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Vasoactive intestinal polypeptide-containing elements in the olfactory bulb of the hedgehog (*Erinaceus europaeus*)

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Abstract

The distribution of vasoactive intestinal polypeptide (VIP)-immunopositive elements was analyzed in the olfactory bulb (OB) of the Western European hedgehog (*Erinaceus europaeus*) under light and electron microscopy. The immunoreactivity appeared in an abundant population of periglomerular cells of the glomerular layer, in interneurons of the external plexiform layer, and in a restricted group of deep short-axon cells of the internal plexiform layer, the granule cell layer and the white matter. In the glomerular layer, VIP-containing periglomerular cells constituted a population of non-GABAergic neurons and did not receive synapses from olfactory axons. In the EPL, VIP-immunoreactivity appeared in a morphologically heterogeneous population of GABAergic interneurons, most of them identified as satellite cells and Van Gehuchten cells. These interneurons exerted an abundant and selective innervation of the somata, primary and secondary dendrites of the principal mitral and tufted cells, but did not contact granule cells. Perisomatic innervation of the principal cells followed two different patterns. The first included 'normal' basket-like arrangements of VIP-containing varicosities surrounding the somata of mitral and tufted cells. In the second, a set of satellite cells gave rise to short dendritic shafts that embraced the somata of principal cells in an 'exuberant' basket-like arrangement. These two morphological patterns of perisomatic innervation of principal cells were correlated with a neurochemical specificity of the target. In this sense, the 'exuberant' basket-like structures were always found surrounding a subpopulation of principal cells that did not contain the calcium-binding protein parvalbumin (PV). By contrast, they were never found surrounding the subpopulation of PV-containing principal cells, which only showed 'normal' basket-like structures. This study provides new data on the connectivity and neurochemical features of the hedgehog olfactory bulb and suggests that the olfactory circuits in this species are more complex than those described in other mammals.

Keywords: GABAergic interneurons; Mitral cells; Olfaction; Periglomerular cells; Perisomatic innervation; Tufted cells

1. Introduction

The insectivorous hedgehog (*Erinaceus europaeus*) is one of the most direct descendants of the protoinsectivores, considered to be the progenitors of all recent mammalian orders (Simpson, 1945). The olfactory structures of this species show an extraordinary degree of development and its olfactory bulbs (OB) represent 17.6% of the total volume of the telencephalon (Stephan and Andy, 1970). Since allometric studies have demonstrated a link between the size of the OB and the functional significance of olfaction, this species is considered a paradigm for an 'olfactory brain', having special relevance in the study of olfaction from the phylogenetic and comparative points of view. In this sense, previous reports focused on the neuroanatomy of the hedgehog OB (López-Mascaraque et al., 1986, 1989, 1990; De Carlos et al., 1989; Valverde and López-Mascaraque, 1991; Palmieri et al., 1993; Alonso et al., 1995; Crespo et al., 1999; Briñón et al., 2001) revealed that the cytoarchitecture of the hedgehog OB shows many similarities to the OB of other mammals. However, important differences were also reported for the morphology (López-Mascaraque et al., 1986, 1990),

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connectivity (De Carlos et al., 1989), and neurochemical features (Alonso et al., 1995; Crespo et al., 1999; Briñón et al., 2001) of the hedgehog bulbar circuits.

A previous report by López-Mascaraque et al. (1989) described the distribution of vasoactive intestinal polypeptide (VIP) in the hedgehog OB. These authors found an extensive VIP-containing bulbar system in this animal, suggesting a relevant involvement of this peptide in the processing of olfactory information. They also reported that such VIP-immunoreactivity appears mainly in periglomerular cells of the glomerular layer (GL) and in interneurons of the external plexiform layer (EPL).

Recently, detailed electron microscopy studies performed on the rat OB have clearly demonstrated that the connectivity of the periglomerular cells and interneurons of the EPL is more complex than earlier suspected (Kosaka et al., 1997, 1998). The authors of those studies propose quite important changes in the classical scheme of bulbar circuits. For example, it was reported that periglomerular cells do not constitute a homogeneous population of interneurons receiving sensory afferences; instead, they display two different patterns of connectivity with the olfactory axons. Whereas one subset of periglomerular cells does not receive synaptic contacts from olfactory axons and is apparently non-GABAergic, the other subset of periglomerular cells receives sensory afferences and the cells are GABAergic (Kosaka et al., 1997, 1998; Toida et al., 1998, 2000; Crespo et al., 2000). Regarding the interneurons of the EPL, these have classically been assumed to contact granule cells (Schneider and Macrides, 1978; Macrides et al., 1985). Despite this, it has recently been reported that these interneurons innervate the perisomatic region and proximal dendritic trunks of the principal neurons—mitral and tufted cells (Toida et al., 1994, 1996)—and that they never synapse with granule cells or other interneurons (Crespo et al., 2001).

Taking these data together, it seems necessary to reappraise the bulbar circuitry in other macrosmatic species other than rodents in order to seek general patterns of neuronal connectivity in the OB and hence understand the processing of olfactory information. In this respect, it is interesting to analyze whether the segregation of two different populations of periglomerular cells reported in the rat is a conserved feature of mammals that appears in other macrosmatic species, such as the hedgehog. In this line, VIP is an excellent neuroanatomical tool that can be used to stain a set of periglomerular cells in the hedgehog. Moreover, the investigation of whether the VIP-containing periglomerular cells of the hedgehog OB belong to a putative GABAergic group that receives sensory afferences or not could provide insight into the involvement of VIP in olfaction. It is also necessary to re-analyze the connectivity of EPL interneurons with mitral and tufted cells, taking into account that mitral and tufted cells do not constitute a homogeneous population of projection neurons (Macrides et al., 1985; Shipley et al., 1995; Shepherd and Greer, 1998). For this purpose, VIP is a useful marker of the interneurons of the EPL and the hedgehog is an excellent model that shows a neurochemical heterogeneity of principal cells (Briñón et al., 2001).

Here, to investigate the VIPergic circuits in the olfactory glomeruli and in the EPL we combined preembedding VIP-immunocytochemistry and post-embedding immunogold staining for GABA at the electron microscope. To analyze whether the VIP-containing interneurons of the EPL innervate the population of principal cells homogeneously or not, we combined VIP- and parvalbumin (PV)-double immunocytochemistry, because the calcium-binding protein PV is an excellent marker for a specific subpopulation for principal cells in the hedgehog OB, as recently demonstrated (Briñón et al., 2001).

2. Material and methods

2.1. Animals and tissue preparations

Six adult male hedgehogs were used in this study. Animals were deeply anaesthetized with 4% chloral hydrate in physiological saline (1 ml/100 g body weight) and perfused intra-aortically, first with saline for one minute and then with 700 ml of fixative, following the experimental procedures explained below. All procedures 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 animals, and conformed to NIH guidelines. Three animals were used for the analysis of VIP distribution and for VIP/PV colocalization experiments. In these animals, the fixative was composed of 4% (w/v) paraformaldehyde and 2% (w/v) picric acid in 0.1M phosphate buffer (PB), pH 7.4. For the colocalization analysis of VIP and GABA and for standard electron microscopy, the three remaining animals were perfused with a fixative composed of 4% (w/v) paraformaldehyde, 0.5% glutaraldehyde and 2% (w/v) picric acid in PB.

After perfusion, the brains were removed and the OBs were dissected and carefully rinsed in cold (4 °C) PB overnight. Then, 50 μm thick transverse sections were cut on a vibratome (VT 1000E, Leica, Nussloch, Germany) and collected in cold (4 °C) PB.

2.2. Immunocytochemical detection of VIP for light and electron microscopy

Free-floating sections were cryoprotected by overnight immersion in a mixture of 25% sucrose and 10%

glycerol in 0.05 M PB at 4 °C. They were then freeze-thawed three times in liquid nitrogen to enhance antibody penetration. Following extensive washes in PB, sections from animals perfused with fixative containing glutaraldehyde were treated with 1% $NaBH_4$ in PB for 30 min and extensively rinsed in PB. For VIP immunocytochemical staining, the avidin–biotin–immunoperoxidase method (Hsu et al., 1981) was used. In this procedure, sections were sequentially incubated in: (i) blocking solution, containing 10% normal goat serum (Vector Labs., Burlingame, CA, USA) and 2% bovine serum albumin (Sigma, St. Louis, MO, USA; #A9647) for 45 min at room temperature; (ii) polyclonal rabbit anti-VIP (Gulyás et al., 1990) for 48 h at 4 °C; (iii) biotinylated goat anti-rabbit IgG (Vector Labs.; 1:200 in PB) for 2 h at room temperature; (iv) avidin–biotinylated horseradish peroxidase complex (Vector; 1:200 in PB) for 2 h at room temperature. After each step, sections were carefully rinsed in PB (3 × 10 min). The peroxidase reaction was developed using 0.05% 3,3′-diaminobenzidine tetrahydrochloride (DAB) (Sigma, #D5637) and 0.003% hydrogen peroxide in PB for 10–20 min until VIP-specific immunostaining could be clearly visualized under the microscope. Sections were then rinsed in PB (3 × 10 min), treated with 1% osmium tetroxide (Sigma, #O5500) for 1 h, and washed repeatedly in PB. Finally, they were stained with 1% uranyl acetate for 90 min, dehydrated through a graded ethanol series, cleared with propylene oxide and flat-embedded in Durcupan (ACM, Fluka AG, Switzerland) between slides and coverslips. The Durcupan was polymerized overnight at 60 °C. After all these steps, sections were carefully examined microscopically. After light microscopic analysis, a few sections containing VIP-positive profiles from the animals perfused with 0.5% glutaraldehyde were re-embedded in Durcupan. The sections selected were cut on an ultramicrotome and serial ultrathin sections were mounted on single-slot formvar-coated copper or nickel grids. Some of them were stained with lead citrate and analyzed by the electron microscopy while others were further processed for post-embedding GABA-immunocytochemistry, as indicated below.

The polyclonal rabbit anti-VIP antibody used in the study has been exhaustively characterized and previously used in the rat nervous system (Gulyás et al., 1990; Acsády et al., 1996; Blasco-Ibáñez et al., 1998; Martínez-Guijarro et al., 1998). Additional controls of immunocytochemical specificity were carried out by omitting the first or second antibody in each step, and by incubating some sections exclusively in 0.05% DAB and 0.003% hydrogen peroxide in PB in order to rule out the presence of endogenous peroxidase in the tissue. No residual reactivity was found in the controls.

2.3. Post-embedding immunogold staining for GABA

In order to analyze the GABAergic nature of the VIP-containing elements, pre-embedding VIP immunocytochemistry was combined with post-embedding immunogold staining for GABA. Post-embedding GABA immunostaining was carried out according to Somogyi and Hodgson (1985) with slight variations as previously reported (Crespo et al., 2000). All steps were carried out on droplets of solution in humid Petri dishes as follows: 1% periodic acid (H_5IO_6) for 10 min; washing in distilled water (3 × 3 min); 2% sodium metaperiodate ($NaIO_4$) for 10 min; washing in distilled water (3 × 3 min); washing in 0.05 M Tris–HCl buffer saline (TBS), pH 7.4, for 3 min; blocking solution containing 1% chicken egg albumin in TBS for 30 min; washing in TBS (2 × 10 min); rabbit anti-GABA antiserum (Sigma, #A2052; 1:2,500 in 1% normal goat serum in TBS) for 2 h; washing in TBS (2 × 10 min); 1% bovine serum albumin and 0.05% Tween 20 in 0.05 M Tris–HCl buffer (TB), pH 7.4, for 10 min; goat anti-rabbit IgG-coated colloidal gold (Sigma, #G7402, 10 nm, 1:15 in TB containing 1% bovine serum albumin and 0.05% Tween 20) for 2 h; washing in distilled water (3 × 5 min) and 0.1 M phosphate buffer saline (PBS), pH 7.4, (4 × 5 min); 2% glutaraldehyde in PBS for 2 min; washing in distilled water (4 × 5 min); silver enhancement for 1 min; washing in distilled water (4 × 5 min); saturated uranyl acetate for 30 min; washing in distilled water (4 × 5 min); staining with lead citrate; washing in distilled water.

Profiles showing a density of colloidal gold particles ten-fold that of the background level in two or three adjacent sections were considered GABA-immunoreactive. Profiles of olfactory nerve terminals or mitral/tufted cells (presumed to be glutamatergic elements) were used to establish background density.

Omission or substitution of the GABA antiserum with normal rabbit serum resulted in a loss of specific staining, i.e. no signs of colloidal gold accumulation could be detected.

2.4. VIP and PV double immunocytochemistry for light microscopy

For VIP and PV double-immunostaining, sections from the animals perfused with the fixative without glutaraldehyde were first processed for VIP-immunostaining as described above. The peroxidase reaction was developed using 0.05% DAB intensified by 0.05 M ammonium–nickel sulfate (NH_4NiSO_4) (DAB–Nickel), and 0.003% hydrogen peroxide in PB for 10–20 min. This chromogen provides a black reaction product. After carefully rinsing the sections in cold (4 °C) PB, they were subjected to a new immunostaining procedure to detect PV. For this second procedure, sections were

sequentially incubated in: (i) blocking solution containing 10% normal goat serum in PB for 45 min at room temperature; (ii) polyclonal rabbit anti-PV (Swant, Bellinzona, Switzerland; 1:10 000 in PB) for 48 h at 4 °C; (iii) biotinylated goat anti-rabbit IgG (Vector; 1:200 in PB) for 2 h at room temperature; (iv) avidin–biotinylated horseradish peroxidase complex (Vector; 1:200 in PB) for 2 h at room temperature. After each step, sections were carefully rinsed in PB (3 × 10 min). The peroxidase reaction was developed using 0.05% DAB and 0.003% hydrogen peroxide in PB for 5–10 min, until specific PV-immunostaining was clearly visualized, providing a brown precipitate. Sections were then washed in PB (3 × 10 min), dehydrated through a graded alcohol series, cleared with xylene and mounted with Eukitt (Kindler GmbH, Freiburg, Germany). Under the microscope, the black and the brown precipitates of the DAB-Nickel and DAB, respectively, were readily distinguishable, as previously reported (Martínez-Guijarro et al., 1994), thus allowing clear identification of the VIP- and PV-containing elements.

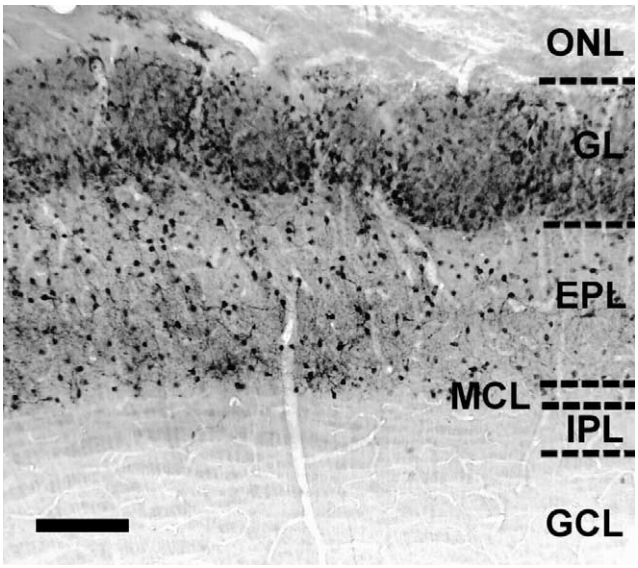

Fig. 1. Panoramic view showing the distribution of VIP-immunostaining in the hedgehog OB. VIP-containing elements are abundant in the periglomerular region of the GL and in the EPL. The ONL lacks VIP-immunoreactivity and the inframitral regions, IPL and GCL only contain a few VIP-immunopositive elements. EPL, External plexiform layer; GCL, granule cell layer; GL, glomerular layer; IPL, internal plexiform layer; MCL, mitral cell layer; ONL, olfactory nerve layer. Scale bar = 200 μm.

3. Results

The pattern of VIP-immunostaining found in this study is in good consonance with previously reported data in the hedgehog OB (López-Mascaraque et al., 1989). We found extensive VIP-immunoreactivity and the distribution of positive cells and fibers followed a laminar pattern (Fig. 1). The VIP-containing elements found in this species belonged to bulbar interneurons. Sensory axons, centrifugal fibers and mitral/tufted cells were immunonegative. VIP-immunoreactive elements were located in all OB layers, with the exception of the olfactory nerve layer (ONL). The highest density of positive neurons was found in the GL and in the EPL, whereas the internal plexiform layer (IPL), the granule cell layer (GCL) and the white matter (WM) displayed only a few VIP-immunoreactive cells.

The VIP-immunocytochemical procedure provides a 'Golgi-like' staining, which allows the visualization of the somata and extensive details of the dendrites and axonal processes of the immunoreactive neurons. This allowed easy identification of these neurons in morphological types, taking as references previous descriptions of the neuronal morphology reported for the hedgehog OB using Golgi, histochemical and immunocytochemical techniques (López-Mascaraque et al., 1986, 1990; Alonso et al., 1995).

3.1. VIP-containing elements in the GL

In the periglomerular region of the GL, an abundant population of small (7–10 μm), round interneurons, easily identifiable as periglomerular cells, displayed VIP-immunoreactivity (Figs. 1 and 2A). The distribution of VIP-immunoreactive periglomerular cells was homogeneous among all the olfactory glomeruli, and no regional differences were observed in the number of VIP-containing neurons that surrounded olfactory glomeruli located in the rostral or caudal portion of the OB or located in the lateral, medial, dorsal or ventral parts of the GL. Most of the VIP-immunopositive periglomerular cells displayed one dendritic trunk that branched within a single glomerulus. The dendritic branches of these cells were not homogeneously distributed within the glomerular neuropil (Fig. 2A). Instead, they appeared to be restricted to strands surrounding neuropil compartments, where no VIP-immunoreactivity was found (Fig. 2B).

The neuropil of the olfactory glomeruli has been previously divided into two different sub-compartments: the *sensory-synaptic compartment*, which includes all sensory axons, and the *central-synaptic compartment*, which is devoid of sensory elements (Chao et al., 1997; Kasowski et al., 1999). Detailed analysis of the dendrites of VIP-immunopositive periglomerular cells at the electron microscope demonstrated that they avoided the *sensory-synaptic compartment* and were exclusively restricted to the *central-synaptic* one (Fig. 2C). Thus, VIP-containing periglomerular cells did not receive synapses from olfactory axons. Their synaptic relationships within the glomerular neuropil only included symmetrical contacts made from VIP-containing pro-

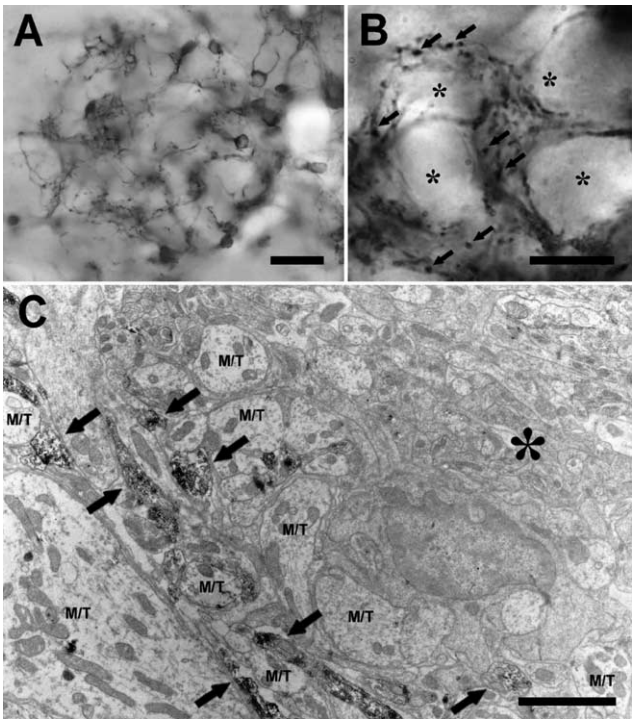

Fig. 2. VIP-immunoreactive periglomerular cells in the glomerular layer. (A) VIP-containing periglomerular cells surrounding an olfactory glomerulus. (B) High magnification demonstrates that the dendritic branches of VIP-containing periglomerular cells are not homogeneously distributed within the glomerular neuropil. They appear restricted to strands surrounding neuropil compartments where no VIP-containing elements are found (asterisks). Arrows point to dendritic gemmules. (C) Electron microscopy demonstrates that VIP-immunolabeled dendritic profiles (arrows) are restricted to the *central-synaptic compartment* of the olfactory glomeruli, which also contains profiles of mitral/tufted cells (M/T) and does not receive sensory afferences. The *sensory-synaptic compartment* (asterisk), which includes olfactory axons, does not contain VIP-containing elements. Scale bars = 25 μm in (A); 10 μm in (B); 2.5 μm in (C).

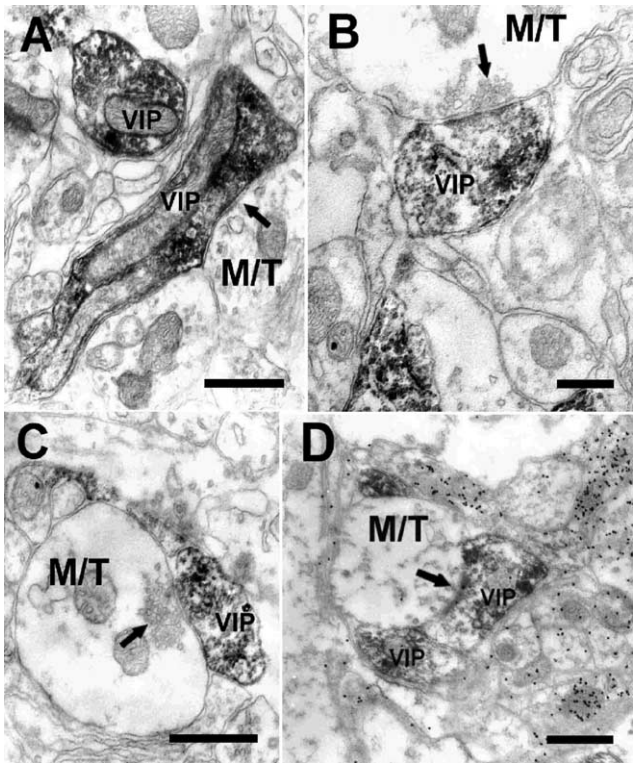

Fig. 3. Synaptic contacts of VIP-containing periglomerular cells in the glomerular neuropil. (A) A VIP-immunopositive dendritic profile of a periglomerular cell makes a symmetrical synaptic contact (arrow) on a dendritic profile of a mitral/tufted cell (M/T). B and C: VIP-immunopositive dendritic profiles receive asymmetrical synaptic contacts (arrows) from dendrites of mitral/tufted cells (M/T). (D) Post-embedding immunogold staining for GABA demonstrates that the dendritic profiles of the VIP-containing periglomerular cells are not GABAergic. Arrow points to an asymmetrical synaptic contact from a dendritic profile of a mitral/tufted cell (M/T) on a VIP-immunoreactive dendritic profile. Scale bars = 0.5 μm in (A), (C) and (D); 0.25 μm in (B).

files onto mitral/tufted cell dendrites and asymmetrical synaptic contacts received from the intraglomerular dendritic profiles of mitral/tufted cells (Fig. 3).

To investigate the GABAergic nature of the population of VIP-containing periglomerular cells in the hedgehog, we combined pre-embedding VIP-immunocytochemistry and post-embedding immunogold staining for GABA. These experiments clearly demonstrated that VIP-containing periglomerular cells, which do not receive synapses from olfactory axons, did not accumulate gold particles (Fig. 3D), indicating that they are non-GABAergic interneurons.

3.2. Distribution of VIP-immunoreactive elements in the EPL

In the hedgehog OB, a large population of interneurons displayed VIP-immunoreactivity in the EPL (Fig. 1). These cells constituted a morphologically heterogeneous group and different neuronal types could be distinguished (Figs. 4 and 5). At the GL/EPL boundary, a few VIP-containing cells had fusiform or ovoid somata and two or more smooth dendrites that extended horizontally in opposite directions (Fig. 4A). These dendrites and their branches were either restricted to the GL/EPL border or coursed throughout the periglomerular region of the olfactory glomeruli, but did not enter the glomerular neuropil. This morphology and location corresponded to that of superficial short-axon cells or horizontal cells previously described using Golgi impregnation techniques or immunocytochemistry (López-Mascaraque et al., 1990; Alonso et al., 1995). In the same region, a few round neurons, similar in size to periglomerular cells, were found to surround small portions of the neuropil close to the periglomerular region of the olfactory glomeruli (Fig. 4B). These cells showed short dendritic trunks that formed dense tufts close to their cell bodies, in a similar way to the perinidal neurons described in the hedgehog OB by Alonso et al. (1995).

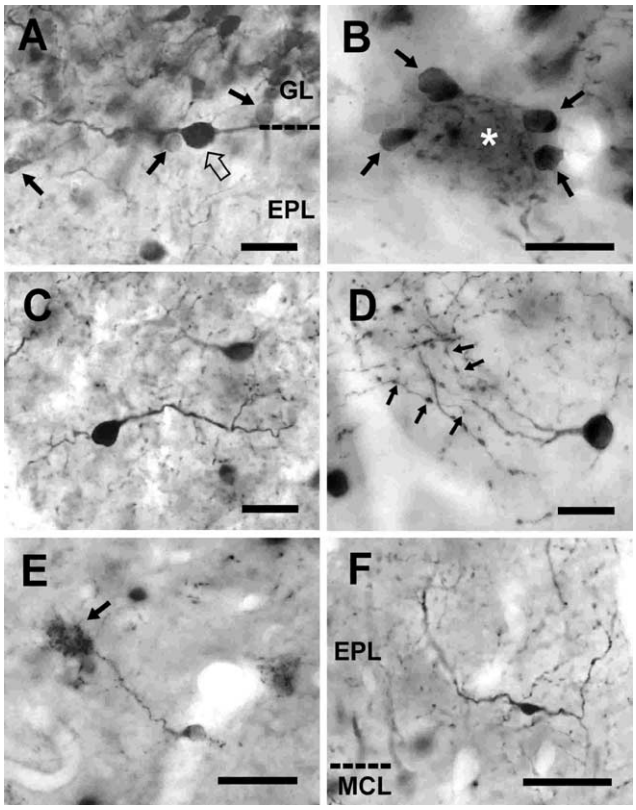

Fig. 4. VIP-containing interneurons found in the external plexiform layer (EPL). (A) Superficial short-axon cell (open arrow) at the boundary between the glomerular layer (GL) and the EPL. Its dendrites course in parallel with the bulbar lamination and are restricted to the GL/EPL border. Compare the size of the superficial short-axon cell with the size of the adjacent periglomerular cells (arrows). (B) VIP-containing perinidal cells (arrows) surrounding a nidus (asterisk) in the most superficial portion of the EPL. C and D: Van Gehuchten cells in the EPL. Note in D that the thinnest dendritic branches of these cells have a similar morphology to axons and are extremely varicose (arrows). (E) A satellite cell emits a single dendrite that ends as a dense tuft (arrow). (F) Small fusiform cell located in the inner portion of the EPL, close to the mitral cell layer (MCL). Scale bars = 25 μm in (A)–(D) and (F); 50 μm in (E).

Most VIP-containing interneurons located throughout the EPL showed round or pyriform small and medium-sized cell bodies (9–15 μm), with one to four thin dendritic trunks densely ramified in this layer, most of them being extremely varicose (Fig. 4C and D). These interneurons corresponded to the satellite cells and Van Gehuchten cells previously described in the hedgehog EPL (López-Mascaraque et al., 1990). A few of the satellite cells showed a single dendrite that formed a dense dendritic tuft around a small field of the neuropil of the EPL (Fig. 4E). Finally, a few small fusiform neurons (5–8 μm) were located in the inner EPL, close to the MCL. These displayed two short poorly ramified dendrites and coursed in opposite directions (Fig. 4F).

The varicose processes of most of the VIP-containing interneurons of the EPL, mainly from satellite cells and Van Gehuchten cells, extended throughout the whole

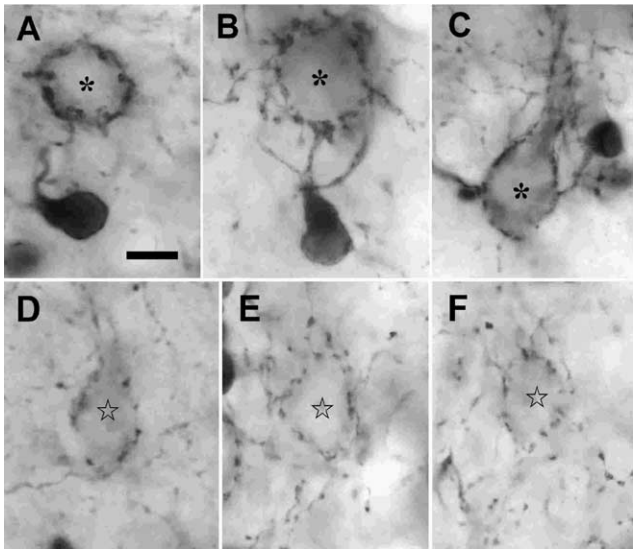

Fig. 5. Perisomatic innervation of mitral/tufted cells by the VIP-containing interneurons of the external plexiform layer (EPL). A, B and C: Some satellite cells, located close to the cell body of the mitral/tufted cells, give rise to short dendritic shafts that embrace the somata of the mitral/tufted cells (asterisk) in an 'exuberant' basket-like fashion. D, E and F: Other mitral/tufted cells are surrounded by 'normal' basket-like arrangements of VIP-containing boutons (stars). Scale bar = 25 μm.

EPL and formed basket-like arrays around isolated large cell bodies located throughout this layer and in the adjacent MCL. Since the cell bodies contacted by the basket-like structures were not VIP-immunostained, their morphological characterization was difficult. Nevertheless, the size of the innervated somata (20–45 μm) and their distribution -throughout the EPL and in the MCL-suggested that they belonged to the mitral/tufted cell population. Electron microscopy confirmed this assumption through their ultrastructural features.

In a detailed analysis of the perisomatic innervation of mitral/tufted cells by the VIP-containing interneurons of the EPL, we found two different types of basket-like arrangements. The first was produced by a set of satellite cells that were always located close to the cell body of the mitral/tufted cells and gave rise to one or two short and varicose dendritic shafts that completely embraced the cell body of the principal neuron in an 'exuberant' basket-like fashion (Fig. 5A–C). This 'exuberant' perisomatic innervation contrasted with the 'normal' pattern of the perisomatic arrangements of VIP-containing boutons found around most of the mitral/tufted cells (Fig. 5D–F).

Both, the 'exuberant' and the 'normal' basket-like structures were analyzed under the electron microscope to explore the possible presence of synaptic contacts between the VIP-containing buttons and the somata of the mitral/tufted cells (Fig. 6). This analysis revealed that the VIP-containing varicosities forming both basket-like structures had large profiles with abundant mitochondria, and that they made symmetrical synaptic

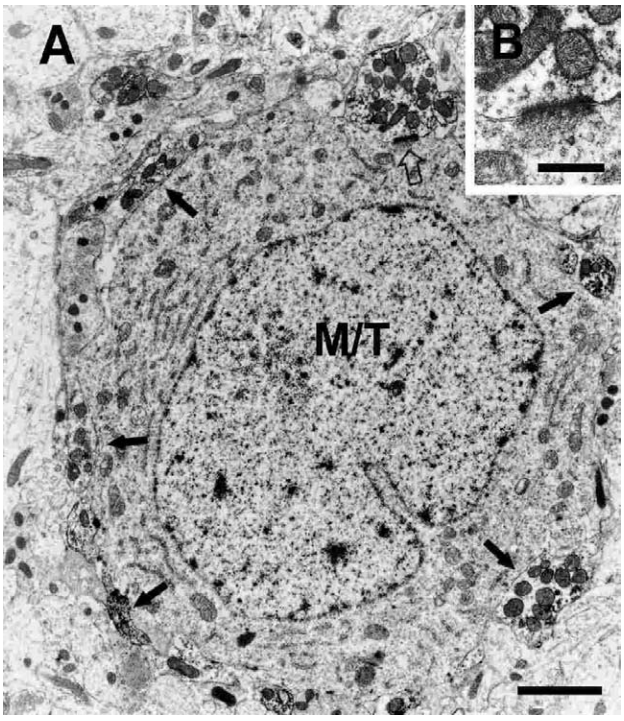

Fig. 6. Perisomatic innervation of mitral/tufted cells by VIP-containing interneurons of the external plexiform layer (EPL) under the electron microscope. (A) Low-magnification electron micrograph showing an 'exuberant' basket-like arrangement surrounding a mitral/tufted cell (M/T). Note contacts between the large varicosities of VIP-containing satellite cells (arrows) and the soma of the M/T. (B) High magnification of the synapses depicted by an open arrow in A. Note asymmetrical synaptic contact from the M/T somata on the VIP-containing bouton. Scale bars = 2 μm in (A); 0.5 μm in (B).

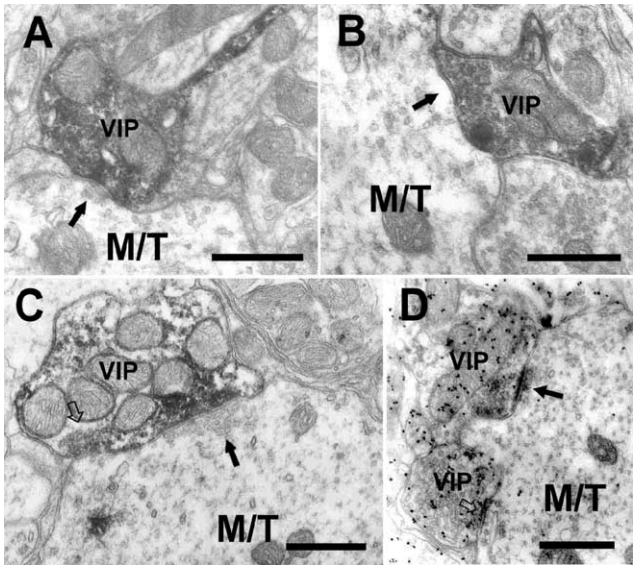

Fig. 7. Synaptic contacts of the VIP-containing interneurons of the external plexiform layer (EPL) with dendritic profiles of mitral/tufted cells (M/T). (A) Asymmetrical synaptic contact from a dendrite of a M/T to a VIP-containing profile (arrow). (B) Symmetrical synaptic contact from a VIP-containing profile to a dendrite of a M/T (arrow). (C) Occasionally, asymmetrical (arrow) and asymmetrical (open arrow) synaptic contacts between M/T and VIP-containing processes form reciprocal pairs. (D) Post-embedding immunogold staining for GABA demonstrates that the VIP-containing interneurons of the EPL are GABAergic. By contrast, their postsynaptic elements are always GABA-immunonegative, showing that they belong to non-GABAergic M/T and ruling out synaptic contacts from VIP-containing interneurons to granule cells. Arrow shows a symmetrical synapse from an M/T to a VIP-containing interneuron. Open arrow shows a symmetrical synapses from a VIP-containing interneuron to the M/T. Scale bars = 0.5 μm.

contacts with the cell bodies of the principal cells. Moreover, these varicosities also received asymmetrical synaptic contacts from the somata of the mitral/tufted cells (Fig. 6B). Frequently, the asymmetrical and the symmetrical contacts were found as reciprocal synapses.

Besides the perisomatic innervation of mitral/tufted cells from VIP-containing interneurons of the EPL, abundant varicose processes of these interneurons were scattered throughout the layer, suggesting additional synaptic contacts in its neuropil: mainly in the middle and deep portions. Electron microscopy demonstrated the presence of synapses between VIP-containing processes and VIP-immunonegative dendritic profiles. In these synaptic contacts, when the postsynaptic VIP-immunonegative element was a large dendritic trunk, it could be clearly identified, on the basis of its ultrastructural features, as a proximal dendrite from a mitral/tufted cell. Synapses between VIP-containing elements and the proximal dendrites of mitral/tufted cells included asymmetrical contacts from the mitral/tufted cell dendrites to the VIP-containing ones (Fig. 7A) and symmetrical contacts from the VIP-containing profiles to the mitral/tufted cell dendrites (Fig. 7B). Occasionally, symmetrical and asymmetrical contacts formed reciprocal pairs (Fig. 7C).

In addition to the synapses found on the large proximal dendrites of mitral/tufted cells, the processes of the VIP-containing interneurons of the EPL also established synaptic contacts with thin VIP-immunonegative dendritic profiles. In these contacts, it was not possible to discriminate, at least on the basis of ultrastructural criteria, whether the postsynaptic VIP-immunonegative dendrites belonged to distal dendritic portions of mitral/tufted cells or, by contrast, they belonged to the dendritic shafts of interneurons. To address this issue, we combined pre-embedding immunostaining for VIP and post-embedding immunogold staining for GABA in ultrathin sections, since the immunodetection of GABA would allow us to distinguish the processes of the non-GABAergic mitral/tufted cells from those of the GABAergic local circuits. These experiments clearly indicated that the postsynaptic elements ($n = 200$) of the VIP-containing profiles were always non-GABAergic (Fig. 7D). Contrariwise, all the VIP-containing elements in the EPL accumulated gold particles. Therefore, they belonged to GABAergic

interneurons (Fig. 7D). These results demonstrated that the population of GABAergic VIP-containing interneurons of the EPL innervates only principal cells and does not contact granule cells.

3.3. VIP-containing interneurons of the EPL exert a heterogeneous perisomatic innervation of mitral/tufted cells

Mitral and tufted cells do not constitute a homogeneous population of principal cells in the OB. By contrast, there are different morphological, neurochemical and functional subsets of mitral/tufted cells. It has recently been demonstrated that the calcium-binding protein PV appears in a specific group of principal cells of the hedgehog OB (Briñón et al., 2001). Since principal cells are the targets for the VIP-containing interneurons of the EPL, we decided to investigate whether the PV-immunopositive and the PV-immunonegative subsets of mitral/tufted cells were homogeneously innervated by VIP-containing interneurons or not. To address this point, we combined VIP- and PV-immunocytochemistry in the same sections using DAB-Nickel as chromogen to visualize the VIP-immunoprecipitate and DAB to visualize PV-immunolabeling (Fig. 8).

These experiments indicated that the population of principal cells was not homogeneously contacted by the VIP-containing interneurons of the EPL. In this sense, the somata of the external tufted cells, located in the periglomerular region of the GL and at the GL/EPL boundary, did not receive innervation from VIP-containing cells (Fig. 8A and B). By contrast, the somata of the mitral cells as well as those of the middle and internal tufted cells were densely innervated by VIP-containing processes (Fig. 8C–F). Although basket-like arrangements were found on PV-positive and PV-negative mitral/tufted cells, in a more detailed analysis it was observed that the 'exuberant' basket-like structures were only found on PV-immunonegative somata (Fig. 8E and F) and were never found on PV-immunopositive cell bodies ($n = 200$), which displayed 'normal' basket-like arrays (Fig. 8C–E).

The PV-immunostaining allowed visualization of the cell body and large portions of the primary and secondary dendrites of the stained mitral/tufted cells. Its combination with VIP-immunolabeling demonstrated that the processes of the VIP-containing interneurons of the EPL innervated not only the cell body, but also large portions of the primary and secondary dendrites of the PV-containing mitral/tufted cells (Fig. 9).

Although PV-immunostaining was used as a tool for the identification of a specific subset of mitral/tufted cells, this calcium-binding protein also appeared in the vast majority of the interneurons of the EPL, colocalizing with VIP in these cells.

3.4. VIP-containing elements in the inframitral layers

In the inframitral layers, granule cells were VIP-immunonegative and only a few interneurons showed VIP-immunoreactivity in this area. The VIP-containing neurons were mainly located in the IPL and in superficial portions of the GCL, although a few were also found in the deep GCL or in the WM. They constituted a morphologically heterogeneous group that included medium-sized cells (10–15 μm) with round or ovoid somata and pyriform, bipolar or multipolar morphologies (Fig. 10). These cells showed one to four dendrites running and branching in different directions throughout the GCL and the IPL. The primary dendrites were smooth, but their thinnest branches became varicose or displayed scarce spines in their terminal portions. In some of these interneurons, the initial portions of the axons and their collaterals could be seen running throughout the GCL or in the superficial portions of the WM. The location and morphology of these neurons corresponded to those of different morphological sets of deep short-axon cells previously described in this species with Golgi (López-Mascaraque et al., 1986), histochemical, and immunocytochemical approaches (Alonso et al., 1995).

4. Discussion

In the present study, we were able to gain insight into the distribution and connectivity of the VIP-containing circuits of the hedgehog OB. The main findings are: (i) VIP-containing periglomerular cells constitute a population of non-GABAergic neurons that do not receive sensory afferences; (ii) unlike VIP-immunopositive periglomerular cells, the VIP-containing cells of the EPL are GABAergic; these interneurons exert an exclusive in-

Fig. 8. VIP- and PV-double immunocytochemistry reveals a heterogeneous perisomatic innervation of principal cells by VIP-containing interneurons of the external plexiform layer (EPL). The black precipitate of the DAB–Nickel shows up the VIP immunoreactivity and the brown precipitate of the DAB depicts the PV immunoreactivity. A and B: PV-containing external tufted cells (open arrows) at the boundary between the glomerular layer (GL) and the EPL do not receive perisomatic innervation from the VIP-containing interneurons of the EPL. C and D Contrary to the PV-containing external tufted cells, the PV-containing middle (C) and deep (D) tufted cell somata are innervated by boutons of the VIP-containing interneurons of the EPL (arrows). Note in D that a similar innervation pattern is found in some PV-immunonegative tufted cells (open arrows). E and F: The 'exuberant' basket-like innervation of mitral/tufted cells from a set of satellite cells is always found on PV-immunonegative mitral/tufted cells (asterisks). Compare this innervation pattern with the 'normal' one found on a PV-containing mitral/tufted cell (arrows in E). Scale bars = 50 μm in (A)–(E); 20 μm in (F).

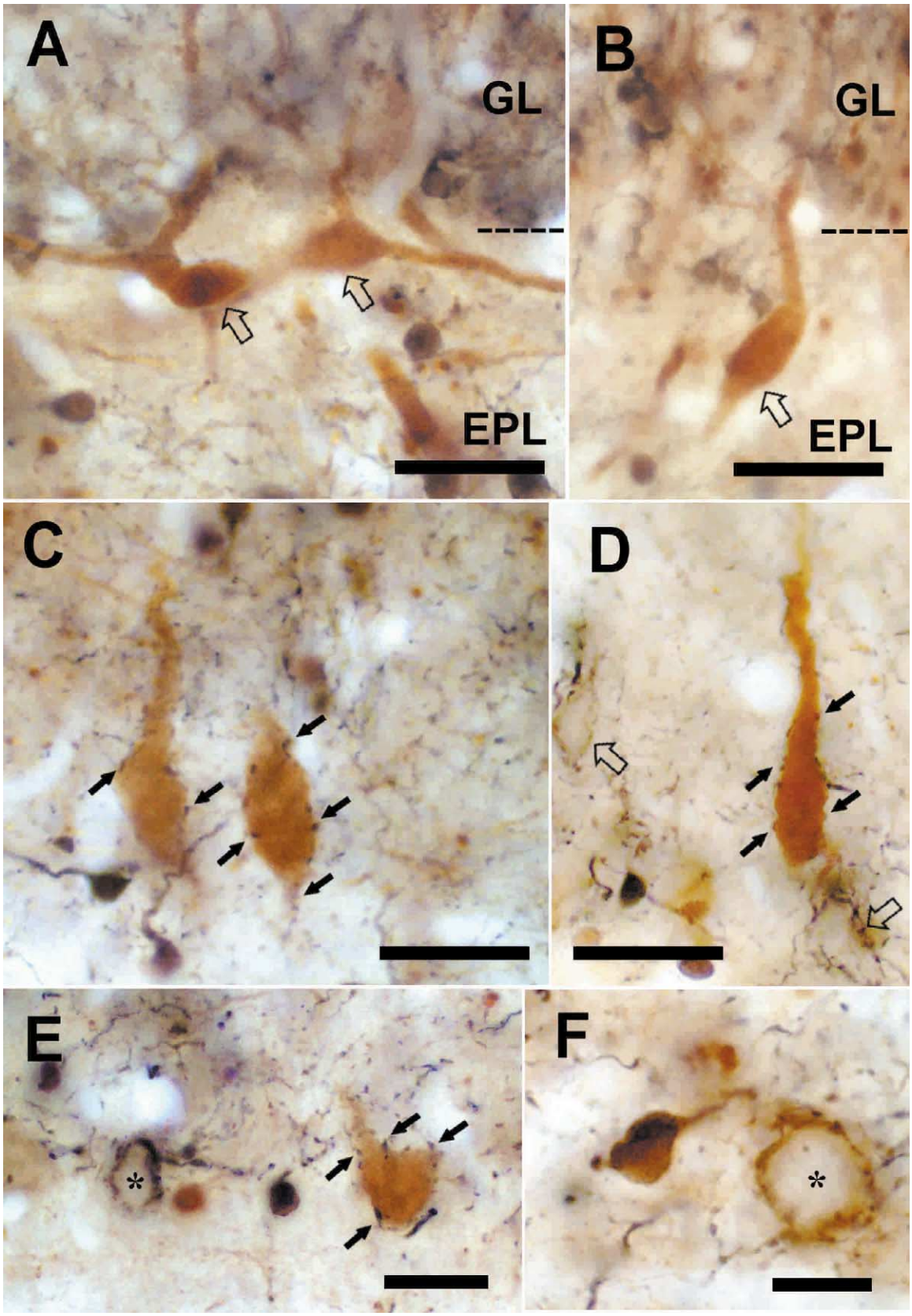

Fig. 8

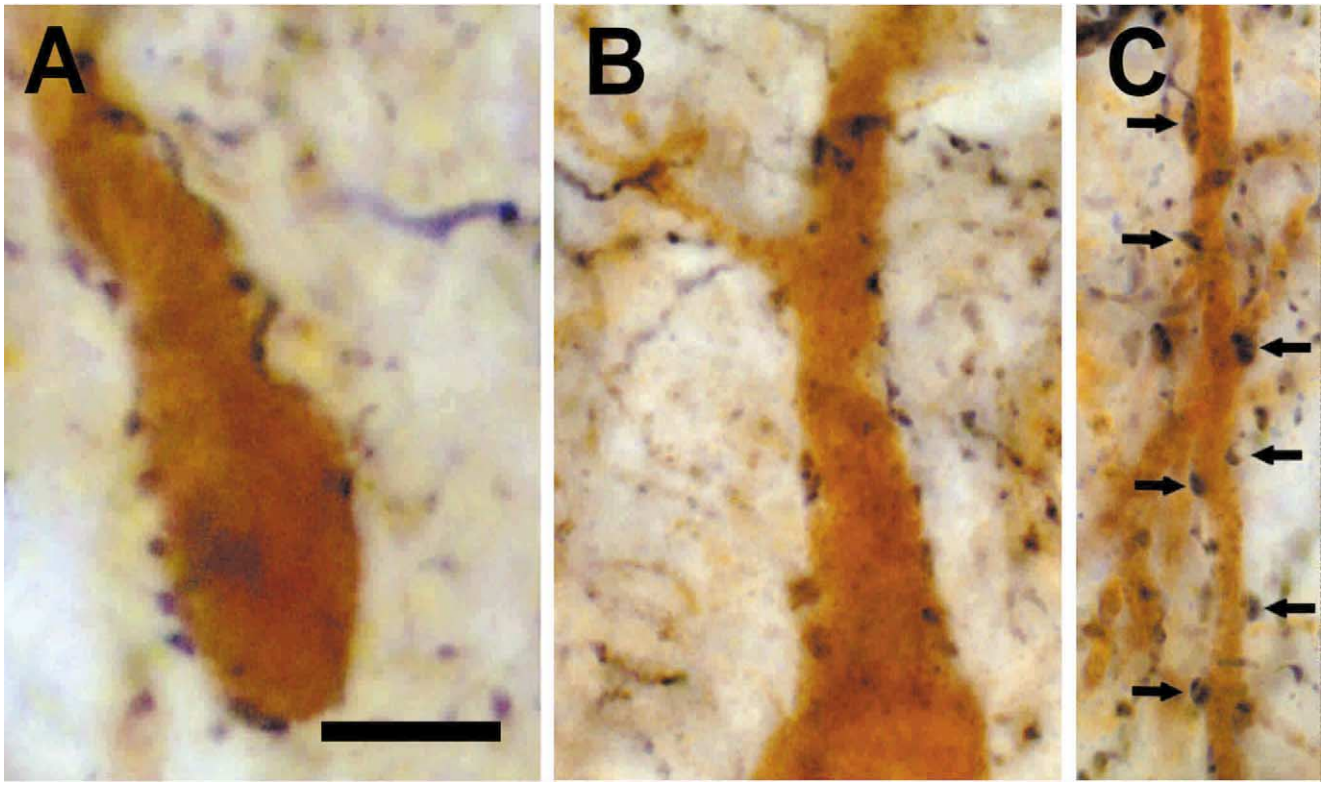

Fig. 9. The VIP-containing interneurons of the external plexiform layer (EPL) not only innervate the perisomatic region (A), but also the proximal primary dendrite (B) and large portions of the secondary dendrites (C) (arrows) of the PV-containing mitral/tufted cells. Note the presence of multiple contacts from a VIP-containing process on a cell. Scale bar = 12.5 μm.

nervation of principal cells; (iii) one group of these interneurons establishes basket-like structures on the somata of principal mitral/tufted cells; (iv) the perisomatic innervation of mitral/tufted cells by the VIP-containing interneurons of the EPL shows a clear selectivity, since it demonstrates that the 'exuberant' basket-like structures from a set of satellite cells are always found on PV-immunonegative mitral/tufted cells and never on PV-containing ones.

4.1. Distribution of VIP-containing elements in the hedgehog OB

A broad distribution of VIP-containing elements was found in the hedgehog OB, and the distribution pattern found for this peptide is in close agreement with the previous description reported by López-Mascaraque et al. (1989). We found immunoreactivity for VIP in three main categories of bulbar interneurons: periglomerular cells, interneurons of the EPL, and deep short-axon cells of the inframitral layers. There are some points of convergence between the results obtained in the hedgehog and data previously reported for different mammals, all of them macrosmatic, including the rat (Gall et al., 1986), golden hamster (Nakajima et al., 1996) and cat (Sanides-Kohlrausch and Wahle, 1990). All these species display an abundant population of VIP-containing interneurons in the EPL and hence this aspect seems to be quite conservative and could indicate an important role for VIP in olfaction, probably related to the functional involvement of these interneurons in the bulbar circuits, as discussed below. The presence of VIP-containing deep short-axon cells in the inframitral layers of the OB is also common to the hedgehog, rat, golden hamster and cat. Nevertheless, the scarce number of deep short-axon cells found in all of these species argues for a minor involvement of VIP in the processing of olfactory information at inframitral level.

Despite the similarities found for the distribution of VIP in the interneurons of the EPL and deep short-axon cells, some discrepancies about the presence of VIP-immunoreactivity in the periglomerular cells of the hedgehog, cat and rodents have been documented. Thus, whereas the hedgehog (López-Mascaraque et al., 1989, present data) and the cat (Sanides-Kohlrausch and Wahle, 1990) display a population of VIP-containing periglomerular cells, this neuronal type has not been found to be VIP-immunoreactive in the rat or in the golden hamster (Gall et al., 1986; Nakajima et al., 1996). These interspecies variations could reflect differences between the VIP-containing circuits of rodents and other groups of macrosmatic mammals. Nevertheless, it is also possible that methodological differences or the sensitivity of the different antibodies used might account

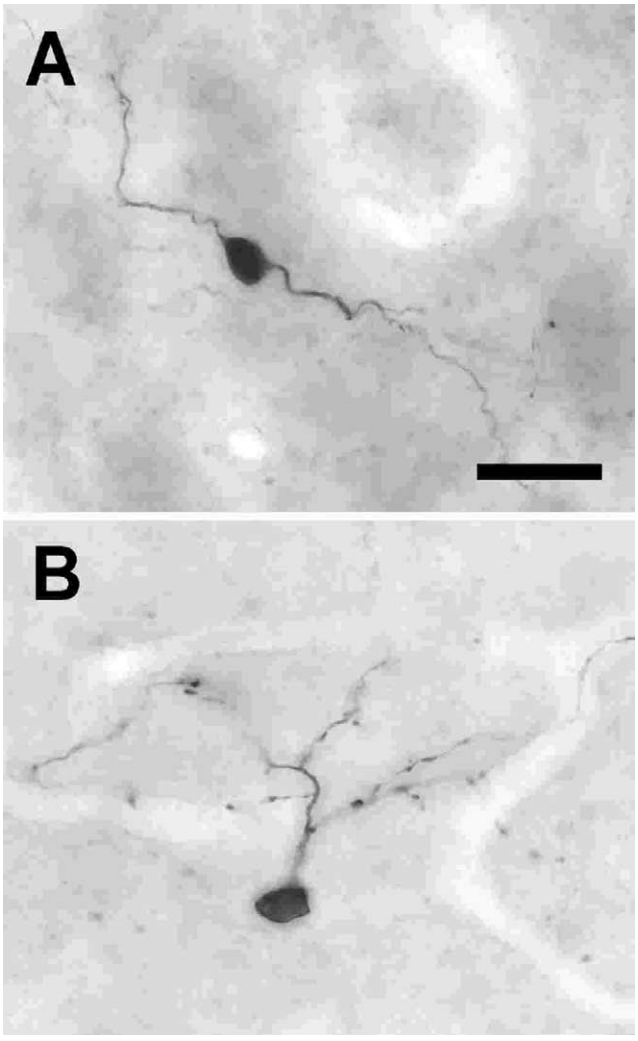

Fig. 10. VIP-containing interneurons found in the inframitral layers. (A) Deep short-axon cell with a bipolar morphology located in the middle portion of the granule cell layer. This cell shows two opposite smooth dendrites running through the VIP-immunonegative granule cells. (B) Deep short-axon cell with pyriform morphology located in the deep portion of the granule cell layer. Note that the thinnest dendritic branches of this cell show varicosities. Scale bar = 25 μm.

for this discrepancy. To avoid these possibilities, some sections of rat OB were processed at the same time as the hedgehog OB sections were stained for VIP-immunocytochemistry. The absence of VIP-containing periglomerular cells in the rat is in close agreement with the results reported by (Gall et al., 1986) and confirms the existence of these interspecies differences between rodents and insectivores.

It is important to note that, although Sanides-Kohlrausch and Wahle (1990) found a population of VIP-immunostained periglomerular cells in the cat OB, these authors reported that the number of periglomerular cells containing this peptide do not reach a density of 50 cells per 80 μm-thick section. In this respect, we estimate, in a semi-quantitative approach, more than 600 VIP-containing periglomerular cells per 50 μm-thick section in the hedgehog. With these data, the importance of the VIP-containing circuits at the glomerular level in the macrosmatic hedgehog seems to be evident in comparison with the cat or with rodents.

4.2. VIP-immunoreactive periglomerular cells

In rodents, it has been clearly demonstrated that although periglomerular cells constitute a morphologically homogeneous population of cells, these interneurons are neurochemically heterogeneous (Halász et al., 1985; Sakai et al., 1987; Seroogy et al., 1989; Crespo et al., 1995, 1997; Kosaka et al., 1995; Shipley et al., 1995 for a review; Briñón et al., 1997, 1999). Moreover, recent studies have demonstrated that the neurochemical differences found between different subsets of periglomerular cells could reflect variations in the connectivity of these interneurons within the glomerular neuropil. In this regard, in the rat OB a subset of GABAergic periglomerular cells that contains tyrosine hydroxylase and the m2 muscarinic receptor subtype receives synaptic contacts from olfactory axons (Kosaka et al., 1997; Crespo et al., 2000; Toida et al. 2000). By contrast, a subset of non-GABAergic periglomerular cells that contain the calcium-binding proteins calretinin or calbindin D-28k does not receive sensory afferences (Kosaka et al., 1997; Toida et al., 1998). These data are quite relevant for an understanding of the processing of olfactory signals at the level of the first synaptic relay, taking place in the glomerular neuropil.

Although previous studies have demonstrated a neurochemical heterogeneity of periglomerular cells in the hedgehog (Alonso et al., 1995; Briñón et al., 2001), at present there are no detailed analysis in this animal demonstrating a correlation between different subsets of periglomerular cells and putative differences in their synaptic connectivity. The present results provide some evidence in this sense. We demonstrate here that the non-GABAergic VIP-containing periglomerular cells do not receive sensory afferences. By contrast, GABAergic profiles of VIP-immunonegative periglomerular cells were frequently found in the *sensory-synaptic compartment*, where they received asymmetrical synaptic contacts from olfactory axons.

In light of the present findings, it would appear that the presence of two major groups of periglomerular cells—i.e. GABAergic periglomerular cells contacted by olfactory axons, and non-GABAergic periglomerular cells that do not receive synapses from sensory afferences—is a striking feature of the OB and remains highly conserved, at least in macrosmatic mammals. What the functional involvement of these two groups of periglomerular cells in olfactory processing is remains currently obscure. Considering their connectivity, it seems possible that GABAergic periglomerular cells could modulate the firing of principal cells as a direct

response to the reception of sensory input. However, the modulation of principal cells from non-GABAergic periglomerular cells seems to be independent of a direct olfactory input and could be dependent on other afferences, such as the centrifugal type. In this sense, it is interesting to note that centrifugal axons do not enter the *sensory-synaptic compartment* and are restricted to the *central-synaptic* one (Chao et al., 1997; Kasowski et al., 1999), the glomerular region to which the dendrites of non-GABAergic periglomerular cells are also restricted (Kosaka et al., 1997; Toida et al., 1998, present data).

The high number of VIP-containing periglomerular cells in the hedgehog argues in favor of an important function for this peptide in glomerular circuits. It has been widely demonstrated that VIP acts by metabotropic mechanisms, via G-protein-coupled receptors that activate adenylate-cyclase and phospholipase-C cascades. Owing to this metabotropic action, Kohlmeier and Reiner (1999) have recently reported that the peptide can excite neurons through activation of a sodium current. Moreover, it has been documented that VIP causes neuron depolarization in vitro (Liu and Morris, 1999). These authors reported that in some cases the depolarizations produced by VIP were very large and caused depolarization block and prolonged desensitization. A similar effect of VIP in the hedgehog olfactory glomeruli might affect the sensitivity of mitral/tufted cells to olfactory signals and it would be interesting to modulate the signal-to-noise ratio in olfactory processing.

In addition to these effects as a neurotransmitter, it has been recently reported that VIP has interesting neurotrophic actions. For example, recent experiments have shown that VIP induces the release of neurotrophic factors that enhance neurite outgrowth (White et al., 2000). In cultures of hippocampal neurons, VIP promotes the secretion of 'activity-dependent neurotrophic factor' from VIP-stimulated astroglia, and this trophic factor acts directly on neurons to promote morphological development and neuronal differentiation (Blondel et al., 2000). A neurotrophic action of VIP in the olfactory glomeruli of the hedgehog cannot be ruled out, as suggested by two interesting observations. The first is that olfactory glomeruli continuously receive new olfactory axons from the olfactory epithelium (Graziadei and Monti-Graziadei, 1980) and newly generated cells from the rostral migratory stream (Lois et al., 1996) that differentiate into periglomerular cells. Thus, the glomerular neuropil is a plastic structure that constantly reorganizes its synaptic connections. The second aspect is that within the glomerular neuropil, the astroglia is restricted to the *central-synaptic compartment*, where VIP-containing periglomerular cells extend their dendrites, and is absent from the *sensory-synaptic* compartment, which is devoid of VIP-containing processes. Nevertheless, further studies should be designed to contrast the hypothetical trophic action of VIP in the non-GABAergic periglomerular cells of the hedgehog.

4.3. VIP-immunoreactive interneurons of the EPL

One of the most conserved aspects of the VIP distribution pattern found in the mammalian OB is the presence of this peptide in an abundant population of interneurons of the EPL. Classically, it was considered that the major populations of interneurons in the OB of mammals were periglomerular cells and granule cells. Other populations of local neurons, such as the interneurons of the EPL or the deep short-axon cells of the inframitral regions, have received considerably less attention (see Shipley et al., 1995; Shepherd and Greer, 1998). Although pioneering Golgi studies by Van Gehuchten and Martin (1891) described the presence of a population of neurons in the EPL, their connectivity in the bulbar circuits has not been determined until recently (Toida et al., 1994, 1996; Crespo et al., 2001). Historically, it was assumed that the interneurons of the EPL could interact with the dendrites of granule cells (Schneider and Macrides, 1978; Macrides et al., 1985). Contrary to this assumption, (Toida et al., 1994, 1996) demonstrated by electron microscopy that the interneurons of the EPL contact mitral/tufted cells in the rat OB and, more recently, we have discarded synaptic relationships of these interneurons with granule cells or other GABAergic interneurons (Crespo et al., 2001). The present results confirm that the interneurons of the EPL do not contact granule cells and corroborate the notion that they are exclusively specialized in the direct innervation of principal cells, not only in the rat but also in the hedgehog. Although the interneurons of the EPL constitute a morphologically heterogeneous population of cells, the distinct neuronal types described in the rat (Kosaka et al., 1994) and hedgehog (López-Mascaraque et al., 1989; Briñón et al., 2001; present data) follow a similar pattern of synaptic relationships in the neuropil of this layer. We thus conclude that the connectivity of these interneurons is conserved in mammals, at least in the macrosmatic rodents and insectivores studied so far. This indicates that the interneurons of the EPL must play a key role in the bulbar circuits, probably exerting a strong modulation of bulbar firing and thereby controlling bulbar transmission to higher order olfactory brain areas. Consequently, these interneurons should not be further neglected.

We have recently discussed the possibility that the interneurons of the EPL in the rat resemble the GABAergic 'basket cells' of the cortex in the OB (Crespo et al., 2001). Taking into account the evident basket-like arrangements of VIP-containing boutons of these interneurons that were observed around principal cells (see Figs. 5 and 6), our present data in the hedgehog

strongly support this idea. Nevertheless, despite the similarities observed among the interneurons of the EPL in the rat and the hedgehog, some important differences between both species should also be considered carefully. The first is that the perisomatic innervation of principal cells from interneurons of the EPL is more powerful and selective in the hedgehog OB than in the rat OB. This is clearly seen from the elevated density of basket-like structures found in the EPL in the hedgehog as compared to the situation in the rat. Moreover, the presence of the 'exuberant' basket-like arrays of VIP-containing processes detected in the hedgehog (see Fig. 5A–C) has not been previously described in the rat or in any other species. The second difference is the morphological and functional specialization of a group of interneurons of the EPL in the hedgehog OB that has no counterpart in the rat OB. These neurons are the satellite cells. Satellite cells were first identified in the hedgehog OB by Golgi techniques (López-Mascaraque et al., 1990). In the original paper, these authors described satellite cells as 'neurons closely associated with the secondary dendrites of mitral cells in a way that suggested a functional relationship'. We agree with this description, and add evidence for the presence of a group of satellite cells closely attached to the cell bodies of principal cells that are clearly specialized in their perisomatic innervation. To date, we have no knowledge about the presence of these neurons in other mammalian species.

From a functional point of view, perisomatic inhibition is believed to exert a strong control over the timing of the action potential in the innervated target (Miles et al., 1996). Thus, the GABAergic VIP-containing satellite cells of the hedgehog could achieve a potent control of the firing of principal cells, and then modulate the timing of the transmission of olfactory information towards superior olfactory centers. Since the synaptic relationships of GABAergic VIP-containing satellite cells and principal neurons include reciprocal synaptic contacts, the satellite cells could then exert a rapid feedback inhibitory action, controlling the output of different sets of the mitral/tufted cells innervated by them. Taking into account that the hedgehog is one of the mammals with the most highly developed olfactory structures, the presence of a population of satellite cells in this animal may indicate the presence of a powerful feed-back system that is quite effective in controlling bulbar output, which is not so selective in other macrosmatic mammals such as rodents.

Despite the above-discussed differences between rodents and insectivores, the GABAergic interneurons of the EPL display common neurochemical features in both macrosmatic groups. For example, they contain PV in the rat (Kosaka et al., 1994) and in the hedgehog (Briñón et al., 2001) and they also contain VIP in both species (Gall et al., 1986; López-Mascaraque et al., 1989; present results). Moreover, using the DAB–Nickel/DAB double-immunocytochemical method, in the hedgehog we found that PV and VIP do not appear in different subsets of interneurons of the EPL, but instead colocalize in the same cells. The conserved expression of PV and VIP in the interneurons of the EPL argues for an essential physiological role of both substances in these GABAergic cells.

In the circuits of the EPL, VIP might act as a neurotransmitter or neuromodulator involved in the GABAergic inhibition of mitral/tufted cells taking place in this layer. In this respect, VIP has been previously related to GABAergic transmission in cultured hippocampal neurons (Wang et al., 1997). These authors reported that activation of presynaptic VIP receptors enhances GABAergic synaptic transmission through a metabotropic action, including activation of adenylate-cyclase and phosphokinase-A. Moreover, it has been suggested that VIP could increase GABA release through stimulation of the vesicular exocytotic apparatus (Wang et al., 1997).

4.4. Heterogeneous innervation of principal cells by VIP-containing interneurons

To date, it is known that the principal cells in the OB constitute a morphologically, neurochemically and functionally heterogeneous population of neurons. Regarding their connectivity, mitral and internal tufted cells display the most extensive projections to caudal regions of the piriform lobe, whereas axons of middle tufted cells are preferentially restricted to the anterior olfactory nucleus, the anterior regions of the piriform cortex, and to the olfactory tubercle (see Macrides et al., 1985; Shepherd and Greer, 1998 for a review). Finally, external tufted cells are integrated in the intrabulbar associational system and send their axons to the IPL of the opposite side of the same OB (Liu and Shipley, 1994). The specific connectivity of principal cells implies that they have distinct electrophysiological properties and local circuits and/or centrifugal afferences should modulate them differentially. In this report, we provide interesting information about the different modulation of different sets of principal cells by GABAergic VIP-containing interneurons of the EPL. For example, we demonstrate that the external tufted cells that constitute the intrabulbar associational system (Liu and Shipley, 1994) do not have the perisomatic basket-like structures from VIP-containing interneurons. Moreover, the 'exuberant' basket-like structures from perisomatic satellite cells always appear on PV-negative tufted cells, but never on PV-positive ones. Taking these data together, it seems that the macrosmatic hedgehog has a complex circuitry of specialized interneurons that are probably able to control different sets of principal cells and hence achieve a degree of tuning of the olfactory information

that is finer than that achieved in other mammalian groups such as rodents. The bulbar circuits found in the hedgehog suggest that this is the most complex olfactory system ever described in mammals.

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References

Acsády, L., Görcs, T.J., Freund, T.F. 1996. Different populations of vasoactive intestinal polypeptide-immunoreactive interneurons are specialized to control pyramidal cells or interneurons in the hippocampus. Neuroscience 732, 317–334.

Alonso, J.R., Arévalo, R., García-Ojeda, E., Porteros, A., Briñón, J.G., Aijón, J. 1995. NADPH-diaphorase active and calbindin D-28k-immunoreactive neurons and fibers in the olfactory bulb of the hedgehog (*Erinaceus europaeus*). J. Comp. Neurol. 351, 307–327.

Blasco-Ibáñez, J.M., Martínez-Guijarro, F.J., Freund, T.F. 1998. Enkephalin-containing interneurons are specialized to innervate other interneurons in the hippocampal CA1 region of the rat and guinea-pig. Eur. J. Neurosci. 10, 1784–1795.

Blondel, O., Collin, C., McCarran, W.J., Zhu, S., Zamostiano, R., Gozes, I., Brenneman, D.E., McKay, R.D. 2000. A glia-derived signal regulating neuronal differentiation. J. Neurosci. 20, 8012–8020.

Briñón, J.G., Alonso, J.R., García-Ojeda, E., Crespo, C., Arévalo, R., Aijón, J. 1997. Calretinin- and parvalbumin-immunoreactive neurons in the rat main olfactory bulb do not express NADPH-diaphorase activity. J. Chem. Neuroanat. 13, 253–264.

Briñón, J.G., Martínez, G.F., Bravo, I.G., Arévalo, R., Crespo, C., Okazaki, K., Hidaka, H., Aijón, J., Alonso, J.R. 1999. Coexpression of neurocalcin with other calcium-binding proteins in the rat main olfactory bulb. J. Comp. Neurol. 407, 404–414.

Briñón, J.G., Weruaga, E., Crespo, C., Porteros, A., Arévalo, R., Aijón, J., Alonso, J.R. 2001. Calretinin-, neurocalcin-, and parvalbumin-immunoreactive elements in the olfactory bulb of the hedgehog (*Erinaceus europaeus*). J. Comp. Neurol. 429, 554–570.

Chao, T.I., Kasa, P., Wolff, J.R. 1997. Distribution of astroglia in glomeruli of the rat main olfactory bulb: exclusion from the sensory subcompartment of neuropil. J. Comp. Neurol. 388, 191–210.

Crespo, C., Arévalo, R., Briñón, J.G., Porteros, A., Bravo, I.G., Aijón, J., Alonso, J.R. 1995. Colocalization of NADPH-diaphorase and acetylcholinesterase in the rat olfactory bulb. J. Chem. Neuroanat. 9, 207–216.

Crespo, C., Alonso, J.R., Briñón, J.G., Weruaga, E., Porteros, A., Arévalo, R., Aijón, J. 1997. Calcium-binding proteins in the periglomerular region of typical and typical olfactory glomeruli. Brain Res. 745, 293–302.

Crespo, C., Briñón, J.G., Porteros, A., Arévalo, R., Rico, B., Aijón, J., Alonso, J.R. 1999. Distribution of acetylcholinesterase and choline acetyltransferase in the main and accessory olfactory bulbs of the hedgehog (*Erinaceus europaeus*). J. Comp. Neurol. 403, 53–67.

Crespo, C., Blasco-Ibáñez, J.M., Briñón, J.G., Alonso, J.R., Dominguez, M.I., Martínez-Guijarro, F.J. 2000. Subcellular localization of m2 muscarinic receptors in GABAergic interneurons of the olfactory bulb. Eur J. Neurosci. 12, 3963–3974.

Crespo, C., Blasco-Ibáñez, J.M., Marqués-Marí, A.I., Martínez-Guijarro, F.J. 2001. Parvalbumin-containing interneurons do not innervate granule cells in the olfactory bulb. Neuroreport 12, 2553–2556.

De Carlos, J.A., López-Mascaraque, L., Valverde, F. 1989. Connections of the olfactory bulb and nucleus olfactorius anterior in the hedgehog (*Erinaceus europaeus*): fluorescent tracers and HRP study. J. Comp. Neurol. 279, 601–618.

Gall, C., Seroogy, K.B., Brecha, N. 1986. Distribution of VIP- and NPY-like immunoreactivities in rat main olfactory bulb. Brain Res. 374, 389–394.

Graziadei, P.P., Monti-Graziadei, G.A. 1980. Neurogenesis and neuron regeneration in the olfactory system of mammals. III. Deafferentation and reinnervation of the olfactory bulb following section of the fila olfactoria in rat. J. Neurocytol. 9, 145–162.

Gulyás, A., Görcs, T.J., Freund, T.F. 1990. Innervation of different peptide-containing neurons in the hippocampus by GABAergic septal afferents. Neuroscience 37, 31–44.

Halász, N., Hökfelt, T., Norman, A.W., Goldstein, M. 1985. Tyrosine hydroxylase and 28k-vitamin D-dependent calcium binding protein are localized in different subpopulations of periglomerular cells of the rat olfactory bulb. Neurosci. Lett. 61, 103–107.

Hsu, S.M., Raine, L., Fanger, H. 1981. Use of avidin–biotin-peroxidase complex (ABC) in immunoperoxidase techniques: a comparison between ABC and unlabeled antibody (PAP) procedures. J. Histochem. Cytochem. 29, 577–580.

Kasowski, H.J., Kim, H., Greer, C.A. 1999. Compartmental organization of the olfactory bulb glomerulus. J. Comp. Neurol. 407, 261–274.

Kohlmeier, K.A., Reiner, P.B. 1999. Vasoactive intestinal polypeptide excites medial pontine reticular formation neurons in the brainstem rapid eye movement sleep-induction zone. J. Neurosci. 19, 4073–4081.

Kosaka, K., Heizmann, C.W., Kosaka, T. 1994. Calcium-binding protein parvalbumin-immunoreactive neurons in the rat olfactory bulb. 1. Distribution and structural features in adult rat. Exp. Brain Res. 99, 191–204.

Kosaka, K., Aika, Y., Toida, K., Heizmann, C.W., Hunziker, W., Jacobowitz, D.M., Nagatsu, I., Streit, P., Visser, T.J., Kosaka, T. 1995. Chemically defined neuron groups and their subpopulations in the glomerular layer of the rat main olfactory bulb. Neurosci. Res. 23, 73–88.

Kosaka, K., Toida, K., Margolis, F.L., Kosaka, T. 1997. Chemically defined neuron groups and their subpopulations in the glomerular layer of the rat main olfactory bulb. II. Prominent differences in the intraglomerular dendritic arborization and their relationship to olfactory nerve terminals. Neuroscience 76, 775–786.

Kosaka, K., Toida, K., Aika, Y., Kosaka, T. 1998. How simple is the organization of the olfactory glomerulus: the heterogeneity of so-called periglomerular cells. Neurosci. Res. 30, 101–110.

Liu, W.L., Shipley, M.T. 1994. Intrabulbar associational system in the rat olfactory bulb comprises cholecystokinin-containing tufted cells that synapse onto the dendrites of GABAergic granule cells. J. Comp. Neurol. 346, 541–558.

Liu, X.H., Morris, R. 1999. Vasoactive intestinal polypeptide produces depolarization and facilitation of C-fibre evoked synaptic responses in superficial dorsal horn neurones (laminae I–IV) of the rat lumbar spinal cord in vitro. Neurosci. Lett. 276, 1–4.

Lois, C., Garcia-Verdugo, J.M., Álvarez-Buylla, A. 1996. Chain migration of neuronal precursors. Science 271, 978–981.

López-Mascaraque, L., De Carlos, J.A., Valverde, F. 1986. Structure of the olfactory bulb of the hedgehog (*Erinaceus europaeus*):

description of cell types in the granular layer. J. Comp. Neurol. 253, 135–152.
López-Mascaraque, L., Villalba, R.M., De Carlos, J.A. 1989. Vasoactive intestinal polypeptide-immunoreactive neurons in the main olfactory bulb of the hedgehog. Neuroci. Lett. 98, 19–24.
López-Mascaraque, L., De Carlos, J.A., Valverde, F. 1990. Structure of the olfactory bulb of the hedgehog (*Erinaceus europaeus*): a Golgi study of the intrinsic organization of the superficial layers. J. Comp. Neurol. 301, 243–261.
Macrides, F., Schoenfeld, T.A., Marchand, J.E., Clancy, A.N. 1985. Evidence for morphologically, neurochemically and functionally heterogeneous classes of mitral and tufted cells in the olfactory bulb. Chem. Senses 10, 175–202.
Martínez-Guijarro, F.J., Blasco-Ibáñez, J.M., Freund, T.F. 1994. Serotoninergic innervation of nonprincipal cells in the cerebral cortex of the lizard *Podarcis hispanica*. J. Comp. Neurol. 343, 542–553.
Martínez-Guijarro, F.J., Briñón, J.G., Blasco-Ibáñez, J.M., Okazaki, K., Hidaka, H., Alonso, J.R. 1998. Neurocalcin-immunoreactive cells in the rat hippocampus are GABAergic interneurons. Hippocampus 8, 2–23.
Miles, R., Toth, K., Gulyas, A.I., Hajos, N., Freund, T.F. 1996. Differences between somatic and dendritic inhibition in the hippocampus. Neuron 16, 815–823.
Nakajima, T., Okamura, M., Ogawa, K., Taniguchi, K. 1996. Immunohistochemical and enzyme histochemical characteristics of short axon cells in the olfactory bulb of the golden hamster. J. Vet. Med. Sci. 58, 903–908.
Palmieri, G., Farina, V., Sanna, L., Asole, A., Zedda, M., Panu, R., De Riu, P.L. 1993. The pyramidal tract of the hedgehog (*Erinaceus europaeus*) and its relationship with the olfactory bulb. Arch. Ital. Biol. 131, 147–158.
Sakai, M., Yoshida, M., Karasawa, N., Teramura-Ito, M., Nagatsu, I. 1987. Coexistence of taurine-like and tyrosine hydroxylase-like immunoreactivities in some periglomerular cells of the rat main olfactory bulb. Biog. Amines 4, 457–464.
Sanides-Kohlrausch, C., Wahle, P. 1990. VIP- and PHI-immunoreactivity in olfactory centers of the adult cat. J. Comp. Neurol. 294, 325–339.
Schneider, S.P., Macrides, F. 1978. Laminar distributions of internuerons in the main olfactory bulb of the adult hamster. Brain Res. Bull. 3, 73–82.
Seroogy, K.B., Hökfelt, T., Buchan, A., Brown, J.C., Terenius, L., Norman, A.W. 1989. Somatostatin-like immunoreactivity in rat main olfactory bulb: extent of coexistence with neuropeptide Y-, tyrosine hydroxylase- and vitamin D-dependent calcium binding protein-like immunoreactivities. Brain Res. 496, 389–396.
Shepherd, G.M., Greer, C.A. 1998. Olfactory bulb. In: Shepherd, G.M. (Ed.), The Synaptic Organization of the Brain, Fourth ed.. Oxford University Press, New York, pp. 159–203.
Shipley, M.T., McLean, J.H., Ennis, M. 1995. Olfactory system. In: Paxinos, G. (Ed.), The Rat Nervous System, Second ed.. Academic Press, San Diego, pp. 899–926.
Simpson, C.G. 1945. The principles of classification and a classification of mammals. Bull. Am. Mus. Nat. Hist. 85, 1–350.
Somogyi, P., Hodgson, A.J. 1985. Antisera to gamma-aminobutyric acid. III. Demonstration of GABA in Golgi-impregnated neurons and in conventional electron microscopic sections of cat striate cortex. J. Histochem. Cytochem. 33, 249–257.
Stephan, H., Andy, O.J. 1970. The allocortes in primates. In: Noback, C.R., Montagna, W. (Eds.), The Primate Brain. Appleton-Century Crofts, New York, pp. 109–135.
Toida, K., Kosaka, K., Heizmann, C.W., Kosaka, T. 1994. Synaptic contacts between mitral/tufted cells and GABAergic neurons containing calcium-binding protein parvalbumin in the rat olfactory bulb, with special reference to reciprocal synapses between them. Brain Res. 650, 347–352.
Toida, K., Kosaka, K., Heizmann, C.W., Kosaka, T. 1996. Electron microscopic serial-sectioning/reconstruction study of parvalbumin-containing neurons in the external plexiform layer of the rat olfactory bulb. Neuroscience 72, 449–466.
Toida, K., Kosaka, K., Heizmann, C.W., Kosaka, T. 1998. Chemically defined neuron groups and their subpopulations in the glomerular layer of the rat main olfactory bulb: III. Structural features of calbindin D28K-immunoreactive neurons. J. Comp. Neurol. 392, 179–198.
Toida, K., Kosaka, K., Aika, Y., Kosaka, T. 2000. Chemically defined neuron groups and their subpopulations in the glomerular layer of the rat main olfactory bulb—IV. Intraglomerular synapses of tyrosine hydroxylase-immunoreactive neurons. Neuroscience 101, 11–17.
Valverde, F., López-Mascaraque, L. 1991. Neuroglial arrangements in the olfactory glomeruli of the hedgehog. J. Comp. Neurol. 307, 658–674.
Van Gehuchten, A., Martin, I. 1891. Le bulbe olfactif chez quelques mammiferes. La Cellule 5, 205–237.
Wang, H.L., Li, A., Wu, T. 1997. Vasoactive intestinal polypeptide enhances the GABAergic synaptic transmission in cultured hippocampal neurons. Brain Res. 746, 294–300.
White, D.M., Walker, S., Brenneman, D.E., Gozes, I. 2000. CREB contributes to the increased neurite outgrowth of sensory neurons induced by vasoactive intestinal polypeptide and activity-dependent neurotrophic factor. Brain Res. 868, 31–38.