macrides (1978) and Scott *et al.* (1987). However, superficial short-axon cells seem to be a heterogeneous population both morphologically and biochemically (Halász, 1990). This agrees with our results indicating the presence of two groups (ND-active and CaBP-positive) of superficial short-axon cells differing not only in these chemical markers but also in their somal sizes and their processes branching pattern. On the other hand, most CaBP-positive short-axon cells are located farther from the glomeruli, their dendritic fields are smaller, not entering between the glomeruli, and we observed a highly developed system of local collaterals. We identified these CaBP-positive neurons as Van Gehuchten cells. Thus, the double-labelling study suggested that three different groups of these interneurons can be differentiated: ND-positive CaBP-negative superficial short-axon cells, ND-negative CaBP-positive superficial short-axon cells and ND-negative CaBP-positive Van Gehuchten cells.

The low number of positive short-axon neurons found for both markers suggests that only subpopulations of both Van Gehuchten and superficial short-axon cells were stained. This coincides with our observations for the periglomerular cells and with previous reports in other brain regions (see Celio, 1990 for CaBP and Vincent *et al.*, 1983 for ND).

It is hitherto unknown which common denominator shares the neurons showing one of these markers: CaBP and ND. CaBP is a calcium-binding protein which controls the intracellular calcium levels and it has been used as a marker for diffusely projecting unmyelinated systems in combination with different types of interneurons (Celio, 1989). ND has been recently shown to be a nitric oxide synthase (Hope *et al.*, 1991), providing therefore an

easy method to locate neurons producing this enzyme throughout the brain.

ND staining is present in neurons uniquely resistant to toxic damage and neurodegenerative disorders (Dawson *et al.*, 1991). Although the exact biochemical basis of this selective resistance is only now beginning to be elucidated, it has been proposed that the ND-positive neurons might possess high levels of NADPH which could reduce oxidative neurotoxins (Dawson *et al.*, 1991) and, at the same time, the nitric oxide produced by these neurons would have effects—even neurotoxicity—in the surrounding cells (Dawson *et al.*, 1991; Hope *et al.*, 1991). Thus, the olfactory bulb, with ND-positive and ND-negative populations belonging to the same neuronal type, may be a useful experimental model to test this putative protective role of the ND enzyme. The complementary distributions indicate that CaBP can be used as a marker for ND-negative periglomerular cells. However, the possible protective role for CaBP in neurotoxic or pathological disease damage should be considered, since a selective sparing of CaBP-labelled neurons has been found in Parkinson's (Yamada *et al.*, 1990) and Alzheimer's diseases (Hoffman *et al.*, 1991) and after intense seizure activity in an experimental epilepsy model (Sloviter, 1989).

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