Journal of Neuroscience Research 25:103–111 (1990)

Interspecies Differences in the Substance P- and Vasoactive Intestinal Polypeptide-like Immunoreactivities in the Olfactory Bulb of *Salmo gairdneri* and *Barbus meridionalis*

J.R. Alonso, R. Coveñas, J. Lara, M. de León, R. Arévalo, and J. Aijón
Cytology and Histology, Faculty of Biology, University of Salamanca, Spain

The distribution of substance P-like (SP) and vasoactive intestinal polypeptide-like (VIP) structures was studied using an indirect immunoperoxidase technique in the mediterranean barbel *Barbus meridionalis* and in the rainbow trout Salmo gairdneri. SP-like positive fibers were observed in the inner strata of the mediterranean barbel olfactory bulb, mainly the granule cell and the plexiform layers. Ganglion cells and fibers of the terminal nerve were also labeled. No SP-positive structure was found in the olfactory bulb of the rainbow trout. On the contrary, the VIP antiserum used displayed very strong immunostaining in the olfactory nerve fiber layer of *Salmo gairdneri*, whereas those fibers in *Barbus meridionalis* showed no immunoreactivity. After a complete transection of the olfactory tract and a survival time of 20 days, SP-immunostained fibers were not observed. Thus, they can presumably be identified as centrifugal fibers, coursing from the telencephalic hemispheres through the olfactory tracts, into the olfactory bulb. The VIP immunoreactivity was confined to the olfactory fibers, both in the olfactory nerve and the olfactory bulb. The positive immunostaining disappeared after chemical lesion of the olfactory mucosa. These observations demonstrate that the olfactory bulb of freshwater teleosts exhibits a high degree of heterogeneity in its immunocytochemical distribution pattern, this pattern also differing from previous reports on higher vertebrates.

Key words: olfactory bulb, immunocytochemistry, teleost

INTRODUCTION

The olfactory bulb is an excellent model system in which to study the functions of neuropeptides and their interactions with other neuroactive substances in the CNS, owing to its clear lamination and established neuronal typology, our understanding of its synaptic organization, easy access to experimental analysis, and the richness, in amounts and in variety, of neuropeptides and other neurotransmitter candidates identified on it, (Burd et al., 1982a; Hálasz and Shepherd, 1983). Thus, the olfactory bulb has been object of numerous biochemical and immunocytochemical studies (see Margolis, 1981; Macrides et al., 1982; Hálasz and Shepherd, 1983, for reviews). However, all these studies have been carried out in the mammalian olfactory bulb and there are scarce data on the olfactory bulb of lower vertebrates.

It has been reported (Allison, 1953; Andres, 1970) that the basic structure of the olfactory bulb is common in all vertebrates. However, there are some recent works (Kosaka and Hama, 1979, 1982, 1982–1983; Alonso et al., 1987, 1988) indicating that, in addition to its less laminated structure, new neuronal types such as ruffed cells or perinest cells, and variations of neuronal types such as granule cells or mitral cells, are present in the teleostean olfactory bulb. Moreover, other neurons described in mammals, such as tufted cells or periglomerular cells, have not been observed in the fish olfactory bulb. Thus, it has been suggested that the teleost olfactory bulb and that of mammals have followed somewhat different evolutionary directions (Kosaka and Hama, 1982). This makes it necessary to establish whether the pattern of distribution of neuroactive substances is similar between the olfactory bulb of higher and lower vertebrates.

During the course of our continuing effort to characterize the neuronal organization of the teleost olfactory bulb, we have tested the possible immunoreactivity of this structure to several neuropeptides, including substance P, vasoactive intestinal polypeptide, angiotensin II, neuropeptide Y, motilin, cholecystokinin-8, methionine-enkephalin, and leucine-enkephalin.

Received January 30, 1989; accepted June 30, 1989.

Address reprint requests to Dr. J. Aijón, Citología e Histología, Facultad de Biología, Plaza de la Merced s/n, 37008 Salamanca, Spain.

In the present report, we have centered on substance P and vasoactive intestinal polypeptide immunoreactivities since a) both substances have been well characterized in the mammalian olfactory bulb, b) antisera against them showed clear-cut immunostaining patterns, and c) the distribution of immunolabeling for both substances displayed a high degree of interspecies variability.

Substance P (SP), an undecapeptide discovered by Euler and Gaddum (1931), is distributed in different tissues and organs such as the intestine, ovary, salivary glands, autonomic nerves, solitary glands and different regions of the CNS (Cuello and Kanazawa, 1978; Dees et al., 1985; Szabo et al., 1987; Vecino et al., 1989). In the olfactory bulb, the presence of SP has been detected using biological tests, radioimmunoassay, pharmacological, and autoradiographic studies on SP-receptors, and immunocytochemical techniques (Pernow, 1953; Amin et al., 1954; Brownstein et al., 1976; Kanazawa and Jessel, 1976; Hökfelt et al., 1975; Ljungdahl et al., 1978; Inagaki et al., 1982; Kream et al., 1984; Shults et al., 1985; Baker; 1986; Dam and Quirion, 1986; Szabo et al., 1987). These latter techniques have demonstrated different immunolabeled structures in the CNS, between different species, even in a phylogenetically close group of species such as the rodents, suggesting high interspecific variability (Baker, 1986). In a recent report (Szabo et al., 1987), SP-immunoreactivity has been observed in the olfactory and electrosensory cells of gymnotid fish, whereas other two species, *Gnathonemus petersii* and the cypriniform *Carassius auratus,* showed no positive labeling in the olfactory bulb.

Vasoactive intestinal polypeptide (VIP), isolated by Said and Mutt (1970), is another neuropeptide with a broad functionality. It might interact with catecholamine systems; regulate the release of certain pituitary hormones, control the glucose metabolism of cortical cells, and act in neural transmission playing a role in the olfactory system (Lorén et al., 1979; Mutt, 1983). In the olfactory bulb of mammals, VIP has been detected using radioimmunoassay (Lorén et al., 1979; Fahrenkrug, 1980) and immunocytochemical techniques (Gall et al., 1986). However, there are no data on the distribution of VIP in the olfactory bulb of lower vertebrates.

The aim of the present work was to examine the distributions of SP and VIP in the olfactory bulb of two species of teleosts, *Barbus meridionalis* and *Salmo gairdneri,* comparing them between both species and with previous data in higher vertebrates. Both teleost species are phylogenetically distant and the anatomy of their olfactory bulbs is different. Our conclusion may provide further information on the localization of neurotransmitters within the olfactory system of lower vertebrates, on the interspecific variations inside the teleosts, and on the presence and distribution of neuroactive substances on the phylogenetic scale.

MATERIALS AND METHODS

Twelve adult mediterranean barbels (*Barbus meridionalis* Risso) with 320–540 g body weight, and 12 adult rainbow trouts (*Salmo gairdneri* Richardson) with body weight of 210–260 g, were used for the present study. The mediterranean barbels were captured in the river Tormes (Salamanca) and the rainbow trouts were obtained from commercial sources (Fisheries "La Flecha," Salamanca). They were kept under standard laboratory conditions (12/12 hours light-dark cycle).

Six specimens of *Barbus meridionalis* were subjected to a complete transection of the olfactory tracts. After anesthesia with 0.03% tricaine methanesulfonate (MS-222, Sandoz), a flap was opened in the skull, using a dental drill, the overlying fatty tissue was aspirated, and the olfactory tracts were exposed. They were completely sectioned using a blade. The flap was then closed and the animals allow to survive for 5, 10, 15, or 20 days. The olfactory epithelia of six specimens of *Salmo gairdneri* were ablated with a 0.15 M solution of $ZnSO_4$ in distilled water, as previously described (Lidow et al., 1987). This procedure was repeated throughout the week before sacrifice. After this, the lesioned and the nonoperated fish (barbels and trouts) were perfused transcardially, after anesthesia with 0.03% MS-222, with 20–40 ml of Teleost Ringer solution followed by 250 ml of a fixative containing 4% paraformaldehyde in 0.12 M phosphate buffer, pH 7.3. The olfactory bulbs were removed and postfixed overnight in the same fixative solution. Finally, they were rinsed several times in phosphate buffer.

Longitudinal sections (40 μm) of the olfactory bulbs were cut using a vibratome (Campden Instruments), washed for 36 h in several changes of phosphate buffer, and processed for immunostaining as free-floating sections.

The sections were incubated for 24 h at 4°C in antiserum against VIP (Cambridge Research Biochemicals, Cambridge, U.K.) diluted at 1:1000, or for 48 h at 12°C in anti-Substance P antiserum also diluted at 1:1000 (a gift from Dr. R.E. Rodríguez). The sections were preincubated for 30 min in 0.3% Triton X-100 in phosphate buffer 0.1 M with 1% normal sheep serum to enhance antibody penetration. Anti-rabbit IgG coupled to horseradish peroxidase (Pasteur) was used as a second antibody. Dilution was also 1:1000 and the IgG was incubated at 20°C for 4 h. Tissue-bound peroxidase was visualized by incubating the sections with 0.07% 3,3′ diaminobenzidine in Tris buffer (0.1 M, pH 7.6) for 5–10 min.

SP and VIP antisera at the working dilution 1:1000 were immunoabsorbed for 1 h at 37°C and 24 h at 4°C with SP (0.1 mg/ml, Peninsula Lab. Inc.) and VIP (0.1 mg/ml, Peninsula Lab. Inc.). The specificity of the immunostaining was also tested by omitting the SP and the VIP antibodies in the first incubation bath. In no case was immunostaining observed.

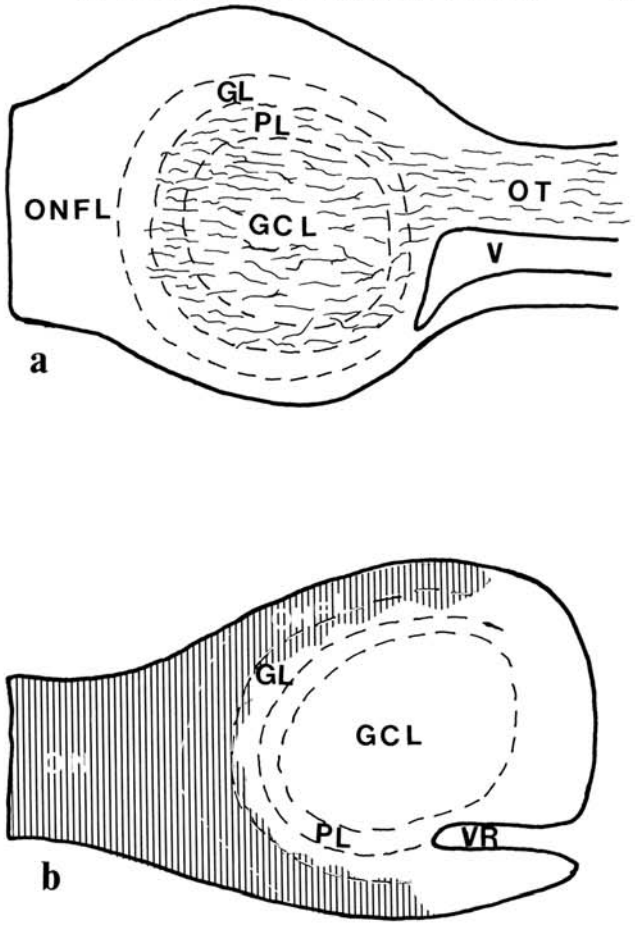
Antisera against angiotensin II, neuropeptide Y, motilin, cholecystokinin-8, methionine-enkephalin and leucine-enkephalin were also tested. However, although a positive immunostaining was observed for some of them, there were no interspecies differences between the immunolabelling in *Salmo gairdneri* and *Barbus meridionalis*.

Finally, we use the term peptide-like (SP-like or VIP-like) immunoreactivity to describe the staining in our material. "Peptide-positive" or "peptide-containing" are used as synonyms of peptide-like immunoreactivity. This is because, in spite of all the controls carried out, we cannot exclude the possibility of the reactive material present in our experimental tissue being immunologically related, but not identical, to the substances under study.

RESULTS

The location of the olfactory bulb is one of the most striking anatomical differences between the brain of *Salmo gairdneri* and that of *Barbus meridionalis*. Thus, in the rainbow trout, the olfactory bulbs are apposed to the telencephalic hemispheres, extending the olfactory nerves for several centimeters, the olfactory tracts being internal and very short. By contrast, the olfactory bulb of *Barbus meridionalis* is adjacent to the olfactory mucosa, the olfactory nerves being very short and the secondary pathway and the olfactory tracts very long. However, according to control techniques, although with small dissimilarities the laminar organization and the neuronal typology seems to be equivalent for both species. In this sense, from the outermost to the innermost, four layers were distinguished in both species: olfactory nerve fiber layer, glomerular layer, plexiform layer, and granule cell layer (Fig. 1). The ventricles of the olfactory bulb showed a different development in *Barbus meridionalis* (Fig. 1a) and in *Salmo gairdneri* (Fig. 1b). In any case, the cytoarchitecture of the olfactory bulb in both species studied is easily comparable.

The pattern of immunoreactivities was completely different between *Barbus meridionalis* and *Salmo gairdneri* for both neuropeptides studied. Numerous SP-immunoreactive fibers were seen in the olfactory bulb of the mediterranean barbel (Fig. 2a,b). No immunolabeling was observed in the olfactory bulb of the rainbow trout. The SP-containing fibers were mainly distributed



1

Fig. 1. Schematic drawings showing the laminar organization of the olfactory bulb of *Barbus meridionalis* (**a**) and *Salmo gairdneri* (**b**). Continuous lines in **a** shows the location of SP-immunoreactivity . Labeled zone in **b** shows the location of VIP-immunoreactivity. GL, glomerular layer; GCL, granule cell layer; ON, olfactory nerve, ONFL, olfactory nerve fiber layer; OT, olfactory tract; PL, Plexiform layer; V, ventricle; VR, ventricular recess.

in the granule cell layer, where they formed a dense plexus. They were also observed in the plexiform layer and, very rarely, in the inner portions of the glomerular layer. The fibers were varicose, small-caliber axons, frequently ramified in the granule cell layer, and were not observed to form pericellular or peridendritic terminal arrays. Ganglion cells and prolongations of the terminal nerve were also positively labeled for SP. However, these neurons and prolongations, which constitute a pathway independent of the olfactory system, were easily distinguished from the above-described SP-immunostained fibers.

The olfactory tract lesions were carried out in order to elucidate whether these latter SP-immunolabeled fibers may be identified as centrifugal fibers, coursing

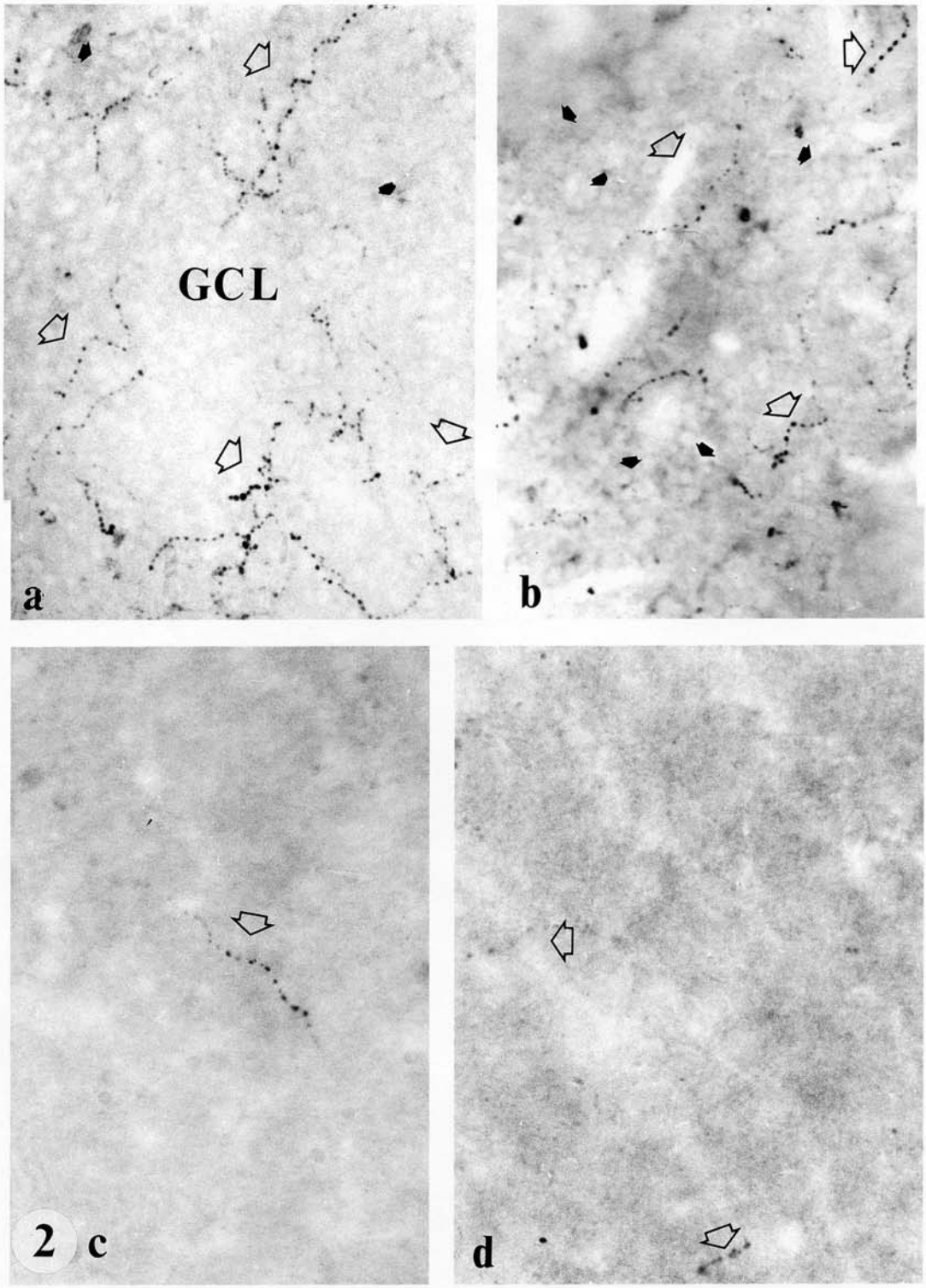


Fig. 2. Sections of the olfactory bulb of *Barbus meridionalis* immunostained for SP. **a**: SP-immunoreactive fibers (open arrows) in the granule cell layer (GCL). ×85. **b**: Section of the olfactory bulb, after olfactory tract transection and 5 days survival time. Note that abundant SP-positive fibers (open arrows) can be observed. ×190. **c**: Section of the olfactory bulb, after olfactory tract transection and 15 days survival time. The SP-positive fibers have practically disappeared and only isolated profiles (open arrows) can be observed. ×425. **d**: Section of the olfactory bulb, after olfactory tract transection and 20 days survival time. Only very weakly immunoreactive and presumably degenerated profiles (open arrows) can be observed. ×425. (Black arrows points to groups of SP-immunonegative granule cells.)

from the telencephalic hemispheres through the olfactory tracts, or whether they belong to interneurons or projecting neurons of the olfactory bulb, such as mitral cells or ruffed cells. After the survival times, which are assumed to originate anterograde degeneration, the great majority of the SP-containing fibers had disappeared (Fig. 2c) and only very scarce profiles which were not possible to follow for a long distance were seen in the granule cell layer (Fig. 2d). In addition, this disappearance of SP-fibers is gradual throughout the different survival times used: 5, 10, 15, and 20 days (Fig. 2). Moreover, in the normal material, the SP-positive fibers were observed coursing in the olfactory tracts and in the telencephalic hemispheres. Finally, no SP-immunoreactive perikarya belonging to the olfactory system were evident in the olfactory bulb of both species.

However, the used antiserum against VIP employed displayed a clear-cut distribution of strong immunostaining in the olfactory bulb of the rainbow trout *Salmo gairdneri* (Fig. 3a), the same antiserum showing no immunoreactivity in the olfactory bulb of the cyprinoid *Barbus meridionalis*. In *Salmo gairdneri,* VIP immunoreactivity was confined to the olfactory fibers in the olfactory nerve, the olfactory nerve fiber layer, and their terminal zone in the glomerular layer (Fig. 3a,b). The olfactory fibers were very strongly labeled, it being possible to follow their courses when they penetrate in different bundles into the glomeruli where they arborize (Fig. 3c,d). A detailed description of the immunostaining of olfactory fibers with different antibodies is in preparation. Other components located in the same region such as displaced neurons, the terminal nerve, and glial cells were not stained, and no immunoreactivity was observed in deeper strata. Finally, the chemical lesions produced in the olfactory epithelia of the rainbow trout, originating a coagulation necrosis of the olfactory receptors, led to disappearance of the VIP-positive immunostaining of the olfactory fibers.

DISCUSSION

We have described interspecific differences in the distribution pattern of SP- and VIP-immunoreactivities in the olfactory bulb of two species of freshwater teleosts. Our observations are not easily comparable with previous studies in which very different immunoreactivity distribution patterns have been described, since Szabo et al. (1987) reported that SP-labeling was located in the olfactory and electrosensory cells. In the olfactory bulb, the pattern of distribution includes the olfactory fibers and their terminal zone in the glomerular layer. This is the same distribution that we have described for the VIP in the rainbow trout olfactory bulb. Moreover, no SP-immunoreactivity was found by these authors in the olfactory bulb of other two teleostean species: *Carassius auratus* and *Gnathonemus petersii* (Szabo et al., 1987). However, our results revealed an additional species, *Salmo gairdneri,* with no positive SP-labeling in its olfactory bulb, and another species, *Barbus meridionalis,* whose SP-distribution pattern is completely different, being restricted to axons located in inner strata of the olfactory bulb, and presumably identified as centrifugal fibers. It is important to note that *Barbus meridionalis* and *Carassius auratus,* where such differences were observed, are phylogenetically close species, both of them belonging to the Order Cypriniforms, family Cyprinidae.

The VIP-labeling in the olfactory fibers of *Salmo gairdneri* coincides with the distribution of SP in the olfactory bulb of four species of gymnotid fish (Szabo et al., 1987), and with the distribution of somatostatin in *Gasterosteus aculeatus* (Honkanen and Ekström, 1988). This heterogeneity in the immunoreactive of the olfactory fibers is even higher since there is considerable evidence that carnosine might be a putative transmitter for the olfactory fibers of the mammalian olfactory bulb (Sakai et al., 1988), and a similar pattern of immunocytochemical staining has been observed for the olfactory marker protein (Monti Graziadei et al., 1977) and β-alanine (Burd et al., 1982b). Data are lacking about possible colocalization of several neurotransmitters in the teleostean olfactory fibers. In some areas of the mammalian CNS, SP has been shown to be colocalized with other neurotransmitters within individual neurons (Chan-Palay et al., 1978; Hökfelt et al., 1978). Although this possibility was not directly tested in the present report, the described distribution pattern of SP- and VIP-immunoreactive patterns does not suggest a colocalization of both peptides in the species studied. Thus, our report demonstrates that SP is not observed in the *Salmo gairdneri* olfactory bulb and similarly VIP is not found in the olfactory bulb of *Barbus meridionalis*. It is therefore possible to conclude that the olfactory fibers, although they have similar structural and ultrastructural characteristics (Alonso et al., 1989), display a very high interspecific variability in their immunocytochemical distribution pattern.

There are abundant data about the localization of SP in the mammalian olfactory bulb. Ljungdahl et al. (1978) reported single SP-positive, varicose nerve terminals distributed in all layers of the main olfactory bulb in the rat, but no SP-positive perikarya. However, other authors such as Inagaki et al. (1981, 1982) in the rat and frog, and Davis et al. (1982) in the hamster have described intrabulbar SP-positive cell bodies. The majority of these perikarya were found in the glomerular layer. This is the case of all the labeled cells in the hamster (Davis et al., 1982), whereas in the frog a few positive neurons were localized in the mitral and granule cell

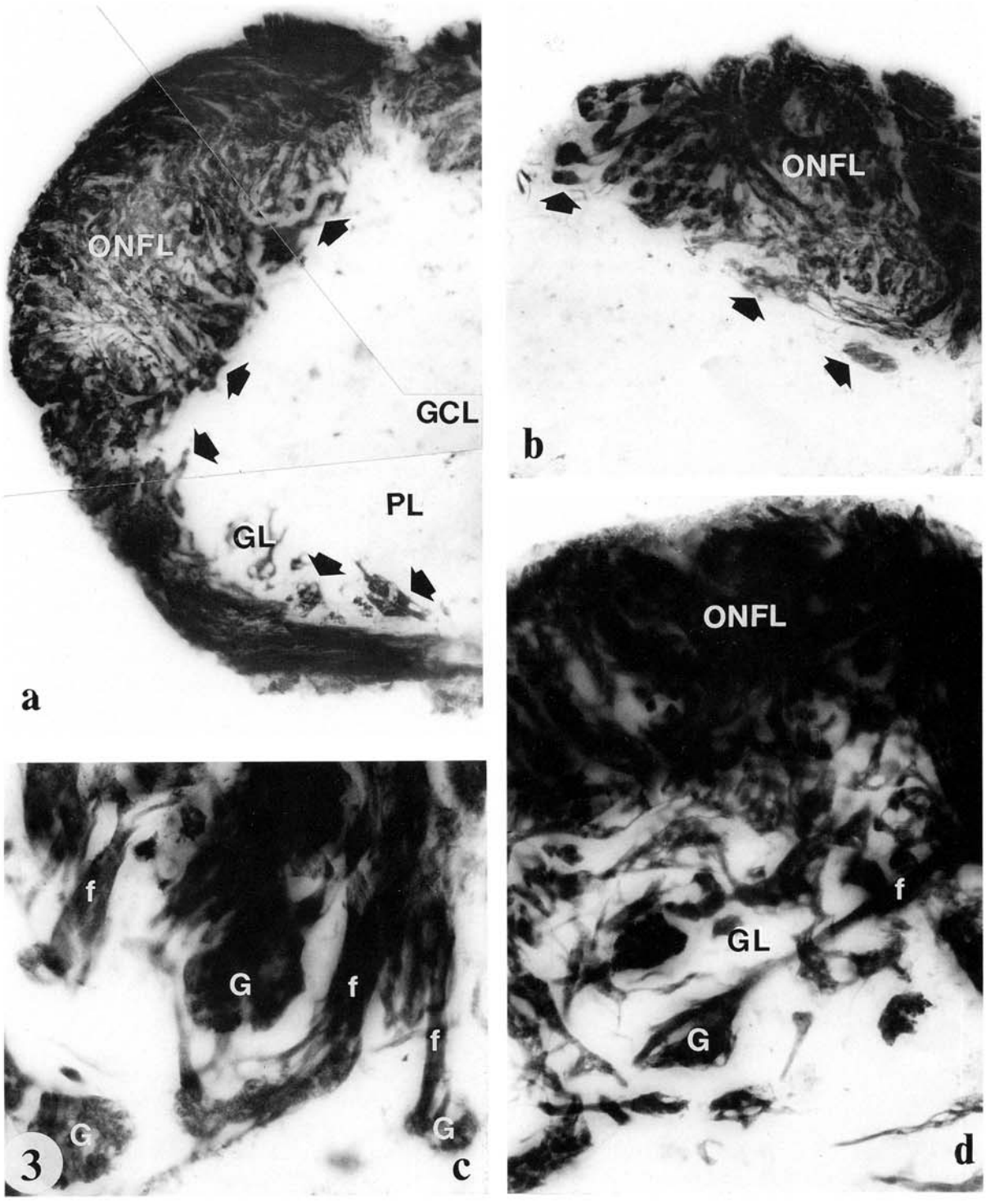


Fig. 3. Sections of the olfactory bulb of *Salmo gairdneri* immunostained for VIP. **a**: Section of the olfactory bulb showing strong VIP-positive labeling in the olfactory nerve fiber layer and glomerular layer. ×275. **b**: Bundles of immunostained fibers (black arrows) entering into the glomerular layer can be observed. ×275. **c–d**: Higher magnifications showing VIP-immunostained fascicles of olfactory fibers ending on glomerular structures. ×515. f, olfactory fibers; G, glomerular structures; GL, glomerular layer; GCL, granule cell layer; ONFL, olfactory nerve fiber layer; PL, plexiform layer.

layer. Labeled neurons included external tufted cells and superficial short-axon cells (Davis et al., 1982). Neither neuronal type has been described in the teleostean olfactory bulb. In the accessory olfactory bulb, which has sometimes been compared with the olfactory bulb of lower vertebrates, labeling of cells and/or processes has been reported in the rat (Inagaki et al., 1982), whereas only processes were seen to be labeled in the hamster (Davis and Macrides, 1983).

The results of experimental lesions described in our report suggest that the SP-positive fibers in the olfactory bulb of the mediterranean barbel are centrifugal fibers in view of the loss of immunostaining throughout the survival time. Moreover, their morphological characteristics and localization in the olfactory bulb coincide with previous observations based on Golgi material (Sheldon, 1912), as well as with their distribution in the olfactory bulb using electron microscopic degeneration techniques (Ichikawa and Ueda, 1979). In addition, we observed SP-containing fibers in the olfactory tracts and the telencephalic hemispheres. These findings could be an explanation for the absence of SP-immunolabeled perikarya originating these fibers in the olfactory bulb. Our suggestion is in agreement with previous studies in the mammalian olfactory bulb since centrifugal fibers containing SP, methionine-enkephalin, and somatostin have also been detected in the hamster (Davis et al., 1982). These fibers pass diffusely through the olfactory tract, entering directly into the granule cell layer, as we have reported in the mediterranean barbel. This homology with a mammalian species is interesting since no immunolabeling in this region has been found in previous reports (Szabo et al., 1987), or in the other teleostean species studied by us, *Salmo gairdneri*.

SP is mainly considered to act in pain transmission (Henry, 1976); however, its functionality in the olfactory bulb is unknown (Burd et al., 1982a). For several authors, SP plays a role as a primary afferent sensory transmitter (Otsuka and Konishi, 1977). However, other observations suggest that SP acts rather as part of a modulating chain of neurotransmitters. Thus, several studies have demonstrated that dopamine, GABA, and methionine-enkephalin inhibit the release of SP (Jessel, 1978; Mudge et al., 1979; Kelley, 1978), the SP-effects being exerted through an interaction with other endogenous peptides (Davies and Dray, 1975; Heike et al., 1986). In the mammalian olfactory bulb, its location on tufted cells suggests an excitatory effect on periglomerular cells. This is in good agreement with other studies proposing an excitatory function for SP in other neuronal systems (Phillis, 1980). Finally, the centrifugal fibers that are shown as SP-positive in the olfactory bulb of *Barbus meridionalis* are considered to exert an excitatory function on the granule cells of the olfactory bulb (Price, 1968; Price and Powell, 1970), which correlates with the proposed excitatory function for SP (Otsuka and Konishi, 1977).

There are no data on the presence of VIP in the olfactory fibers of higher vertebrates. However, the structure and ultrastructure of the olfactory fibers (Alonso et al., 1989) and the VIP amino acid sequence have been highly conserved during evolution (Dimaline et al., 1987). In this way, the strong immunoreactivity shown by the olfactory fibers and the inexistent background of all other components of the olfactory bulb point to a high specificity of the immunostaining in the teleostean olfactory bulb.

VIP has been observed in the rat and mouse olfactory bulb using radioimmunoassay (Lorén et al., 1979) and immunocytochemical techniques (Gall et al., 1986). The latter techniques allow one to identify VIP-immunoreactivity localized within neuronal perikarya scattered across the full depth of the external plexiform layer and varicose processes also in this stratum. More rarely, VIP-positive neurons were observed in the mitral cell layer, the granule cell layer and the external plexiform layer/glomerular layer border region. The VIP-immunostained neurons were identified as superficial short-axon cells such as the Van Gehuchten cells, superficial tufted cells, and deep short-axon cells such as the vertical cells of Cajal and the horizontal cells (Gall et al., 1986). Thus, in the rat olfactory bulb, VIP-immunostained elements are in a position to modulate olfactory bulb output via the modulation of the granule cells and, consequently, of the mitral cells. It should be noted that tufted cells, and the described variants of short-axon cells have not been observed in the teleost olfactory bulb.

VIP displays diverse biological actions such as vasodilatation of various vascular beds (including pial vessels), smooth muscle relaxation, stimulation of exocrine, and endocrine pancreatic secretion and also of water and ion excretion from the intestinal tract, activation of glycogenolysis in hepatocytes, and cerebral cortex slices. Finally, it plays a role in intercellular communication (Magistretti, 1986). All these observations suggest a major role for VIP in the coupling between energy metabolism, blood flow and neuronal activity (Magistretti and Morrison, 1985). In the CNS, it has been suggested (Lorén et al., 1979) that VIP is involved in cortical and limbic circuitries. Thus, Leranth et al. (1984) have reported that the VIP-positive hippocampal neurons are probably local interneurons. This agrees well with reports on the VIP positive neurons in the rat olfactory bulb (Gall et al., 1986). In the rainbow trout, it is evident that the VIP-like immunoreactive elements in the olfactory bulb (the olfactory fibers) are not modulator elements. This suggests that VIP might play a role in the transmission of the olfactory input from the olfactory mucosa to

the main second order neurons of the olfactory bulb, the mitral cells.

It cannot be ruled out that after treatment with intraventricular or intratissue injections of colchicine or using antibodies raised in phylogenetically closer species, new immunoreactive structures, fibers, and/or cell bodies may be labeled in the olfactory bulb of the freshwater teleosts studied. However, the different controls, the strong immunoreactivity of the labeled elements and the practically inexistent background, indicate a high specificity. Additionally, our study used the same antibodies, fixatives, localization procedures, and simultaneous processing for the olfactory bulb sections from *Salmo gairdneri* and *Barbus meridionalis*. Thus, they are true interspecific variations, and other factors such as variation in antibody sensitivity or the localization technique can be excluded. The possibility that such differences may be related to the physiological transduction of chemical inputs in the olfactory mucosa is an attractive hypothesis.

ACKNOWLEDGMENTS

This work was supported by grants from the Junta de Castilla y León (Spain) and the Ministerio de Educación y Ciencia (PM88-0154) (Spain).

LITERATURE CITED

Allison AC (1953): The morphology of the olfactory system in the vertebrates. Biol Rev 28:195–244.

Alonso JR, Lara J, Miguel JJ, and Aijón J (1987): Ruffed cells in the olfactory bulb of freshwater teleosts. I. Golgi impregnation. J Anat 155:101–107.

Alonso JR, Lara J, Coveñas R, and Aijón J (1988): Two types of mitral cells in the teleost olfactory bulb. Neurosci Res Comm 3:113–118.

Alonso JR, Lara J, Coveñas R, Vecino E, Piñuela C, and Aijón J (1989): The outer fibrilary layer in the olfactory bulb of freshwater teleosts. Arch. Biol. in press

Amin AH, Crawford TBB, and Gaddum JH (1954): The distribution of substance P and 5-hydroxytryptamine in the central nervous system of the dog. J Physiol (London) 126:596–618.

Andres KH (1970): Anatomy and ultrastructure of the olfactory bulb in fish, amphibia, reptiles, birds and mammals. In Wolstenholme GEW, Knight J (eds.): "Ciba Foundation Symposium on Taste and Smell in Vertebrates." London: Churchill, pp. 177–196.

Baker H (1986): Species differences in the distribution of substance P and tyrosine hydroxylase immunoreactivity in the olfactory bulb. J Comp Neurol 252:206–226.

Brownstein MJ, Mroz EA, Kizer JS, Palkovits M, and Leeman SE (1976): Regional distribution of substance P in the brain of the rat. Brain Res 116:299–305.

Burd GD, Davis BJ, and Macrides F (1982a): Ultrastructural identification of substance P immunoreactive neurons in the main olfactory bulb of the hamster. Neuroscience 7:2697–2704.

Burd GD, Davis BJ, Macrides F, Grillo M, and Margolis FL (1982b): Carnosine in primary afferents of the olfactory system: An autoradiographic and biochemical study. J Neurosci 2:244–255.

Chan-Palay V, Jonsson G, and Palay S (1978): Serotonin and substance P coexist in neurons of the rat's central nervous system. Proc Natl Acad Sci USA 75:1582–1586.

Cuello AC, and Kanazawa I (1978): The distribution of substance P immunoreactive fibers in the rat central nervous system. J Comp Neurol 178:129–156.

Dam TV, and Quirion R (1986): Pharmacological characterization and autoradiographic localization of substance P receptors in guinea pig brain. Peptides 7:855–864.

Davies J, and Dray A (1975): Substance P and opiate receptors. Nature 268:351–352.

Davis BJ, Burd GD, and Macrides F (1982): Localization of methionine-enkephalin, substance P and somatostatin immunoreactivities in the main olfactory bulb of the hamster. J Comp Neurol 181:213–244.

Davis BJ, and Macrides F (1983): Tyrosine hydroxylase immunoreactive neurons and fibers in the olfactory system of the hamster. J Comp Neurol 214:427–440.

Dees WL, Skelley CW, and Kozlowski GP (1985): Central effects of an antagonist and an antiserum to substance P on serum gonadotropin and prolactin secretion. Life Sci 37:1627–1631.

Dimaline R, Young J, Thwaites DT, Lee CM, Shuttleworth TJ, and Thorndyke MC (1987): A novel vasoactive intestinal peptide (VIP) from elasmobranch intestine has full affinity for mammalian pancreatic VIP receptors. Biochim Biophys Acta 930: 97–100.

Euler US von, and Gaddum JH (1931): An unidentified depressor substance in certain tissue extracts, J Physiol (London) 72: 74–87.

Fahrenkrug J (1980): Vasoactive intestinal polypeptide. Trends Neurosci 3:1–4.

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

Halász N, and Shepherd GM (1983): Neurochemistry of the vertebrate olfactory bulb. Neuroscience 3:579–619.

Heike Y, Hisano S, Tsuruo Y, Katoh S, and Daikoku S (1986) Immunocytochemical evidence for synaptic regulation of paraventricular vasopressin-containing neurons by substance P. Brain Res 369:341–346.

Henry JL (1976): Substance P and pain: An updating. Trends Neurosci 3:95–97.

Hökfelt T, Kellerth JO, Nilsson G, and Pernow B (1975): Substance P: localization in the central nervous system and in some primary sensory neurons. Science 1980:889–890.

Hökfelt T, Ljungdahl A, Steinbusch H, Verhofstad A, Nilsson G, Brodin E, Pernow B, and Goldstein M (1978): Immunohistochemical evidence of substance P-like immunoreactivity in some 5-hydroxytryptamine-containing neurons in the rat central nervous system. Neuroscience 3:517–538.

Honkanen T, and Ekström P (1988): Labelling of olfactory epithelium and olfactory fibers in *Gasterosteus aculeatus* L., Teleostei. Eur. J. Neurosci. Suppl. 1:22.

Ichikawa M, and Ueda K (1979) Electron microscopic study of the termination of the centrifugal fibers in the goldfish olfactory bulb. Cell Tissue Res 197:257–262.

Inagaki S, Sakanaka M, Shiosaka S, Senba E, Takatsuki K, Takagi H, Kawai Y, Minagawa H, and Tohyama M (1982): Ontogeny of substance P-containing neuron system of the rat: Immunohistochemical analysis. I. Forebrain and upper brain stem. Neuroscience 7:251–277.

Inagaki S, Senba E, Shiosaka S, Takatsuki K, Sakanaka M, Matsuzaki

T, and Tohyama M (1981): Regional distribution of substance P-like immunoreactivity in the frog brain and spinal cord: Immunohistochemical analysis. J Comp Neurol 201:243–254.

Jessel TM (1978) Substance P release from the rat substantia nigra. Brain Res 151:469–478.

Kanazawa I, and Jessel T (1976): Postmortem changes and regional distribution of substance P in the rat and mouse nervous system. Brain Res 117:362–367.

Kelley AE (1978): Behavioral response to bilateral injections of substance P into the substantia nigra of the rat. Brain Res. 158: 474–478.

Kosaka T, and Hama K (1979): A new type of neuron with a distinctive axon initial segment. Brain Res 163:119–145.

Kosaka T, and Hama K (1982): Structure of the mitral cell in the olfactory bulb of the goldfish (*Carassius auratus*). J Comp Neurol 212:365–384.

Kosaka T, and Hama K (1982–1983): Synaptic organization in the teleost olfactory bulb. J Physiol (Paris) 78:707–719.

Kream RM, Davis BJ, Kawano T, Margolis FL, and Macrides F (1984): Substance P and catecholaminergic expression in neurons of the hamster main olfactory bulb. J Comp Neurol 222: 140–154.

Leranth C, Frotscher M, Tombol T, and Palkovits M (1984): Ultrastructure and synaptic connections of vasoactive intestinal polypeptide-like immunoreactive non-pyramidal neurons and axon terminals in the rat hippocampus. Neuroscience 12:531–542.

Lidow MS, Gesteland RC, Shipley MT, and Keene SJ (1987): Comparative study of immature and mature olfactory receptor cells in adult frogs. Dev Brain Res 31:243–258.

Ljungdahl A, Hökfelt T, and Nilsson G (1978): Distribution of substance P-like immunoreactivity in the central nervous system of the rat. I. Cell bodies and nerve terminals. Neuroscience 3: 861–943.

Lorén I, Emson PC, Fahrenkrug J, Björklund A, Alumets J, Hakanson R, and Sundler F (1979): Distribution of vasoactive intestinal polypeptide in the rat and mouse brain. Neuroscience 4:1953–1976.

Macrides F, Davis BJ, and Burd GD (1982): Neuropeptide systems in the olfactory bulbs. In Breipohl W (ed.): "Olfaction and Endocrine Regulation." London: IRL Press, pp. 83–91.

Magistretti PJ (1986): Intercellular communication mediated by VIP in the cerebral cortex. Peptides 7:169–173.

Magistretti PJ, and Morrison JH (1985): VIP neurons in the neocortex. Trends Neurosci. 8:7–8.

Margolis FL (1981): Neurotransmitter biochemistry of the mammalian olfactory bulb. In Cagan RH, Hare MR (eds.): Biochemistry of Taste and Olfaction." New York: Academic Press, pp. 369–394.

Monti Graziadei GA, Margolis FL, Harding JW, and Graziadei PPC (1977): Immunocytochemistry of the olfactory marker protein. J Histochem Cytochem 25:1311–1316.

Mudge AW, Leeman SE, and Fischbach GD (1979): Enkephalin inhibits release of substance P from sensory neurons in culture and decreases action potential duration. Proc Natl Acad Sci USA 76:526–530.

Mutt V (1983): VIP, motilin and secretin. In: Brain Peptides. Krieger DT, Brownstein MJ, Martin JB (eds.): John Wiley & Sons, New York: pp. 871–901.

Otsuka M, and Konishi S (1977): Electrophysiological and neurochemical evidence for substance P as a transmitter of primary sensory neurons. In Euler ES von, Pernow B (eds.): "Substance P." New York: Raven Press, pp. 1922–1928.

Pernow B (1953): Studies of substance P. Purification, occurrence, and biological actions. Acta Physiol Scand 29 (Suppl)105:1–90.

Phillis JW (1980): Substance P in the central nervous system. In: Barker JL, Smith TG Jr (eds.): "The Role of Peptides in Neuronal Function." New York: Marcell Dekker Inc., pp. 615–652.

Price JL (1968): The termination of centrifugal fibers in the olfactory bulb. Brain Res 7:483–486.

Price JL, and Powell TPS (1970): An experimental study of the site of origin and the course of the centrifugal fibers to the olfactory bulb in the rat. J Anat 107:215–237.

Said SI, and Mutt V (1970): Polypeptide with broad biological activity: Isolation from small intestine. Science 169:1217–1218.

Sakai M, Kani K, Karasawa N, Yoshida M, and Nagatsu I (1988): Carnosine-like immunoreactivity in the olfactory bulb of the rat: an electron microscopic study. Brain Res 438:335–338.

Sheldon E (1912): The olfactory tracts and centers in teleosts. J Comp Neurol 22:177–339.

Shults CW, Quirion R, Chronwall B, Chase TN, and O'Donahue TL (1985): A comparison of the anatomical distribution of substance P and substance P receptors in the rat central nervous system. Peptides 5:1097–1128.

Szabo T, Bläser S, Denizot JP, and Véron-Ravaille M (1987): High substance P-like immunoreactivity (SPLI) in the olfactory and electrosensory cells of gymnotid fish. Neurosci Lett 81:245–249.

Vecino E, Coveñas R, Alonso JR, Lara J, and Aijón J (1989): Immunocytochemical study of substance P-like cell bodies and fibers in the brain of the rainbow trout, *Salmo gairdneri*. J. Anat. in press