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Substance P-like immunoreactivity in the ganglion cells of the tench terminal nerve

J.R. Alonso, R. Coveñas, J. Lara, M. de León, R. Arévalo and J. Aijón

Department of Cytology and Histology, Faculty of Biology, University of Salamanca, Salamanca (Spain)

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The distribution of substance P (SP) in the olfactory bulb of the tench *Tinca tinca* was studied by using an indirect immunoperoxidase technique. Many perikarya and processes of the ganglion cells of the nervus terminalis (NT) were strongly labeled. In addition, SP-like immunopositive fibers were observed in the proximity of these neurons and extending along the olfactory nerves and the olfactory tracts. The ganglion cells of the NT were not immunoreactive for methionine- and leucine-enkephalin, motilin, vasoactive intestinal polypeptide, neuropeptide Y, cholecystokinin-8, and tyrosine hydroxylase.

The nervus terminalis (NT) has been observed in all classes of vertebrates [2] and consists of ganglion cells and nerve fibers that are closely associated with the olfactory nerve and bulb. However, the NT and the olfactory pathway seem to be independent systems, differing in their connectivity, distribution pattern of immunoreactivity, and function [2, 3, 8].

In mammals, the NT ganglion cells display vasoactive intestinal polypeptide, choline acetyltransferase, acetylcholinesterase and luteinizing hormone-releasing hormone (LHRH) [5, 15, 16]. In fish, LHRH and molluscan cardioexcitatory tetrapeptide (FMRFamide) immunoreactivities have been well characterized in the ganglion cells of the NT [9, 10, 13, 20]. In addition, there is a preliminary observation suggesting that the NT in goldfish also contains substance P (SP) [18]. However, it remains controversial since it has been speculated that it may not represent SP but a portion of the lower vertebrate FRMFamide or its precursor [19]. Moreover, other studies about SP immunoreactivity have not described labeled perikarya in the olfactory bulb of different teleosts including goldfish [21]. Thus, we have systematically searched for the possible presence of SP and other neuroactive substances in NT neurons.

Correspondence: J. Aijón, Dept. of Cytology and Histology, Faculty of Biology, University of Salamanca, Plaza de la Merced s/n, 37008 Salamanca, Spain.

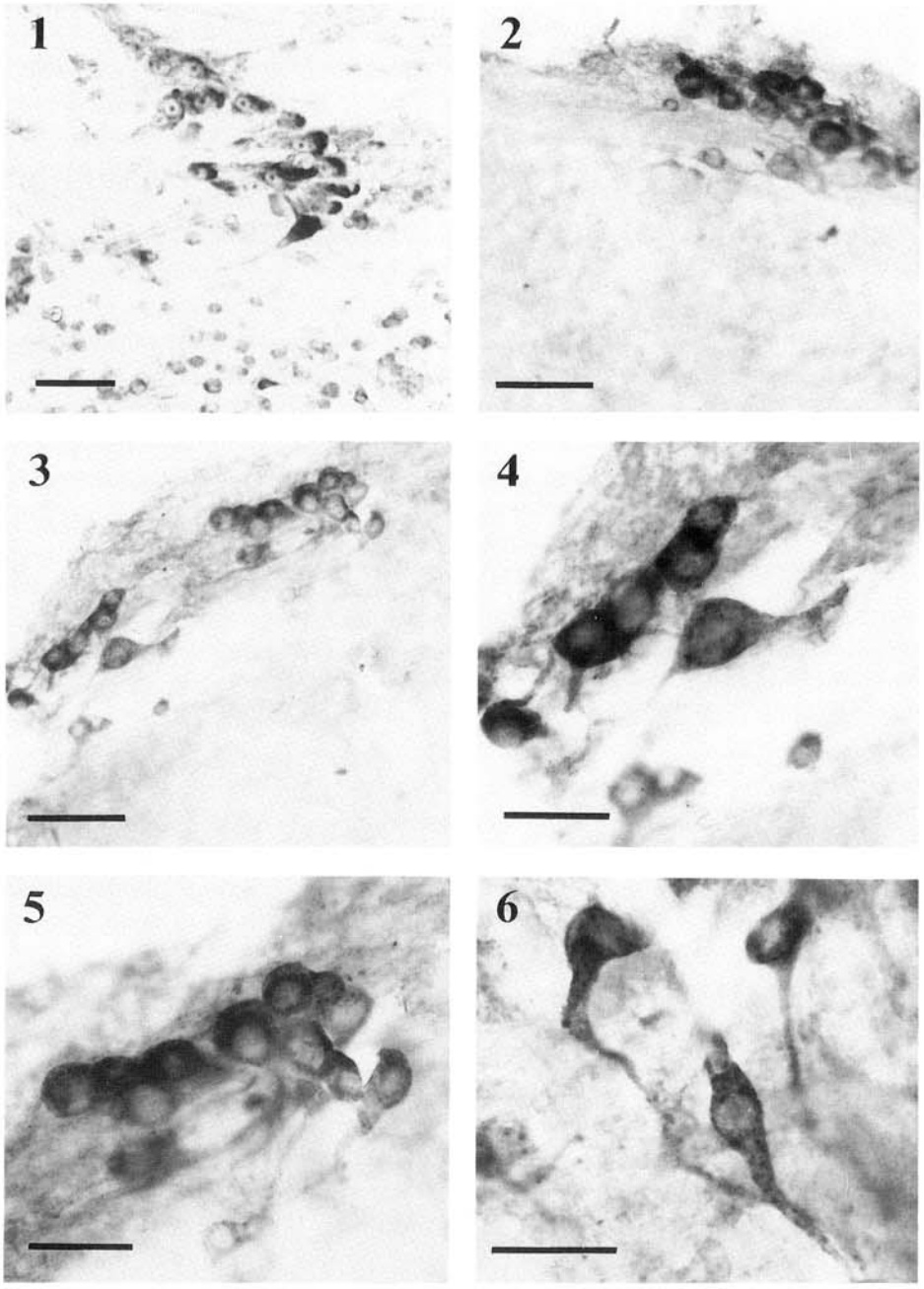

Fig. 1. Ganglion of the NT. Nissl's stain.

Fig. 2. Ganglion of the NT. SP immunostaining.

Fig. 3. Two clusters of SP-positive ganglion cells.

Figs. 4 and 5. Higher magnifications of the neurons showed in Fig. 3.

Fig. 6. SP-immunopositive perikarya located in the olfactory nerve. Bars in Figs. 1–3 = 70 μm; in Figs. 4 and 5 = 35 μm; Fig. 6 = 30 μm).

Five adult tenches (*Tinca tinca* L.) (190–275 g b.wt.) were used. Under deep anesthesia with 0.03% MS-222 (Sandoz), the animals were perfused transcardially with 25 ml of 0.63% saline followed by 250 ml of 4% paraformaldehyde in 0.12 M phosphate buffer, pH 7.2. After perfusion, the olfactory bulbs were removed and post-fixed overnight in the same fixative. With a vibratome, 40 μm sagittal sections were cut and processed for immunostaining as has been described in detail elsewhere [1]. Sections were incubated with SP antiserum (1:1000) for 48 h at 4°C. Anti-rabbit IgG coupled to horseradish peroxidase (Institut Pasteur, Paris) was used as a second antibody (diluted 1:1000 at 20°C for 4 h). Tissue-bound peroxidase was visualized by incubating the sections with 0.07% diaminobenzidine and 0.01% hydrogen peroxide. Antiserum specificity was tested by preabsorption for 1 h at 37°C and 24 h at 4°C with SP (0.1 mg/ml, Peninsula). Specificity of immunostaining was controlled by incubating some sections with phosphate buffer or non-immune rabbit serum instead of the primary antibody. Controls did not show immunostaining. Substance P antiserum was raised in rabbits by multiple intradermal injections for a period of eight months of synthetic SP (Peninsula) coupled to bovine serum albumin via glutaraldehyde. In addition, it has been found using radioimmunoassay [12] that the SP antibody used exhibited only very limited cross-reactivity with the peptides: SP (fragment 5–11) (0.3%), SP (fragment 4–11) (0.01%), physalaemin (0.003%), eledoisin (0.001%), kassinin (0.02%), and substance K (0% at 100 nM).

Antisera against methionine-enkephalin, leucine-enkephalin, vasoactive intestinal polypeptide, motilin, neuropeptide Y, cholecystokinin-8, and tyrosine hydroxylase were tested. No immunoreactivity was found in the ganglion cells of the NT.

In normal histological sections (Fig. 1), the ganglion cells of the NT formed dense clusters of large neurons closely located to the rostral surface of the olfactory bulb. After SP immunostaining, positive perikarya displaying similar characteristics were observed in the same location. Thus, these cells were normally situated in the outer fibrillary layer, the first stratum of the bulbar lamination (Figs. 2–5). Additionally, smaller groups of cells and isolated neurons were observed between the fascicles of the olfactory nerve (Fig. 6). The immunostained ganglion cells of the NT were closely associated in one or two compact clusters (Figs. 2, 3). Each cluster consists of 20–55 neurons that are ovoid, round or pyramidal in shape, and ranged from 18 to 39 μm in diameter. Positive neurons showed a strong SP-like immunostaining in their perikarya and proximal dendrites, which extended parallel to the bulbar surface (Figs. 2–5). In addition, fine immunopositive fibers were observed in the proximity of the immunolabelled neurons (olfactory nerve fiber layer and glomerular layer of the bulbar lamination), and extending along the olfactory nerve and olfactory tract. No immunoreactive cell body was observed in deeper strata such as the plexiform layer, the granule cell layer or the ependym.

The distribution of SP immunoreactivity in the tench is clearly different from that previously reported in the olfactory bulb of teleosts and higher vertebrates. Intrinsic SP-positive perikarya, identified as external tufted cells and superficial short-axon cells, have been detected in the mammalian olfactory bulb [6, 11]. Both of these neuronal types are absent in the teleost olfactory bulb. Previous studies carried out in

teleosts have shown a high interspecies variability for SP immunoreactivity in the olfactory bulb [21]. Thus, Szabo et al. [21] showed labeling of the olfactory fibers in gymnotid fish, whereas two other species, *Gnathonemus petersii* and *Carassius auratus*, showed no positive staining in the olfactory bulb. Our report adds more complexity to this distribution pattern, suggesting a significant role for SP in the fish forebrain.

The function of the NT system remains unclear. However, there is evidence such as the NT central projections, its involvement in sperm release, and the altered LHRH immunoreactivity labeling following hypophysectomy which suggests a role in integrating different brain regions for sexual and reproductive behavior [23]. Thus, the NT system is the only pathway with direct anatomical connections between the nose and regions of the forebrain implicated in reproductive functions [15]. On the other hand, it has been suggested that olfactory inputs mediated through the NT can initiate or modify vision-related behavior [17]. It has also been demonstrated that the NT gives rise to fibers to both the retina and the olfactory epithelium [10]. Thus, our results clearly correlate with previous studies on lower vertebrates that showed the physiological effect of SP on the retinal ganglion cells [22] and the olfactory receptor cells [4]. Additionally, the application of other NT-located neuroactive substances, such as LHRH and FMRFamide also altered the activity in the isolated goldfish retina [22]. In conclusion, it is possible to hypothesize that the effect of SP on the function of the retinal ganglion cells is exerted through the innervation by NT ganglion cells. This is in good agreement with previous studies proposing an excitatory function for SP in different neuronal systems [14]. However, a recent study [7] has shown that the retinopetal NT fibers neither enhance nor inhibit scotopic photosensitivity for large unfocused stimuli.

Neurohistochemical and morphological investigations of the NT system suggested the presence of neuronal subpopulations in the NT ganglion according to the presence of neuroactive substances, cell morphology and diverse projection patterns [3]. The demonstration of SP in the NT ganglion cells of the tench poses new questions such as possible interactions between the different neuroactive substances (LHRH and SP) identified in these neurons.

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