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Distribution of vasoactive intestinal polypeptide-like immunoreactivity in the olfactory bulb of the rainbow trout (*Salmo gairdneri*)

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The distribution of vasoactive intestinal polypeptide-like structures in the olfactory bulb of the rainbow trout was studied using an indirect-immunoperoxidase technique. Olfactory fibres were very strongly labelled, whereas the fibres or cell bodies in the remaining strata of the olfactory bulb showed no immunoreactivity. In addition, the olfactory nerve fibres were not immunoreactive for methionine- and leucine-enkephalins, motilin, neuropeptide Y, substance P, cholecystokinin-8 and tyrosine-hydroxylase.

The vasoactive intestinal polypeptide (VIP) originally isolated from porcine gastrointestinal tract[28] has been detected in several regions of the mammalian central nervous system using biochemical or immunocytochemical techniques[7,10,12–14,16,19–21,26,27,29,32,35]. These studies have pointed to some interspecific differences with respect to the distribution of VIP even in phylogenetically close species[21,24,26,35]. All of these studies were carried out in the brain of mammals, but no data are available on the localisation of VIP-like structures in the CNS of lower vertebrates.

It has further been pointed out that VIP might act in neural transmission: interact with catecholamine systems; influence the release of certain pituitary hormones; regulate the glucose metabolism of cortical cells; regulate blood flow, and also play a role in the olfactory system[21,23]. VIP has been detected in the rat olfactory bulb by radioimmunoassay[11,21] and immunocytochemical techniques[14]. Using the latter methodology, VIP immunoreactivity has been observed in intermediate-sized neurones in the external plexiform layer, in small short-axon neurones in the mitral cell layer and the granule cell layer, and in a few large neurones in the glomerular layer/external plexiform layer border[14]. However, the lamination in the teleostean olfactory bulb is different[1,5,6]. New neuronal types[3,17,18] or variations of neuronal types[4] have been described and other neuronal types observed in higher vertebrates, such as tufted cells or glomerular cells, have not been found in the teleost olfactory bulb. Thus, it seems necessary to establish whether the pattern of distribution of immunoreactivities is similar between the mammalian and teleostean olfactory bulb.

The aim of the present work was to examine the distribution of VIP in the olfactory bulb of the rainbow trout (*Salmo gairdneri*) comparing it with previous data in higher vertebrates. Our conclusions may provide further information on the localization of neurotransmitters within the olfactory system of lower vertebrates and the presence or absence of these neuroactive substances on the phylogenetic scale.

Ten adult rainbow trouts (*S. gairdneri* Richardson) with 200–250 g b. wt. were obtained from commercial sources (Fisheries La Flecha, Salamanca) and used for the present study. They were kept under standard laboratory conditions (12/12 h light/dark cycle). The animals were anaesthetised

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with 0.03% tricaine methanesulfonate (MS-222, Sandoz) and perfused transcardially with 25 ml of 0.63% saline followed by 250 ml of 4% paraformaldehyde in 0.12 M phosphate buffer, pH 7.3. The olfactory bulbs were removed and postfixed in the same fixative for 12 h. Finally, they were rinsed several times in phosphate buffer.

With a vibratome (Campden Instruments), 40 μm sagittal sections were cut, washed for 36 h in several changes of phosphate buffer and processed for immunostaining.

Rabbit Vasoactive Intestinal Polypeptide antiserum was purchased from Cambridge Research Biochemicals, Cambridge, U.K. The VIP antiserum was diluted at 1:1000 and the sections were incubated for 24 h at 12 °C. The sections were preincubated for 30 min in 0.3% Triton X-100 in 0.1 M phosphate buffer with 1% normal goat 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 2 h. Tissue-bound peroxidase was visualised by incubating the sections with 3,3′-diaminobenzidine (0.07%) in Tris buffer (0.1 M, pH 7.6) for 10 min.

At the working dilution (1:1000) the VIP antiserum was immunoabsorbed for 1 h at 37 °C and 24 h at 4 °C with VIP (0.1 mg/ml, Peninsula Lab.). Specific absorption was also tested by omitting the VIP antibody in the first incubation bath. In both cases, no immunostaining was observed.

In addition, antisera against methionine–enkephalin, leucine–enkephalin, motilin (Cambridge Research Biochemicals, Cambridge, U.K.), neuropeptide Y (gift from Dr. J.M. Polak), tyrosine–hydroxylase (gift from Dr. J. Thibault), cholecystokinin-8 and substance P (gifts from Dr. R.E. Rodríguez) were tested on the rainbow trout olfactory bulb. In all the cases, previously described immunocytochemical controls were carried out and no immunoreactivity was found.

Fig. 1a shows the general structure of the rainbow trout olfactory bulb, which from the outermost to the innermost parts includes the following strata:

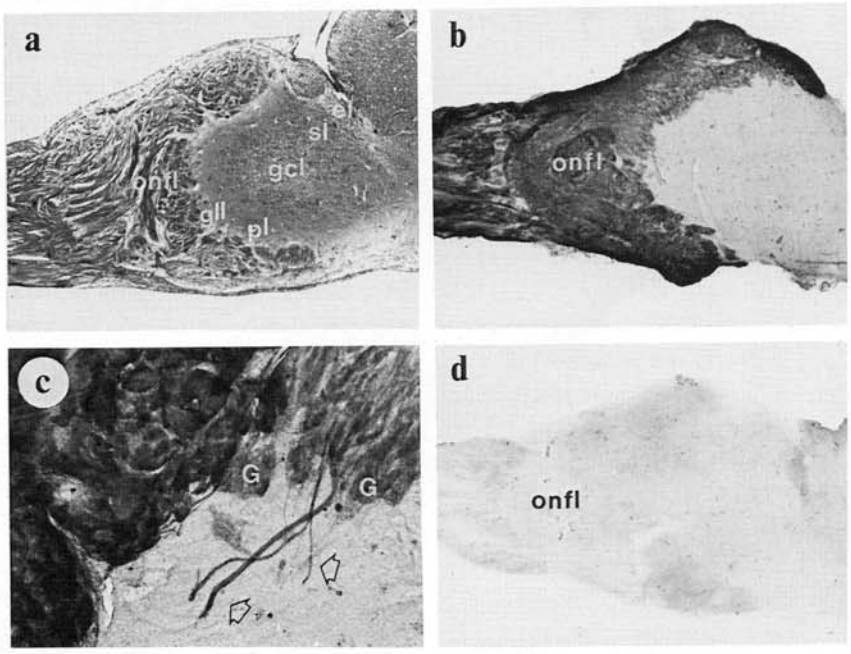

Fig. 1. a: photomicrograph of a Holmes-stained sagittal section of the rainbow trout olfactory bulb. The following strata can be observed: el, ependimary layer; gcl, granule cell layer; gll, glomerular layer; onfl, olfactory nerve fibre layer; pl, plexiform layer; sl, subependimary layer. (×62.5). b: sagittal section of the olfactory bulb showing VIP-like immunoreactivity. Note the strong immunolabelling of the onfl and the lack of reaction in the remaining strata. (×62.5). c: a higher magnification of Fig. 1b. Note VIP-immunoreactive glomeruli (G) and fibrous bundles (open arrows) entering into different regions of the glomerular layer. (×375). d: control section after immunoabsorption of the VIP antiserum with VIP. No immunoreactivity was observed. (×62.5).

olfactory nerve fibre layer, glomerular layer, plexiform layer, granule cell layer, subependimary layer and ependimary layer.

VIP-immunoreactive structures in the olfactory bulb of the rainbow trout were only located in the olfactory fibres comprising the olfactory nerve and the first stratum in the bulbar lamination. The olfactory fibres were very strongly labelled (Fig. 1b), and it was possible to follow their courses when they penetrated, in different bundles, into the second stratum of the olfactory bulb, the glomerular layer (Fig. 1c). Other components of the olfactory nerve and the olfactory nerve fibre layer in the rainbow trout, such as displaced mitral cells, terminal nerve fibres, ganglionary cells, and glial cells, were not stained.

Moreover, according to the density and distribution of the immunoreactivity in the olfactory fibres, no significant differences were observed between the sections of the different animals, between the left and right olfactory bulbs, or between the dorsal and ventral, medial and lateral, or rostral and caudal regions. Finally, no immunoreactivity was observed in deeper layers such as the plexiform, granule cell, subependimary or ependimary layers or in the glomerular layer, with the exception of the olfactory fibres. Thus, in those more internal strata neither fibres nor cell bodies containing VIP were observed.

On the other hand, the olfactory fibres were not immunoreactive for the following peptides assayed: methionine–enkephalin, leucine–enkephalin, motilin, neuropeptide Y, cholecystokinin-8, and substance P. The presence of tyrosine hydroxylase was also negative.

It seems that the immunoreactivity found in the olfactory fibres was due to the VIP since the immunostaining with antiserum against VIP was blocked by addition of the homologous antigen (Fig. 1d). The remaining controls previously described were also negative.

The general structure of the olfactory nerve fibre layer and the ultrastructural characteristics of the olfactory fibres in teleosts coincides with those reported for higher vertebrates[2]. The olfactory nerve fibre layer is one of the few regions in the vertebrate brain reported to contain a pure population of a single neuronal element: the olfactory fibre[15]. In teleosts, we have reported in this stratum the presence of displaced elements and components of the terminal nerve[2]. However, they contribute minimally to this layer[5]. In addition, in the present study they were not labelled.

In mammals, VIP has not been reported in the olfactory nerve fibre layer and there is considerable evidence to suggest that carnosine could be a putative transmitter for the olfactory fibres. This compound appeared in all regions of the primary olfactory neurons of the olfactory mucosa, i.e. apical dendrites, somata, axons, and axon terminals[30]. A similar pattern of immunocytochemical staining has been observed for the olfactory marker protein[22] and tritiated β-alanine[8]. Sharma[34] has also reported monoamine oxidase in this layer, pointing to the presence of catecholamines. However, receptor cells display no immunoreactivity[33], suggesting that components other than olfactory nerve fibres might be associated with monoamine oxidase activity[15]. In any case, there are no data on the presence of VIP in the olfactory fibres of higher vertebrates, the present report being the first study which shows VIP immunoreactivity in this structure in vertebrates. On the other hand, it cannot be ruled out that after treatment with intraventricular or intratissue injections of colchicine or using an antibody raised in a phylogenetically closer species, new immunoreactive structures, fibres and/or cell bodies may be labelled in the olfactory bulb of the rainbow trout. However, the VIP amino acid sequence, studied in mammals, birds and elasmobranchs, suggests that the biologically active conformation of this peptide has been highly conserved during evolution[9]. Moreover, as may be inferred from our results, the olfactory fibres display a strong degree of immunoreactivity and all other components of the olfactory bulb show a practically non-existent background, indicating a high specificity.

Lorén et al.[21] have suggested that VIP is involved in cortical and limbic functions. In the rat hippocampus, Léránth et al.[20] have shown that the VIP-like immunopositive neurones are most probably local circuit neurones. Such modulator functionality is also suggested for the VIP-positive neurones in the rat olfactory bulb. These neurones were identified as tufted cells, and different types of short-axon cells such as horizontal cells, the vertical cells of Cajal[25,31] or superficial short-axon cells[14]. It

should be noted that tufted cells and these variants of short-axon cells have not been observed in the teleost olfactory bulb. In the rainbow trout, it is evident that the VIP-like immunoreactive elements in the olfactory bulb — the olfactory fibres — are not modulator elements.

The differences reported in the present paper between the VIP immunoreactivity in lower and higher vertebrates point to an important functional significance. The labelled VIP immunoreactive elements in the rat olfactory bulb are in a position to influence the bulbar output modulating the activity of the internal granule cells, which are considered to be the most important interneurones of the olfactory bulb in all vertebrates. On the contrary, the VIP-positive elements in the rainbow trout, the olfactory axons, are very long centripetal axons (in other teleosts, such as the cyprinoids, they are comparatively very short) which carry olfactory information from the first (mucosa) to the second (olfactory bulb) station in the olfactory pathway, suggesting that VIP might play a role in the transmission of the olfactory input.

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