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Immunohistochemical Demonstration of Some Putative Neurotransmitters in the Lamprey Spinal Cord and Spinal Ganglia: 5-Hydroxytryptamine-, Tachykinin-, and Neuropeptide-Y-Immunoreactive Neurons and Fibers

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ABSTRACT

The distribution of some putative neurotransmitters was investigated in the spinal cord and spinal ganglia of the lamprey, a primitive vertebrate, by using immunohistochemical methods. In the spinal cord a midline row of 5-hydroxytryptamine (5-HT)-immunoreactive neurons was present immediately ventral to the central canal over the entire length of the spinal cord. The ventral processes of these neurons formed a dense ventromedial plexus of varicosities. In the dorsal, lateral, and ventral spinal axon columns, several longitudinal 5-HT fibers were present. After chronic spinal transections the distribution of 5-HT fibers was unchanged; it is therefore concluded that there was no substantial descending 5-HT contribution and that the spinal 5-HT neurons supplied the regional 5-HT innervation. The spinal 5-HT cells sent fibers into the dorsal and ventral roots; 5-HT cell bodies and fibers were also present in the spinal dorsal root ganglia, in their dorsal, ventral, and lateral nerve branches, and in the dorsal and ventral branches of the ventral roots.

Neurons and fibers containing peptides of the tachykinin (TK) family (to which, amongst others, substance P belongs) were found in the spinal cord. TK neurons in the spinal cord supplied the local TK innervation, as well as TK fibers in the dorsal and ventral roots. Fibers have been found containing either TK, or 5-HT, or both compounds.

Neurons containing neuropeptide-Y (NPY)-immunoreactive material were present in a medial column just dorsal to the central canal. The NPY neurons have longitudinal, mainly descending, fibers that provide the local NPY innervation of the lamprey spinal cord.

The present results provide evidence for local spinal systems containing 5-HT, TK, 5-HT and TK, or NPY, but in contrast to mammals, these compounds do not seem to arise from supraspinal neurons.

Key words: lamprey, spinal cord, 5-hydroxytryptamine, substance P, neuropeptide Y, tachykinins

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The location and action of various putative neurotransmitters in the nervous system have been a subject of great interest; during recent years special attention has been given to small peptides as putative neurotransmitters in the nervous system (see Otsuka and Takahashi, '77; Snyder, '80; Hökfelt et al., '80; the concept "neurotransmitter" is used as defined in Van Dongen, '81). In several instances, neurons have been found to contain not only one, but two or three, putative neurotransmitters (Hökfelt et al., '82).

The spinal cord of the lamprey, a primitive vertebrate (Whiting and Tarlo, '65; Bardack and Zangerl, '71), may be a good system for analyzing the presence and actions of putative neurotransmitters (cf. Homma, '83). Lampreys have numerous large neurons, which are easily identified in morphological and electrophysiological studies (cf. Rovainen, '79). Furthermore, the lamprey spinal cord can be studied in vitro with the combined advantages of intact preparations and the now frequently used slice preparation (see Andersen and Langmoen, '80). Thus, under appropriate conditions, parts of the spinal cord can be functioning properly for several days in vitro. Individual neurons are visible, and several different types can be distinguished, allowing physiological analysis to be carried out on identified neurons (Rovainen, '67a,b; Grillner et al., '82).

It is already known that 5-hydroxytryptamine (5-HT)-containing neurons are present in the lamprey brain stem and spinal cord (Honma, '69,'70; Baumgarten, '72; Ochi and Hosoya, '74; Steinbusch et al., '81; Filler et al., '83). In this report, the distribution of 5-HT, peptides of the tachykinin (TK) family (to which, amongst others, substance P belongs; Erspamer et al., '77; Pernow, '83), and neuropeptide-Y (NPY)-like peptides (Tatemoto, '82; Tatemoto et al., '82) in the lamprey spinal cord will be described with the aim of providing a basis for an investigation of the physiological role of these putative neurotransmitters. A preliminary report of this work has appeared (Van Dongen et al., '84).

MATERIALS AND METHODS

Animals

Adult lampreys of two species, the river lamprey (*Lampetra fluviatilis*; 0.15–0.40 m, labeled "*Lamp.*"), and the silver lamprey (*Ichthyomyzon unicuspis*; 0.15–0.25 m, labeled "*Ichth.*") were used. They were caught in the Älvkarleby river in Sweden (*Lamp.*) or in Iowa (USA) (*Ichth.*) and kept in aerated aquaria at a temperature of 5–10°C. Some of the animals were treated with colchicine (8–20 μg), but no differences were found between these and untreated animals. For the operation, animals were anaesthetized with tricaine methanesulfonate (MS 222, Sandoz) (Jolly et al., '72).

Chronic spinal transections

Spinal transections were performed in nine animals (two *Ichth.* and seven *Lamp.*). The animals were operated upon in ice-cold lamprey Ringer solution. The lamprey Ringer solution was prepared according to Wickelgren ('77); it was oxygenated with O_2/CO_2 (95%/5%), adjusting the pH to 7.4. The spinal cords were exposed just caudal to the gill region in all cases. Two different types of transection were made (Fig. 1): (1) a single spinal transection (N = 2, *Ichth.* and N = 2, *Lamp.*), and (2) isolation of a portion of three to five segments of spinal cord by a rostral and caudal spinal transection, and transections of all dorsal and ventral roots of this portion (N = 5, *Lamp.*). After the spinal transections, the cut ends of the spinal cords retracted, leaving an open space of more than 0.5 mm. Thereafter, the wounds were sutured, and the animals were placed into their aquaria (5–10°C). The spinal cords were removed 4 weeks following the transections (see below).

Preparation

To prepare the lamprey spinal cords for immunohistochemistry, the animals were anaesthetized and decapitated. The following portions of the body were cut off: (1) the gill region, (2) the region between the last gill opening and the dorsal fins (called "intermediate cord"), or (3) the region of the dorsal fins (called "caudal cord"). These parts were put in ice-cold Ringer solution (see above), eviscerated, and the muscles were removed. The remaining pieces consisted of notochord, spinal cord with meninx primitiva and perimeningeal tissue, the dorsal haematopoetic tissue, and the connective tissues around the perimeningeal tissue (cf. Fig. 4). Two different types of dissection were made: (1) in most cases, the haematopoetic tissue and the connective tissue between haematopoetic tissue and perimeningeal tissue were removed, the dorsal and ventral roots were cut, and the piece was removed from the notochord; this piece consisted of spinal cord, meninx primitiva, and most of the perimeningeal tissue. (2) For analyzing the spinal ganglia, only the dorsal haematopoetic tissue was removed, leaving the connective tissue lateral and dorsal to the perimeningeal tissue and the spinal cord and notochord in situ ("spinal-cord-notochord preparation", cf. Fig. 4). The average length of the segments in each piece was determined. Both types of preparations were pinned down on Sylgard gel (Dow Corning) at their in situ length and processed for immunohistochemistry.

Immunohistochemistry

The spinal cord tissue was fixed for 1–2 hours in an ice-cold solution of either p-formaldehyde (40 g/L, i.e., "10% formalin") in 0.1 M phosphate buffer, pH 7.2, or of p-formaldehyde (40 g/L) in 0.16 M phosphate buffer, pH 6.9, with picric acid (2 g/L). After fixation, the tissue was rinsed at least 24 hours in 5% sucrose in 0.1 M phosphate buffer, pH 7.2, containing 0.01% Na-azide and 0.02% Bacitracin (Sigma) at 4°C. Transverse and horizontal serial cryostat sections (14 μm) were made and processed according to the indirect immunofluorescence method of Coons and collaborators (Coons, '58) adopted for transmitter histochemistry (Hökfelt et al., '73, '75, '83). The sections were incubated with antisera raised against 5-HT (dilution 1:400), substance P(1:200), or NPY (dilution 1:400), and subsequently with the second antibody (for characteristics of the antisera see below). The second antibodies were conjugated either to fluorescein isothiocyanate (FITC) or to tetramethylrhodamine isothiocyanate (TRITC) (Dakopatts, Copenhagen). To investigate the coexistence of 5-HT and TK, the sections were incubated in a mixture of guinea-pig-anti-5-HT and rat-anti-substance-P, and then first with TRITC-coupled rabbit-anti-rat-immunoglobulins, and thereafter with FITC-coupled rabbit-anti-guinea-pig-immunoglobulins. A Zeiss fluorescence microscope was used to examine the sections. It was equipped with an HBO 200 high-pressure mercury lamp, excitation and emission filters (see below), and an oil darkfield condensor. The following Zeiss filters were used: (1) for FITC: excitation filter KP 500, and sometimes also LP 455 to reduce background fluorescence, and emission filter LP 520 and sometimes also KP 560; (2) for TRITC:

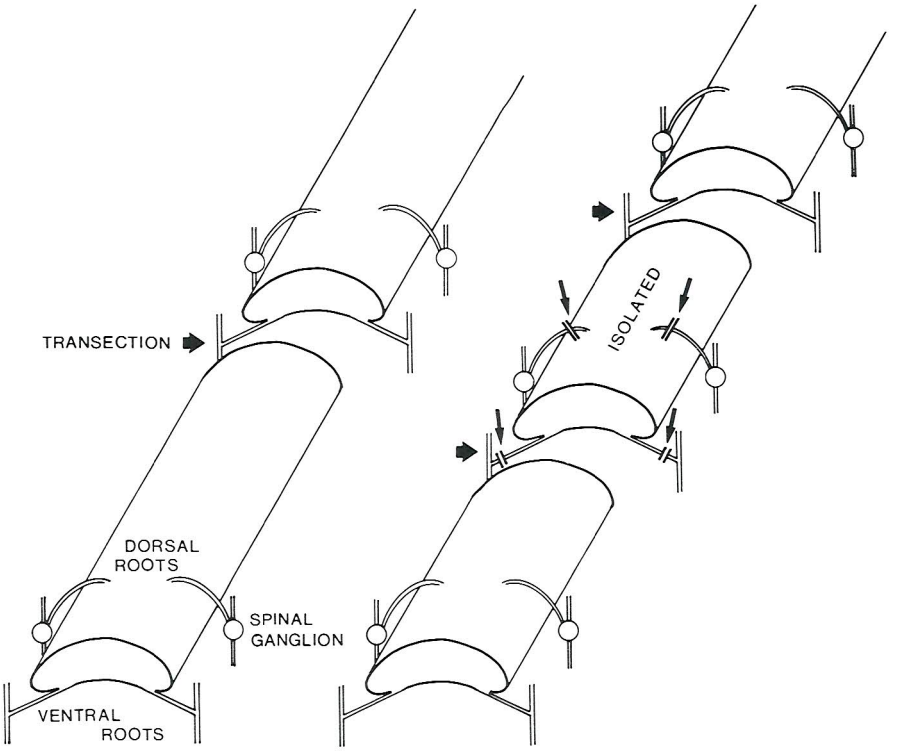

Fig. 1. Schematic survey showing a single spinal transection (left) and a spinal isolation (right). Although pieces of three to five segments were isolated, only one ventral and one dorsal root are shown in the isolated part.

excitation filter BP 546 and emission filter LP 590 (Hökfelt et al., '83). Black-and-white pictures were made with Scopix RP1 (Agfa Gevaert) film for FITC and on Tri-X (Kodak) film for FITC or TRITC. Single or sequentially exposed black-and-white pictures were made with filter settings appropriate for FITC (5-HT, TK, or NPY) or for TRITC (TK) (some of the micrographs were slightly retouched). Fiber counts were made in the rostral and caudal parts of the spinal cords (medium seized (0.17 m) *Ichth.* or longer (0.30 m) *Lamp.*). When fibers could be followed over some distance in a single section, the width of a homogeneous region was measured with an ocular micrometer, and the number of fibers passing through a plane perpendicular to these fibers was counted (unless indicated otherwise, data are obtained from *Ichth.*, and expressed in mean ± S.D.). The fibers in the dorsal and ventral roots were counted in tissues dissected as spinal-cord-notochord preparations.

Antibodies

The following antibodies were used: 5-HT antisera, raised either in rabbits or guinea pigs against 5-HT coupled to bovine serum albumin (Steinbusch et al., '78), rat monoclonal antibodies raised against synthetic substance P coupled to bovine serum albumin (Cuello et al., '79), and antibodies raised in rabbits against NPY coupled to bovine thyroglobulin (Lundberg et al., '84). The peptide made visible with this substance P antiserum is in all probability not substance P (Van Dongen et al., '85b), and therefore, the more general term "tachykinin" (TK) will be used instead of "substance P" for the immunoreactive material detected with this antibody in the lamprey spinal cord.

Subdivision of the lamprey spinal cord

Most of the results will be presented in micrographs of horizontal sections, because these give the most informative surveys. In this study we distinguish five dorsoventral planes (as indicated in Fig. 2B).

(1) dorsal plane (Dp): dorsal to the cell bodies of the sensory dorsal cells;

(2) dorsal cell body plane (DCp): containing the cell bodies of the sensory dorsal cells;

(3) intermediate plane (Ip): containing the cell bodies of the lateral gray column;

(4) Müller-fiber plane (Mp): containing several Müller axons, ventral to the cell bodies of the lateral gray column, (Müller axons are also present in other planes but they are most prominent in this plane);

(5) ventral plane (Vp): containing the ventral and ventrolateral axon columns, where they are no longer connected by more medial spinal cord tissue in the sections (due to the concave ventral surface of the lamprey spinal cord).

These horizontal planes are further subdivided in mediolateral columns as described by Selzer ('79) (cf. Fig. 2B).

RESULTS

The distribution of neurons and fibers containing 5-HT, TK, or NPY-immunoreactive material was similar in *Lampetra fluviatilis* and *Ichthyomyzon unicuspis*. In the text,

both species will be referred to as "lamprey," while the species will be mentioned in the figure legends.

5-Hydroxytryptamine (5-HT)

Spinal cord

Cell bodies. Over the entire length of the spinal cord, a row of 5-HT cell bodies was found in the intermediate plane, just ventral to the central canal (Fig. 2C). A few 5-HT cell bodies were found more lateral or ventral to this cell row. The cell bodies were round or oval (as seen in horizontal and transverse sections). The oval cells showed no predominant orientation of the long axis. The 5-HT cell bodies were small (11 $\pm$ 3 μm in *Ichth.*, 0.17 m, and 17 $\pm$ 3 μm in *Lamp.*, 0.30 m) and their number varied between 22 and 45 per segment; 5-HT cells had processes in all directions, and two different types of processes have been found: nonvaricose, relatively short, dendritelike processes, and varicose fibers originating from the cell body or the dendrites (Fig. 3B). As a rule, 5-HT cells could not be attributed to either the right or the left hemisegment, and cells were often found sending processes both to the left and to the right part of the spinal cord (Fig. 3B).

Types of 5-HT fibers. Three types of 5-HT varicose fibers were found: (1) coarse, comparatively straight fibers with large varicosities (2.8 $\pm$ 0.8 μm) and intervaricose segments of variable and often long lengths (4.1 $\pm$ 2.1 μm), (2) finer fibers with smaller varicosities (1.1 $\pm$ 0.4 μm) and shorter intervaricose segments (2.6 $\pm$ 1.0 μm), and (3) fine varicosities (0.93 $\pm$ 0.26 μm) forming a dense plexus, in which the intervaricose segments could not be determined reliably. We will follow the terminology of Sano et al. ('82) to describe these fibers: "A-fibers" (coarse), "B-fibers" (fine), and "C-fibers" (very fine).

5-HT fibers. In all regions of the lamprey spinal cord 5-HT fibers were present (Fig. 2A). In the regions of the axon tracts longitudinal fibers were most prominent (Figs. 2C, 3A,C). Often the longitudinal fibers were seen to be continuous with laterally directed fibers coming from the 5-HT cell bodies. The longitudinal fibers could be followed for 0.3 mm or more; they travelled without division over some distance and then gave off rostrally or caudally directed collaterals. The highest density was found in the dorsal horns (i.e., the lateral half of the medial columns in the dorsal plane; mainly A-fibers; 8.7 $\pm$ 2.2 $\times$ 10^3 fibers/mm^2 in *Ichth.*, and 6.6 $\pm$ 2.1 $\times$ 10^3 fibers/mm^2 in *Lamp.*), while mainly B-fibers were found at a lower density in the other parts of the axon columns (4.1 $\pm$ 2.1 fibers/mm^2). The density of B-fibers was still lower in the lateral and ventrolateral parts of the axon columns (i.e., the lateral half of the lateral column in the intermediate, Müller-fiber, and ventral planes; 6.5 $\pm$ 1.6 $\times$ 10^2 fibers/mm^2). In the region of the sensory dorsal cell bodies (i.e., in the medial column in the dorsal cell plane), some ventrodorsal but only few longitudinal 5-HT fibers were found (Fig. 3C). In the columns of neuronal cell bodies (i.e., in the gray column in the intermediate plane) some laterally oriented or oblique 5-HT fibers were present, often near neuronal cell bodies (Fig. 2C). However, the cell bodies of most neurons were not associated with 5-HT fibers. The region of the ependymal cells around the central canal was usually devoid of 5-HT fibers, and only a few longitudinal 5-HT fibers were found in this region.

5-HT plexus. The spinal cord 5-HT cell bodies also had ventral processes which gave rise to a dense plexus of 5-HT C-fiber varicosities in the medial columns of the Müller-fiber plane (estimated density 2 $\times$ 10^7 varicosities/mm^3) in the ventromedial spinal cord (Figs. 2A, 3B,D). This 5-HT plexus extended over the medial half to two-thirds of the medial column, sparing the central canal column. The density of 5-HT varicosities in this 5-HT plexus is 10 to 100 times higher than in other regions of the spinal cord.

Spinal ganglia and spinal nerves

5-HT cell bodies. Several small (13 $\pm$ 2 μm, in medium-sized *Ichth.*) 5-HT neurons were found in the peripheral nervous system. The spinal ganglia contained six to 12 5-HT cells scattered between large (38 $\pm$ 11 μm) spinal ganglion cells (Fig. 4B); 5-HT cells were also found in branches connected to the spinal ganglia: in the dorsal and ventral branches of the dorsal roots and in the caudolateral branch, and in the dorsal and ventral branches of the ventral roots (cf. Freud, 1878; Tretjakoff, '27). These 5-HT cells sent their processes in both directions of the nerves where they were located. No 5-HT cell bodies have been found in the most proximal parts of the dorsal or ventral roots, i.e., between the spinal cord and the branching points of these roots after travelling through the connective tissue of the spinal canal (26 dorsal and 50 ventral roots have been inspected).

Spinal ganglia and dorsal roots. Dorsal roots, which often split into two or three rootlets before entering the spinal cord, contained several 5-HT fibers of the A-type (Figs. 3A, 4B). The roots (or rootlets) contained 17.3 $\pm$ 3.7 5-HT fibers per spinal hemisegment. The 5-HT fibers in the dorsal roots were continuous with the longitudinal 5-HT fibers in the lateral part of the medial columns in the dorsal plane. Some dorsal root 5-HT fibers were continuous with longitudinal fibers running in a rostral or a caudal direction from the roots, and other fibers divided forming T-connections with longitudinal 5-HT fibers (Fig. 3A). The spinal ganglia as well as their dorsal, ventral, and lateral branches contained several 5-HT fibers. Only very rarely was a 5-HT fiber found to follow a blood vessel dorsal or ventral to the spinal cord (Fig. 5D).

Ventral roots. Spinal ventral roots contained several varicose 5-HT A-fibers (8.9 $\pm$ 2.6 fibers/root, Fig. 4A). Occasionally, T-connections were found between the ventral root 5-HT fibers and the longitudinal 5-HT fibers in the far ventral plane. The dorsal and ventral branches of the ventral roots contained several 5-HT fibers.

Spinal transections. Four weeks after the spinal transections, considerable axonal degeneration was found; the degenerated material often had a brown fluorescence (cf. Figs. 6A, 14). Rostral to the transection, signs of degenera-

Fig. 2. A. 5-HT immunofluorescence in a transverse section through the intermediate part of the spinal cord of *Ichth.* B. The same section restained with cresyl violet. The dorsoventral planes are indicated as defined in this study, and the mediolateral columns as defined by Selzer ('79). Dp, dorsal plane; DCp, dorsal cell plane; Ip, intermediate plane; Mp, Müller-fiber plane; VP, ventral plane; Cc, central canal column; Mc, medial column; Gc, lateral gray column; Lc, lateral column. C. 5-HT immunofluorescence in a horizontal section through the intermediate spinal cord of *Ichth.*, in the intermediate plane, showing 5-HT cell bodies and 5-HT processes. The mediolateral columns are also indicated. To facilitate an overview, the micrograph is taken with a long exposure time showing also a weak unspecific fluorescence of cell bodies in the lateral gray column. Bars = 50 μm.