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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:

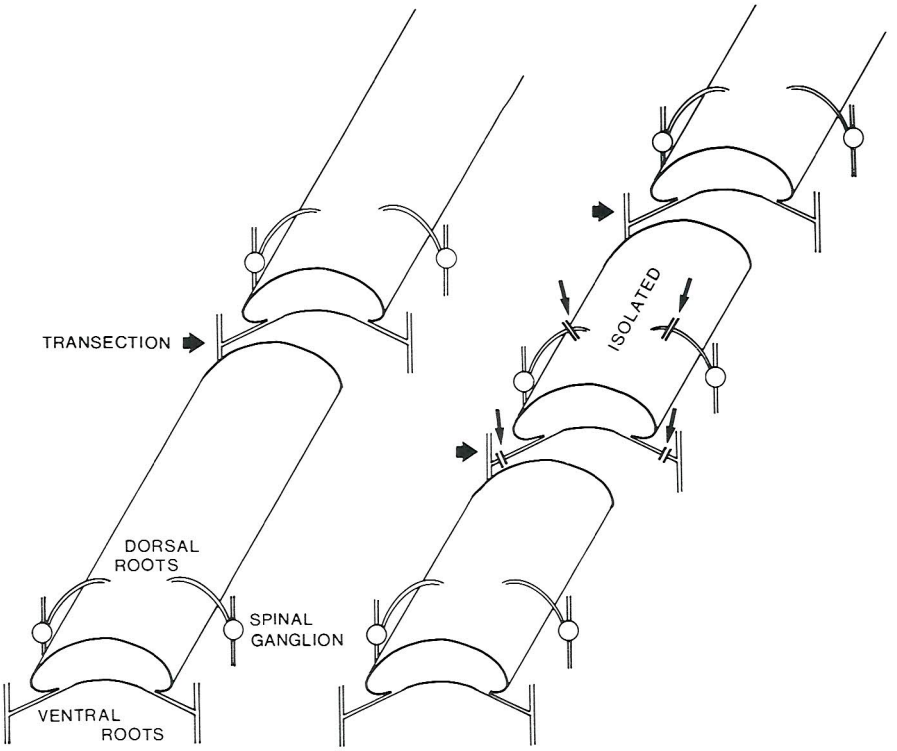

Fig. 1. Schematic survey showing a single spinal transection (left) and a spinal isolation (right). Although pieces of three to five segments were isolated, only one ventral and one dorsal root are shown in the isolated part.

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.

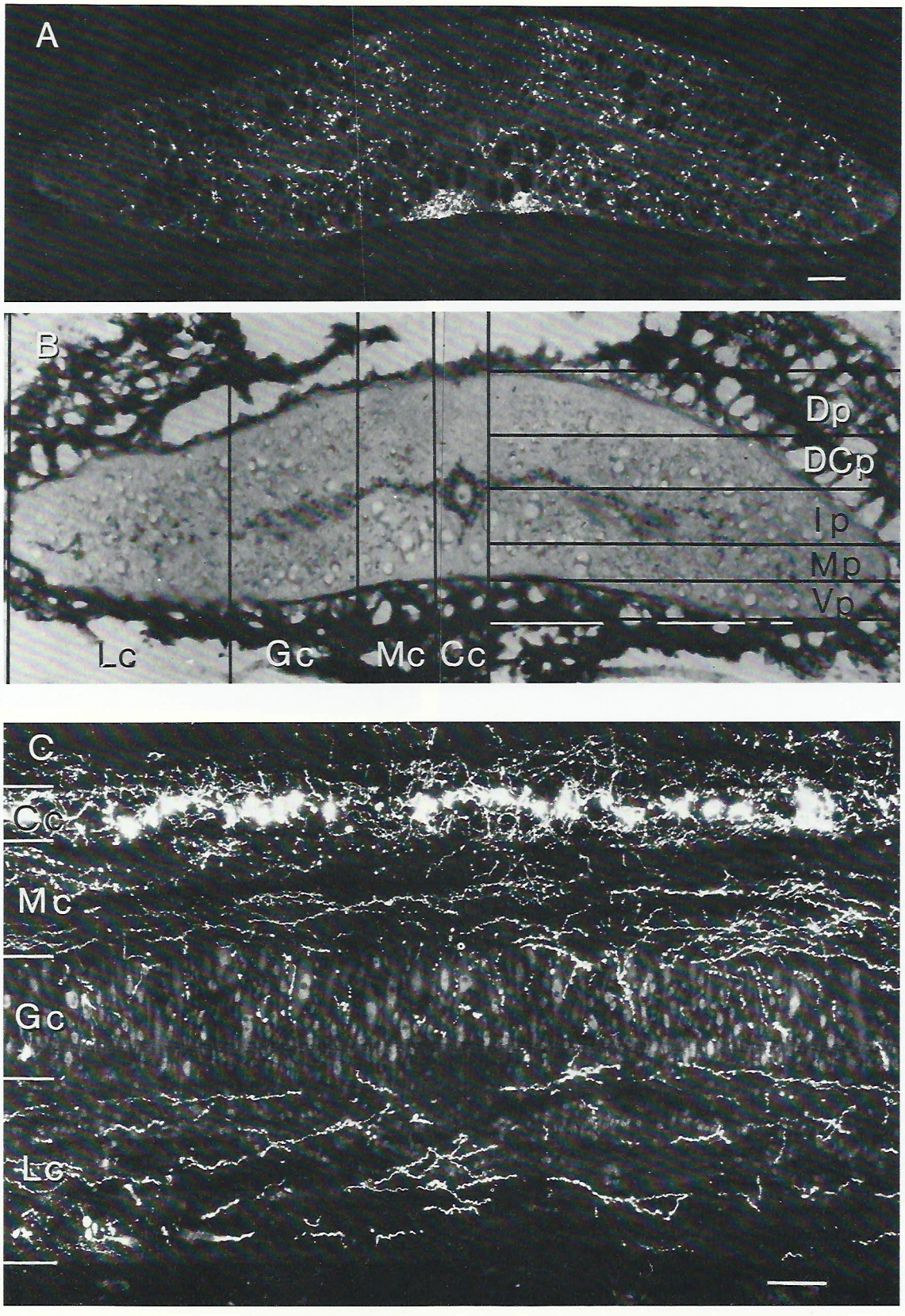

Figure 2

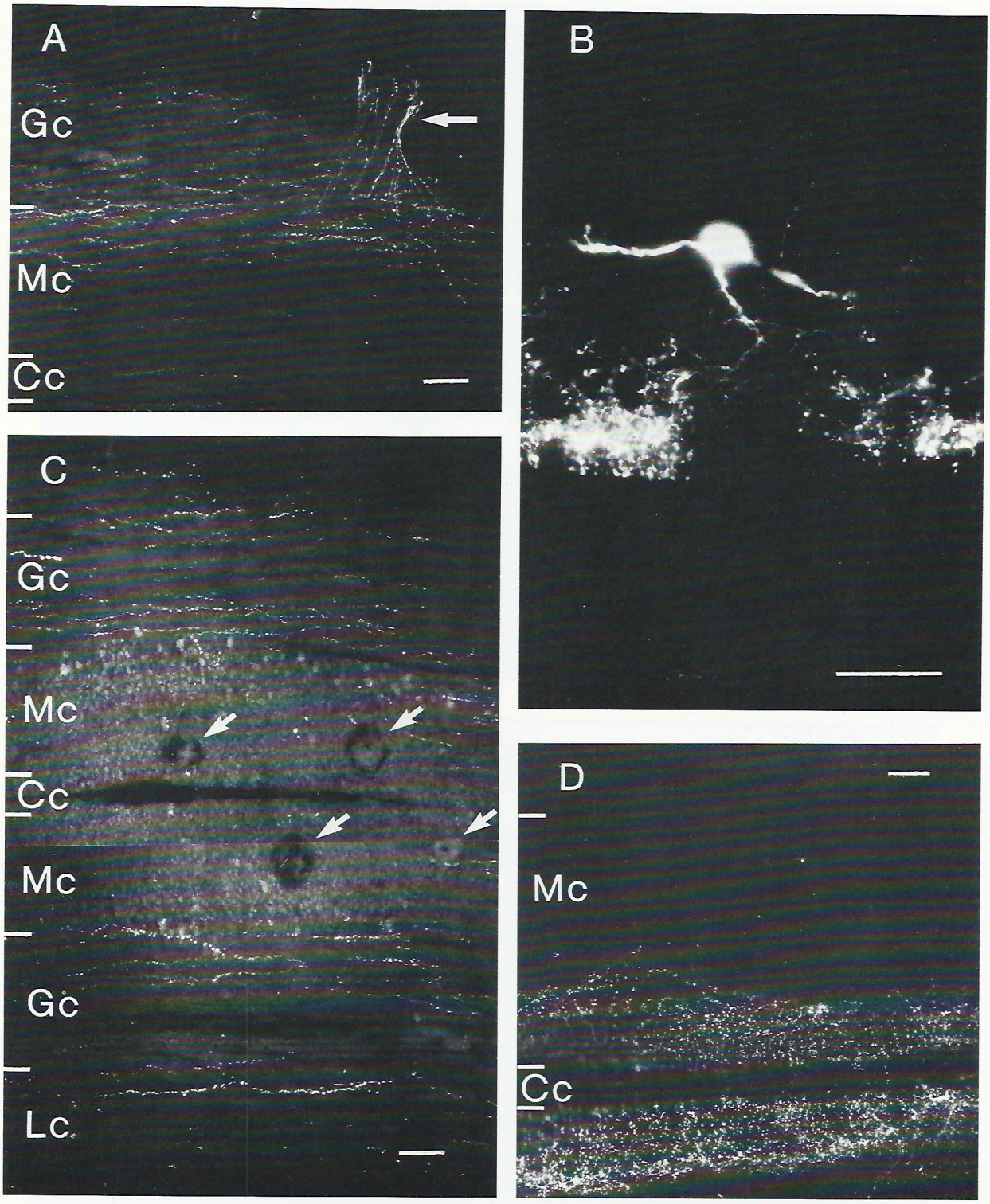

Fig. 3. 5-HT immunofluorescence in the spinal cord of *Ichth.* A. Horizontal section through the dorsal plane showing also a dorsal root (arrow). B. 5-HT cell body with dendrites and varicose processes into the 5-HT plexus (transverse section). C. 5-HT fibers in the dorsal cell plane (horizontal section); in the medial column sensory dorsal cells (arrows) are visible. D. Müller fiber plane through the 5-HT plexus (horizontal section). For the meanings of the abbreviations see legend to Figure 2. Bar = 50 μm.

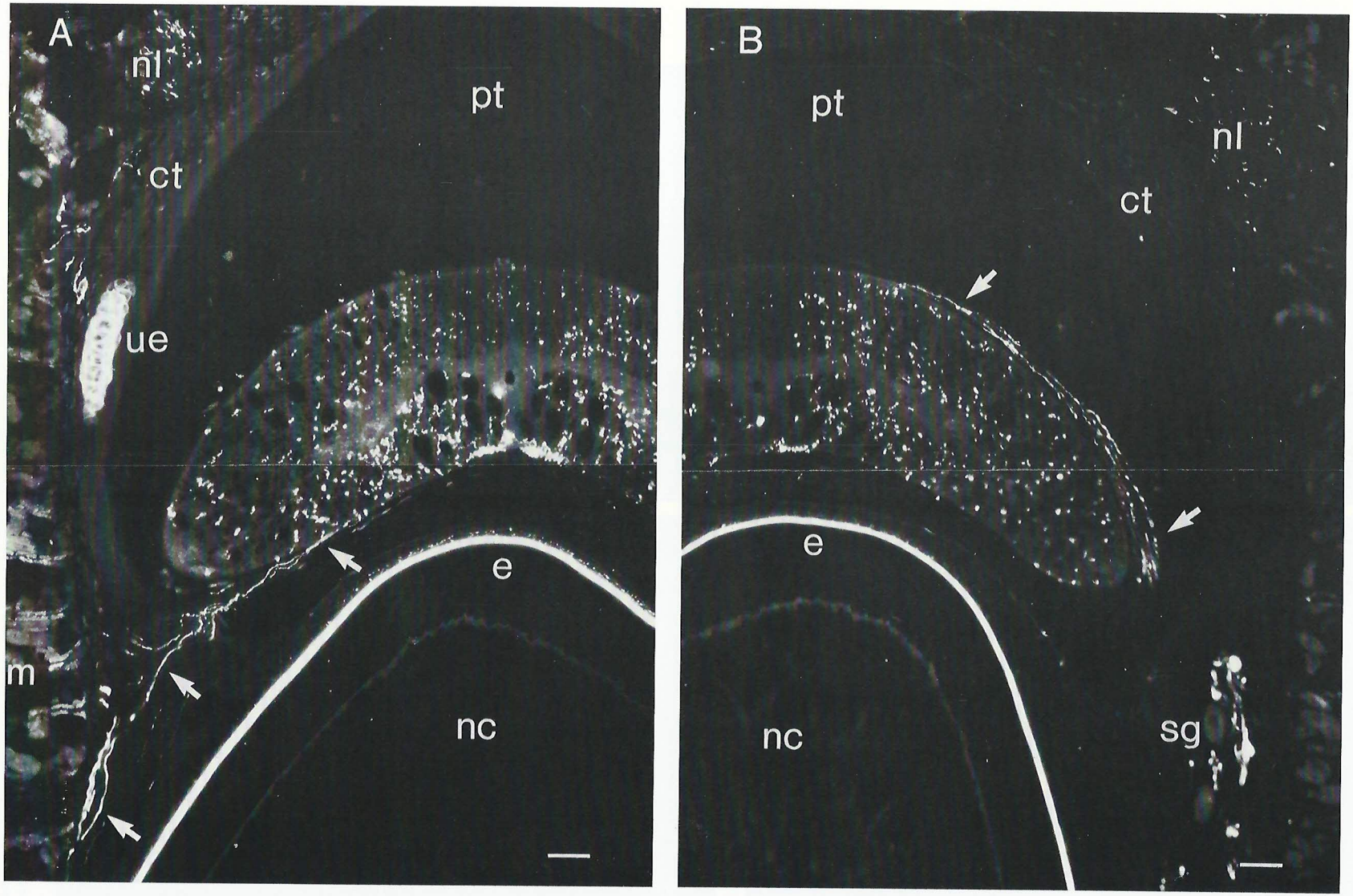

Fig. 4. 5-HT immunofluorescence in transverse sections through the spinal cord and notochord (nc) of *Ichth.*; note also the autofluorescent elastica of the notochord (e), and an unknown autofluorescent element (ue) in the connective tissue (ct). m = muscles, pt = perimeningeal tissue, nl = *nervus lateralis* containing 5-HT fibers. A. Section showing a ventral root (arrows). B. Section showing a dorsal root (arrows) and spinal ganglion (sg) containing two 5-HT cell bodies. Bars = 50 μm.

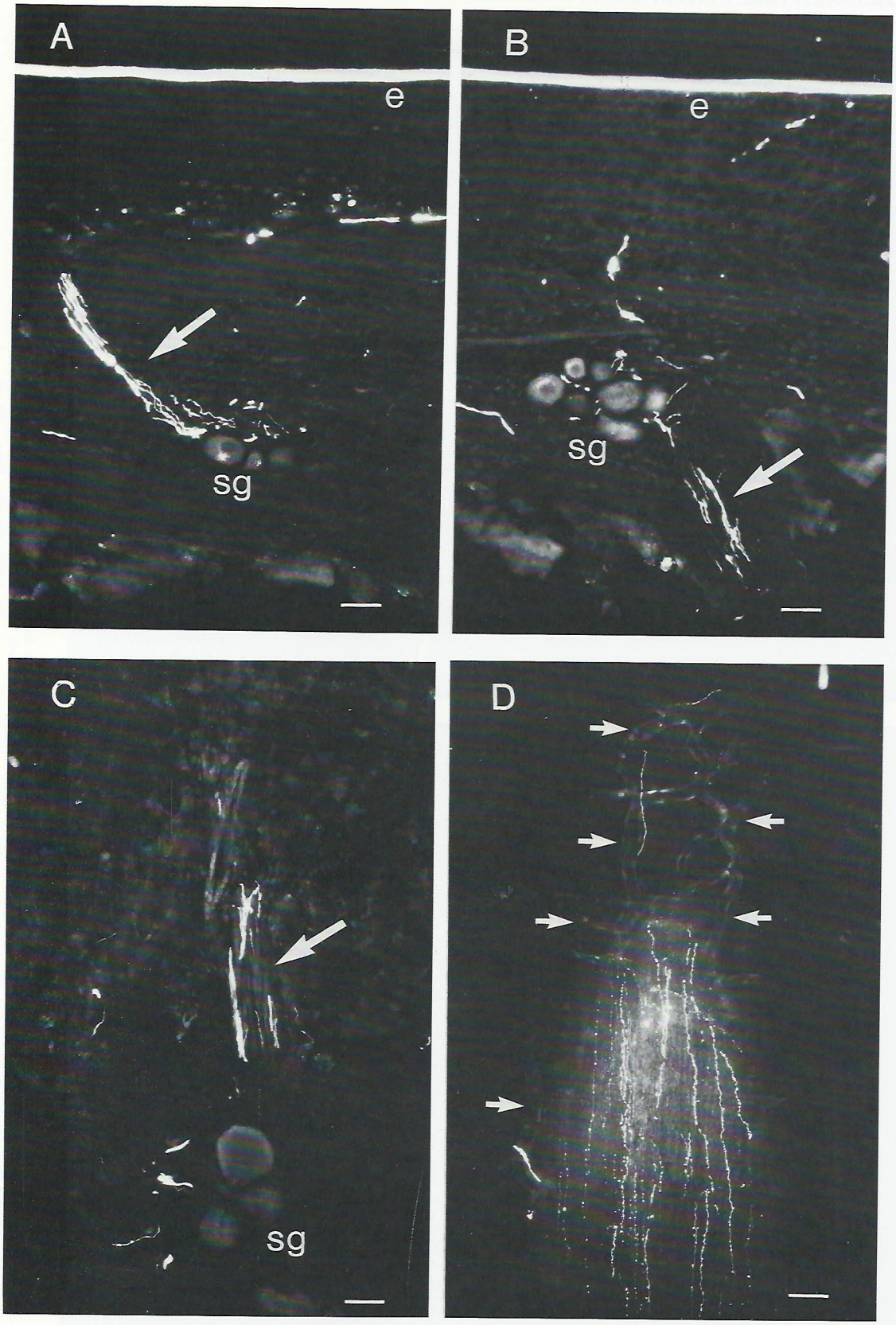

Fig. 5. 5-HT immunofluroescence in horizontal sections; the pictures are taken with long-exposure durations and with filter settings chosen in order to show the autofluorescent large cell bodies in the spinal ganglia (no 5-HT cells are shown in these figures) and the autofluorescent blood vessels. A. Spinal ganglion (sg) with dorsal root (arrow) toward the spinal cord (*Ichth.*). e, elastica. B. Spinal ganglion (sg) with caudolateral nerve (arrow) (*Ichth.*). C. Spinal ganglion as in A but 4 weeks after dorsal rhizotomy (*Lamp.*). D. Dorsal plane with blood vessels (arrows) dorsal to the spinal cord; they are only rarely close to 5-HT fibers (*Lamp.*). Bars = 50 μm.

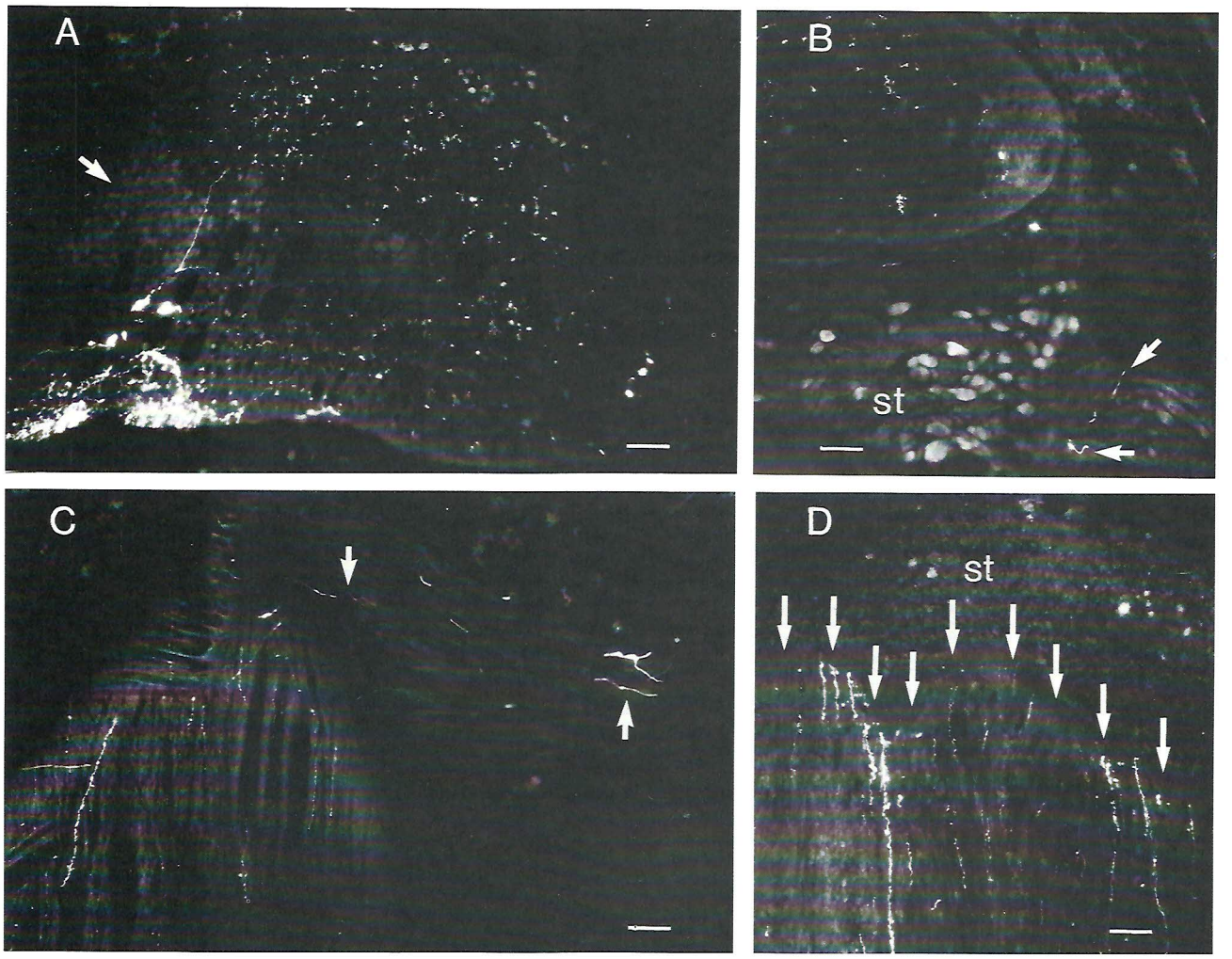

Fig. 6. 5-HT immunofluorescence in the isolated spinal cord of *Lamp.* A. Transverse section showing a distribution of 5-HT cells and fibers as in the intact animal (cf Figs. 2, 5). This picture is taken with emission filter LP 520 and KP 560 to reduce degeneration fluorescence, but a triangle of degenerated material (arrow) is still visible dorsal to the central canal (cf. Fig. 15). B. Transverse section showing 5-HT fibers in the distal part of a transected ventral root (arrows) and autofluorescent scar tissue (st) medial to the ventral root stump (emission filter LP 520). C. Horizontal section showing 5-HT fibers in the proximal part of the transected ventral root (arrows). D. Horizontal section through the dorsal plane showing 5-HT fibers in the spinal cord, not entering the scar tissue (st, arrows at the margin). Bars = 50 μm.

tion were present in the dorsal columns; caudal to the transection several, but not all, Müller axons had disappeared. In the isolated spinal cords both types of degeneration were present.

Spinal cord. Four weeks after the transections no 5-HT fibers were seen entering the scar tissue rostral or caudal to the transection, but 5-HT fibers were found at the border of this tissue (Fig. 6D). No clear differences were found in the 5-HT innervation (Fig. 6A) between the spinal cord sections rostral and caudal to the transections; only some reduction in fiber density was found immediately rostral and caudal to the transection. When a spinal cord section was isolated by two transections, the 5-HT fiber distribution remained unchanged (Fig. 7). For instance, the following densities of 5-HT fibers were found in the lateral half of the medial column in the dorsal plane (in 10^3 fibers/mm^2): rostral to transection 6.4 $\pm$ 0.4; isolated spinal cord 7.2 $\pm$ 0.9; caudal to transection 6.6 $\pm$ 0.6; intact spinal cord 6.6 $\pm$ 0.7 (data from *Lamp.*, mean $\pm$ S.E.M., counted in five-segment-isolated pieces).

Spinal ganglia and spinal nerves. After dorsal rhizotomy 5-HT fibers and 5-HT cells were present in the spinal ganglia (Fig. 5C), and 5-HT fibers were present in the stumps of the dorsal roots proximal to the spinal cord (Fig. 8B). Likewise, after ventral rhizotomy 5-HT fibers were still present in the proximal and distal stumps of the ventral roots, but their numbers could not be determined reliably since only a few complete stumps were present in sections of the isolated cord (Fig. 6B,C).

Tachykinins

Spinal cord

Cell bodies. Up till now single TK-immunoreactive cell bodies have been found in only a few animals, and they were present in the same location as the 5-HT cells. These TK cells also contained 5-HT.

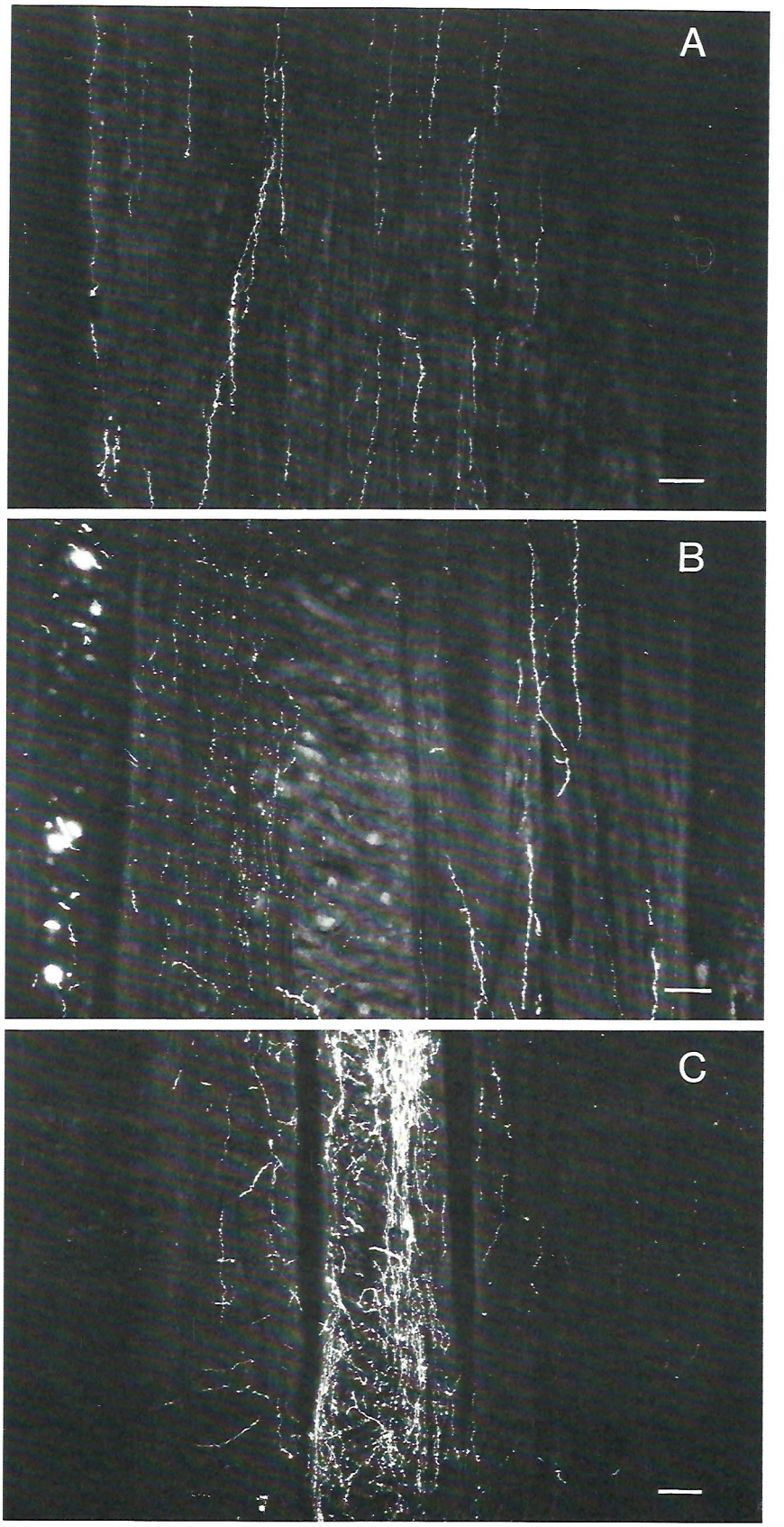

Fig. 7. 5-HT immunofluorescence in horizontal sections of the isolated spinal cord of *Lamp.* showing the same distribution as in the intact spinal cord. A. Dorsal plane. B. Intermediate plane. C. Müller fiber plane. For the meanings of the abbreviations see legends to Figure 2. Bars = 50 μm.

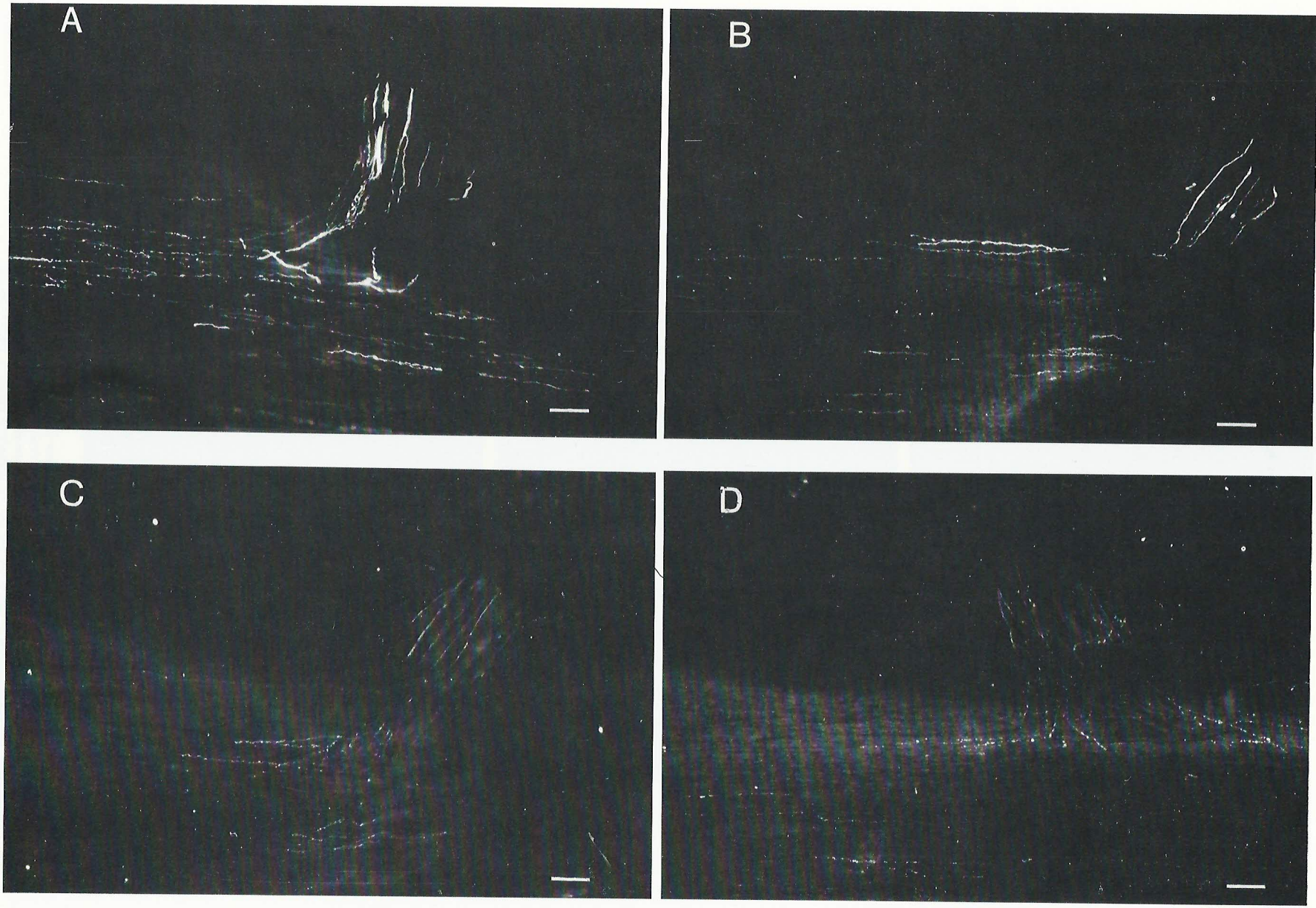

Fig. 8. Horizontal section through the dorsal plane of *Lamp.* showing dorsal roots in the intact and isolated spinal cord. A, B: 5-HT immunofluorescence. C, D: TK immuofluorescence. A, C: Intact dorsal roots. B, D: Transected dorsal roots. Bars = 20 μm.

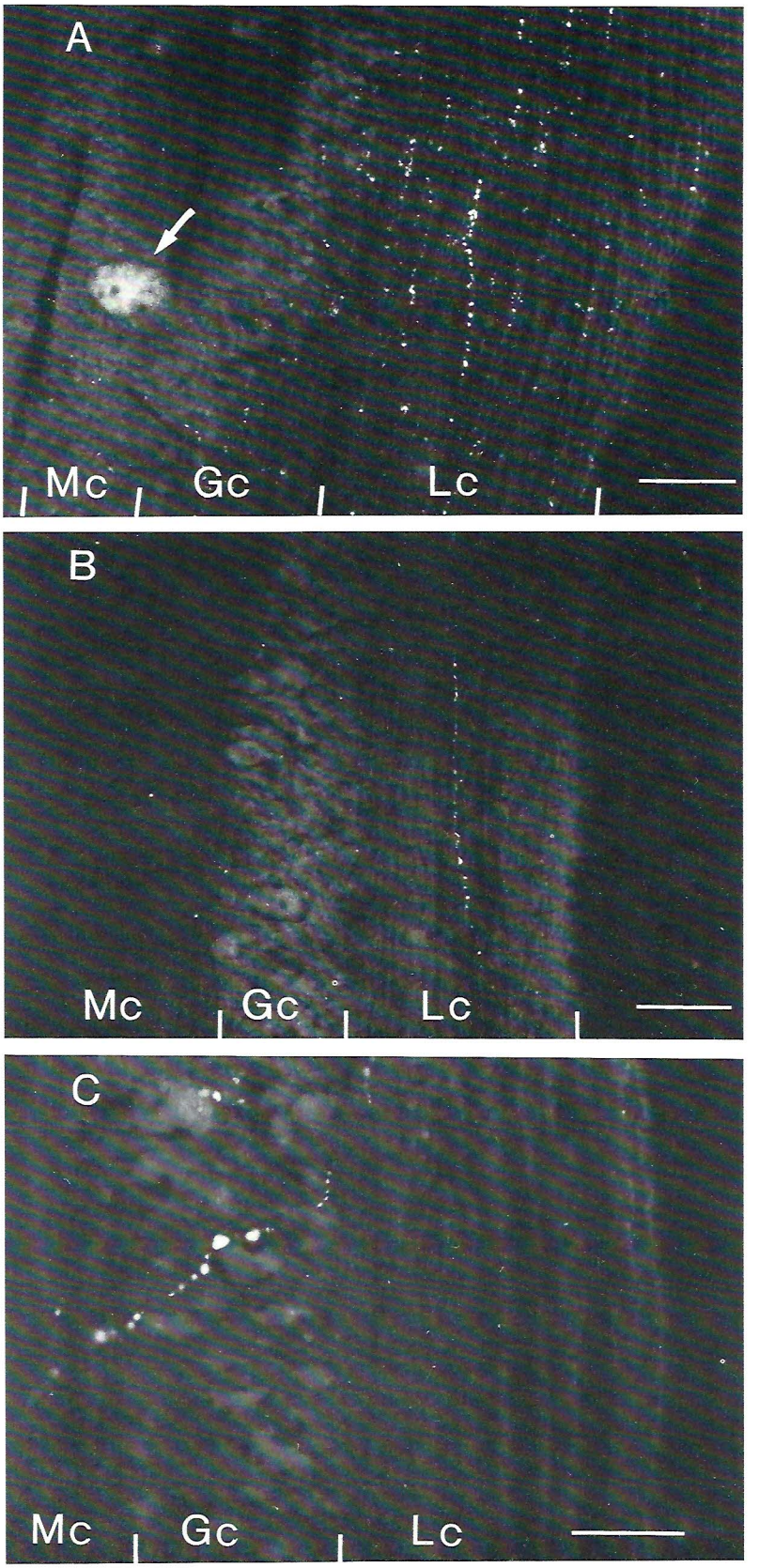

Fig. 9. TK immunofluorescence in horizontal sections through the spinal cord of *Ichth.* A. Dorsal cell plane containing an autofluorescent dorsal cell (arrow). B. Intermediate plane showing longitudinal TK fiber in the lateral column. C. Intermediate plane showing a mediolateral TK fiber in the gray column. For the meanings of abbreviations see the legends to Figure 2. Bars = 50 μm.

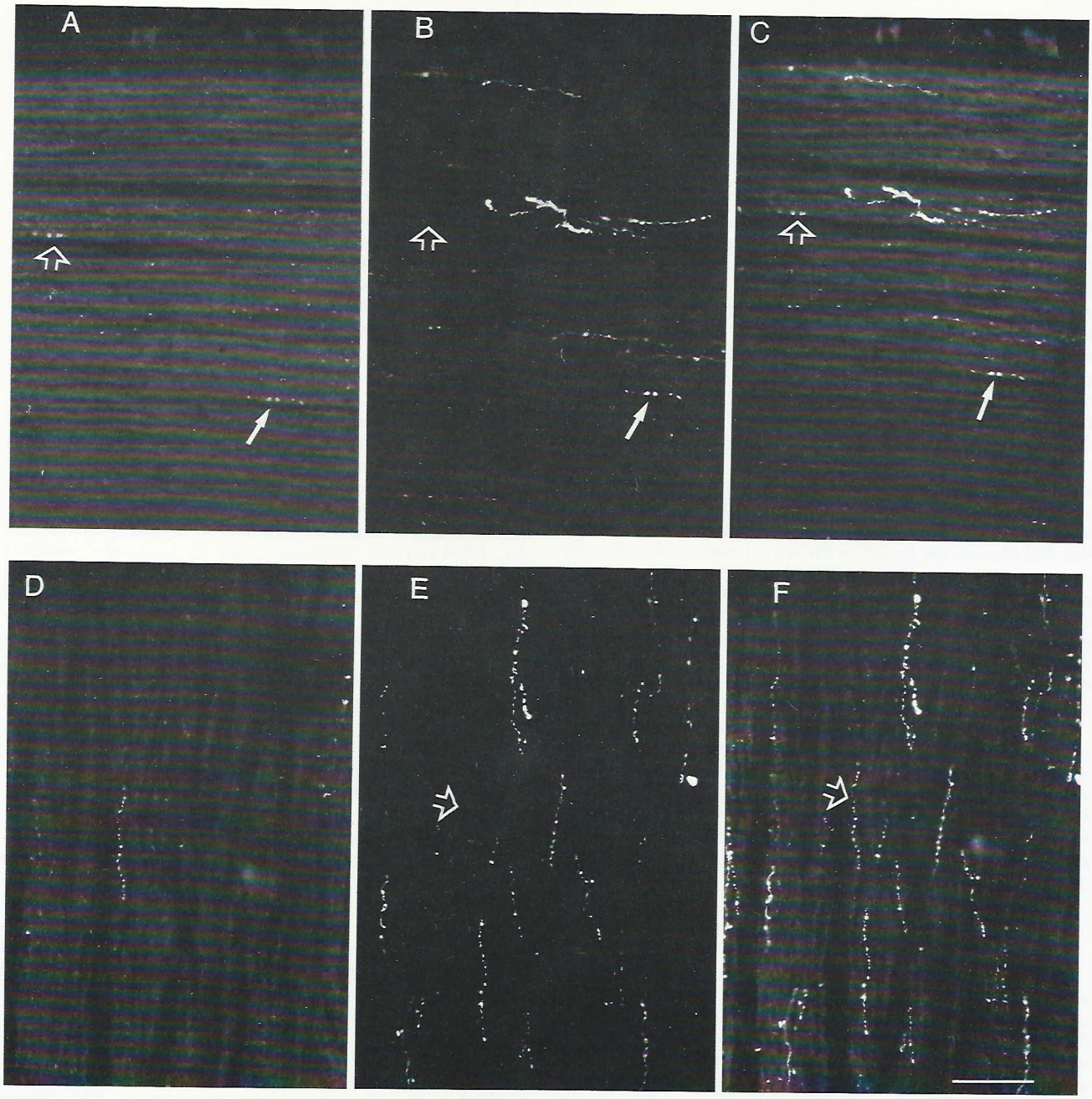

Fig. 10. Horizontal sections through the spinal cord of *Ichth.* A, D: TK immunofluorescence (TRITC, filter setting as in Methods). B, E: 5-HT immunofluorescence (FITC). C, F: Double exposures showing TK fibers containing 5-HT (closed arrows), and not containing 5-HT (open arrows). A–C. One coexistent fiber. D–F. No coexistence. Bar = 50 μm.

Fibers. The density of the TK fibers was usually much less than that of the 5-HT fibers (Fig. 10), while in some animals approximately the same number of fibers of both types were found. In the dorsal plane, almost exclusively longitudinal TK fibers were found (Fig. 8C), and they were mainly present in the lateral half of the dorsal column (density up to $7.6 \pm 2.2 \times 10^3$ fibers/mm^2). The TK fibers were less abundant in the mediodorsal and the dorsolateral axon columns (densities up to 2.7 ± 1.2 and $2.2 \pm 1.3 \times 10^3$ fibers/mm^2, respectively), and they could be followed over a distance of 0.3 mm or more. Ventral to the dorsal plane, the density of TK fibers was lower. In the dorsal cell plane (Fig. 9A), a few TK fibers were found in the medial column (i.e., where the sensory dorsal cells are located), and some longitudinal TK fibers were present in the dorsolateral axon column. No TK fibers were found in the

region of ependymal cells around the central canal. In the intermediate plane, a few predominantly longitudinal TK fibers were present (Fig. 9B). Some fibers ran between the cell bodies in the gray column (Fig. 9C).

5-HT and TK coexistence in fibers. In single sections incubated simultaneously with 5-HT antibodies and substance-P-antibodies, both types of immunofluorescence were simultaneously visible. They could be distinguished unambiguously by their color (Fig. 10D–F). Figure 10 demonstrates that cross-reactions between antibodies were negligible with the dilutions used: between the substance-P antibodies and 5-HT, between the 5-HT-antibodies and TK, between the rabbit-anti-guinea-pig immunoglobulins and rat-anti-substance-P, and between the rabbit-anti-rat immunoglobulins and guinea-pig-anti-5-HT. TK fibers (N = 124) have been inspected on the simultaneous presence of 5-HT. Of the fibers investigated, 119 contained TK but no 5-HT, while five contained both compounds (Fig. 10A–C). In these animals the number of 5-HT fibers was higher than that of TK fibers. Therefore, in even a smaller proportion (about 0.5%) of the 5-HT fibers, TK could be detected with the available antibodies and the technique used.

Spinal ganglia and spinal nerves. In the spinal ganglia some fine TK fibers were found. In some animals several fine TK fibers were found in the dorsal roots with a density comparable to that of the 5-HT fibers (Fig. 8C). Longitudinal TK fibers entered the dorsal roots both from the caudal and the rostral side. Some longitudinal fibers were seen to give off collaterals into the dorsal roots. Also the spinal ventral roots contained some varicose TK fibers. No TK fibers were found associated with the blood vessels around the spinal cord.

Spinal transections. Spinal transections did not cause a clear change in the spinal TK innervation immediately caudal or rostral to the point of transection. In the isolated spinal cord longitudinal TK fibers (Fig. 11B,C) and TK fibers in the proximal and distal stumps of the dorsal (Fig. 8D) and ventral roots were still present. After 4 weeks survival no TK fibers were seen entering the scar tissue (Fig. 11A).

Neuropeptide Y

Spinal Cord

Cell bodies. Several neurons containing NPY immunoreactive material have been found in the dorsal cell plane, in the dorsal parts of the medial and central canal columns (Fig. 12A,B). These neurons were small (12.3 $\pm$ 2.7 μm, in medium-sized *Ichth.*) and bipolar with medially and laterally oriented processes, and were present over the entire length of the spinal cord. The cells were visible in the intact material with a weak or moderately intense fluorescence, but the fluorescence was often stronger 4 weeks after spinal transections. About 20 NPY cells could be identified per segment.

Fibers. The highest density of NPY fibers was found in the dorsal plane in the lateral parts of the medial columns ($1.04 \pm 0.26 \times 10^4$ fibers/mm^2, cf. Figs. 12C, 13). These fibers were almost exclusively longitudinal (Fig. 12C). Longitudinal fibers were also found in the dorsolateral (Fig. 12B) and lateral axon columns (Fig. 12D), where the number was higher in the gill region than in the intermediate and caudal cord (Fig. 13). Some dorsoventral or mediolateral NPY fibers were found along the cell bodies in the gray column. The ventral regions of the spinal cord were virtually devoid of NPY fibers. No NPY fibers were found in the ependymal region around the central canal.

Spinal ganglia and spinal nerves. NPY neurons or fibers were found neither in the spinal ganglia and the spinal nerves nor associated with the blood vessels around the spinal cord.

Spinal transections. Four weeks after the spinal transections, NPY fibers were still present in the spinal cord rostral and caudal to the transections, and also in the isolated portions. Just rostral to the transection, however, both in the rostral portion and in the isolated portion, the NPY fibers were more intensely fluorescent (Fig. 14), indicating an accumulation of NPY in these fibers. Just caudal to the transection the number of NPY fibers and the intensity of the fluorescence were strongly reduced (Fig. 14C).

DISCUSSION

Distribution of 5-HT in the lamprey spinal cord

With the Falck-Hillarp and with immunofluorescence techniques 5-HT-like material has been detected in the lamprey spinal cord (Honma, '70; Baumgarten, '72; Ochi and Hosoya, '74; and this study). This material is probably genuine 5-HT (Filler et al., '83). The distribution of 5-HT-immunoreactive material was similar in *Lampetra fluviatilis*, *Ichthyomyzon unicuspis*, and in *Lampetra japonica* (Honma, '70; Ochi and Hosoya, '74). The 5-HT cell bodies have the characteristic shape and type of processes found in 5-HT neurons of other vertebrates (Sano et al., '82). In this study, we have confirmed and extended earlier findings observed with the Falck-Hillarp technique (Honma, '70; Baumgarten, '72; Ochi and Hosoya, '74) such as the presence of a ventromedial 5-HT plexus and 5-HT fibers in the dorsal and ventral roots (Honma, '70; Baumgarten, '72; Ochi and Hosoya, '74). The density of 5-HT varicosities in the lamprey 5-HT plexus is 10 to 100 times higher than in other regions of the lamprey spinal cord, and about 50 times higher than in the most densely innervated layer of the primate visual cortex (Takeuchi and Sano, '84). Immunohistochemistry is more sensitive than the Falck-Hillarp technique for visualization of 5-HT fibers, and additional fibers were demonstrated in the present study: longitudinal fibers in the dorsal, dorsolateral, lateral, and ventral axon columns (cf. Filler et al., '83). Since these axon columns contain dendrites of spinal neurons (Tretjakov, '09), these 5-HT fibers may make contacts with spinal neurons (Van Dongen et al., '85a).

A local intraspinal 5-HT system

No signs of degeneration of 5-HT or TK fibers have been found 4 weeks after the various transections. This allows two possible interpretations: (1) the number of 5-HT or TK fibers travelling over a distance of several segments beyond the transection in either direction is small, or (2) such fibers are present, but degeneration has not yet been taken place. In mammals and fish, however, the 5-HT fibers cut from the cell body are completely lost after 3–5 weeks (Carlsson et al., '64; Dahlström and Fuxe, '65; Anderson, '72; Oliveras et al., '77; Ritchie et al., '84), while after 4 weeks in the lamprey no signs of beginning deterioration were present in the 5-HT and TK fibers rostral or caudal to the spinal transections nor proximal or distal in the dorsal and ventral roots. With the same survival time massive degeneration has been found in other systems (cf. also Selzer, '78), and also several NPY fibers just caudal to the transections

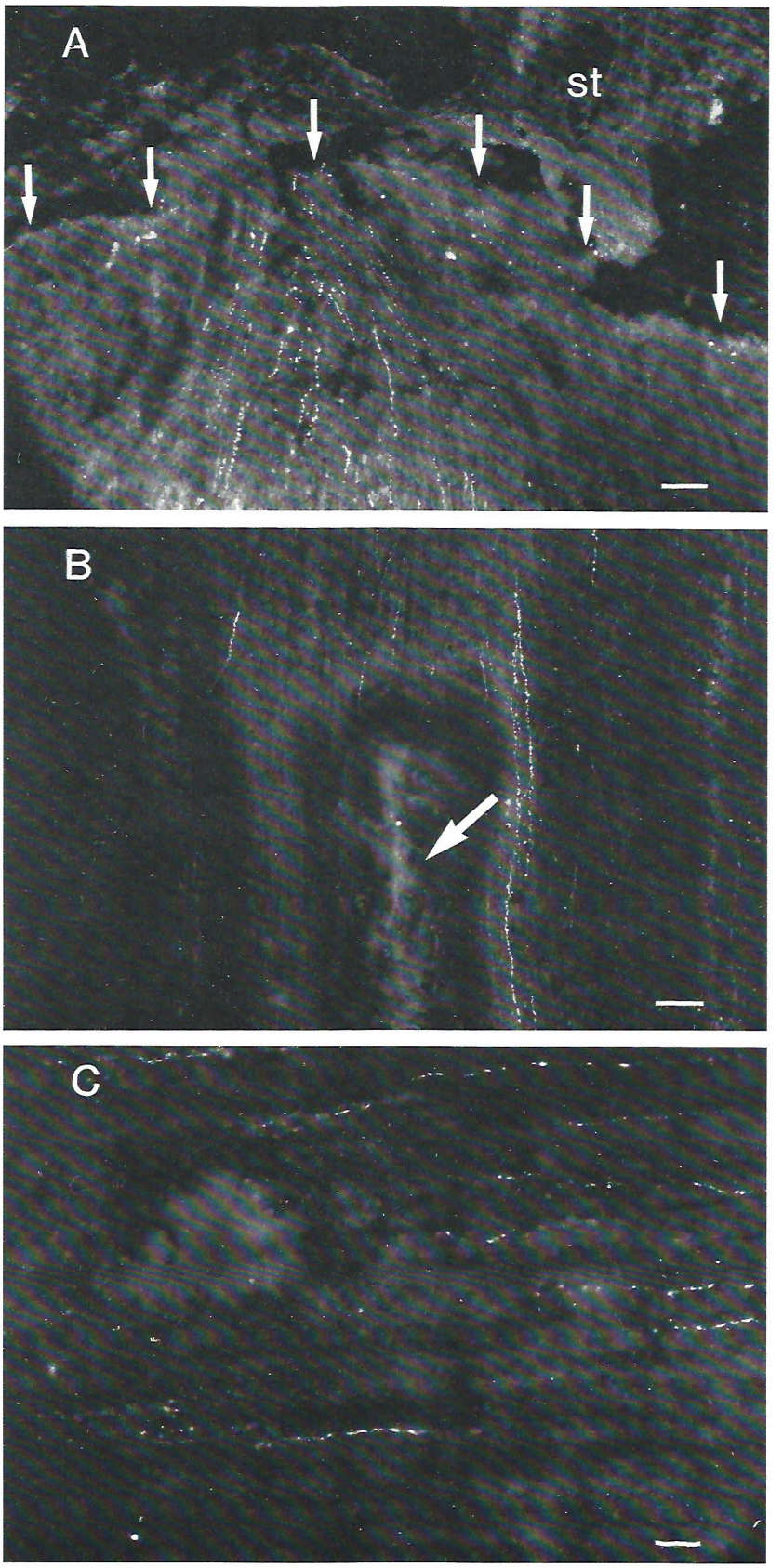

Fig. 11. TK immunofluorescence in horizontal sections of the isolated spinal cord of *Lamp*. A. Dorsal plane showing TK fibers in the spinal cord, not entering the scar tissue (st, arrows at the margin). B. Dorsal plane showing TK fibers and degenerated tissue (arrow). C. Dorsal cell plane showing longitudinal TK fibers. Bars = 50 μm.

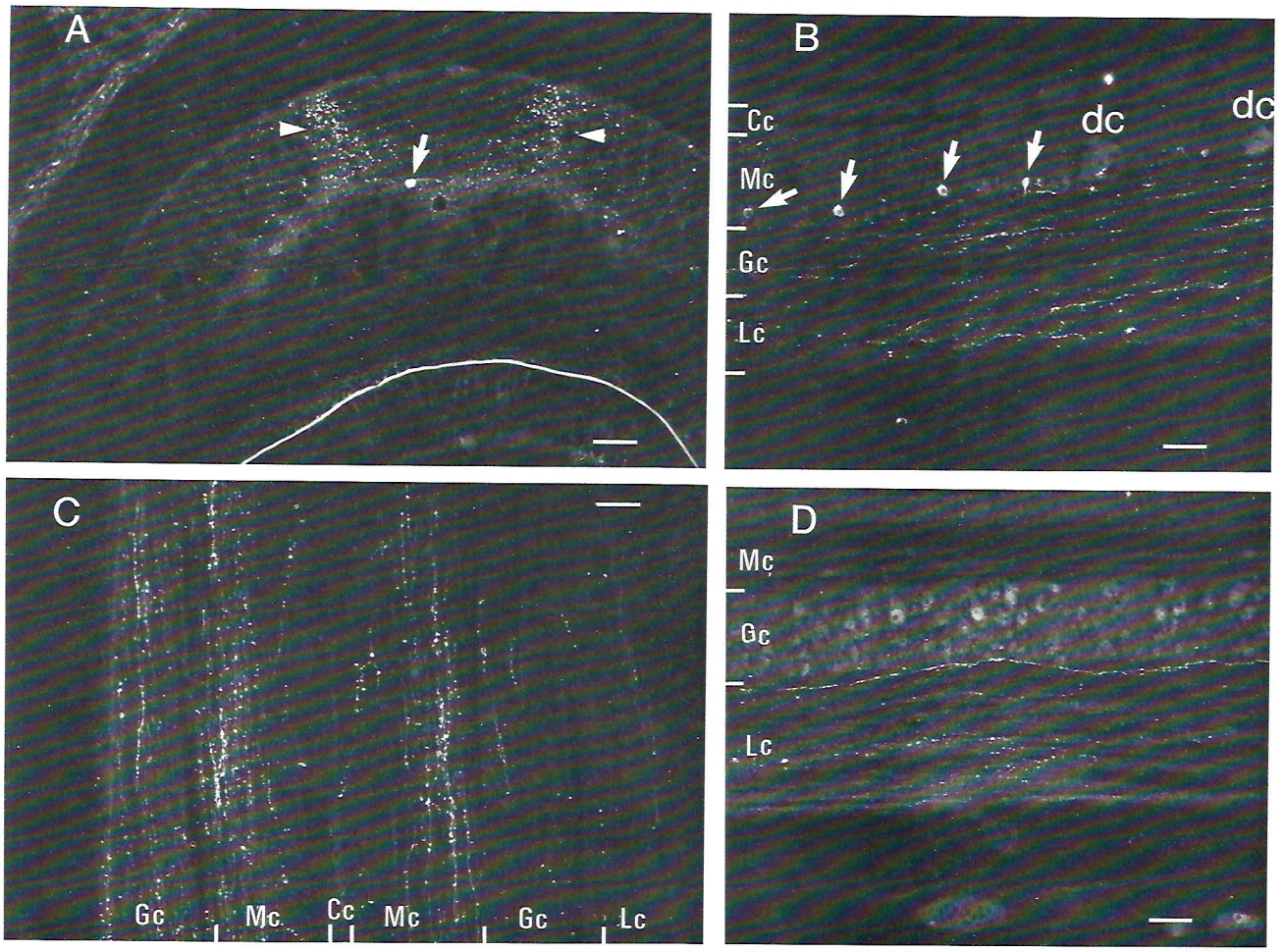

Fig. 12. NPY-like immunofluorescence in the spinal cord of *Ichth.* A. Transverse section showing one NPY immunoreactive neuron (arrow) and fibers mainly in the dorsal horns (arrow heads). B. Horizontal section through the dorsal cell plane showing NPY immunoreactive neurons (arrows) and sensory dorsal cells (dc). C. Fluorescent fibers in a horizontal section through the dorsal plane. D. Horizontal section through the intermediate plane of the gill region showing a few positive fibers in the lateral column. For the meanings of the abbreviations see the legends at Figure 2. Bars = 50 μm.

had disappeared. Moreover, in all regions where 5-HT fibers have been found after transection, also 5-HT cell bodies were present, and processes of these 5-HT cells have been found in all directions where 5-HT fibers were present. For all these reasons it appears likely that the 5-HT neurons in the lamprey spinal cord and ganglia provide the local 5-HT innervation. The proportion of longitudinal fibers travelling over many segments is probably small, since isolations of spinal pieces of five segments did not cause a change in the density of the longitudinal columns. The 5-HT innervation is probably not strictly segmental, since fibers have been observed to continue their longitudinal course beyond the place of entrance of the dorsal roots. In the lamprey we did not find any indication for descending 5-HT fibers originating from the brain.

Comparision with other vertebrates

As in lampreys, the spinal cords of elasmobranchs (Atlantic stingray), holosteans (garfish), some teleosts (eel but not sunfish) and salamanders contain 5-HT neurons (Lefranc et al., '70; Parent et al., '78; Gruberg and Harris, '81; Parent and Northcutt, '82; Ritchie et al., '83). As in the lamprey, they are found ventromedial to the central canal, and only in the eel also lateral and dorsal to the central canal. The 5-HT neurons in the spinal cord of the lamprey, the Atlantic stingray, and the garfish seem to innervate mainly motor parts of the spinal cord (Parent and Northcutt, '82; Ritchie et al., '84; Van Dongen et al., '85a). The majority of the spinal 5-HT fibers in the Atlantic stingray, however, originate in the brainstem (Ritchie et al., '84), and in mammals almost all spinal 5-HT fibers appear to have their cell bodies in the brainstem (cf. Dahlström and Fuxe, '65; Hökfelt et al., '78; Steinbusch, '81; Bowker et al., '82). More recently, however, evidence has been provided for the existence of 5-HT neurons in the spinal cords of young opossums and monkeys treated with tryptophan and pargyline (LaMotte et al., '82; DiTirro et al., '83). Similarities between body segments are usually regarded as primitive, while segmental differentiation is considered to be evolutionary advanced. A decrease in the intraspinal contribution to the

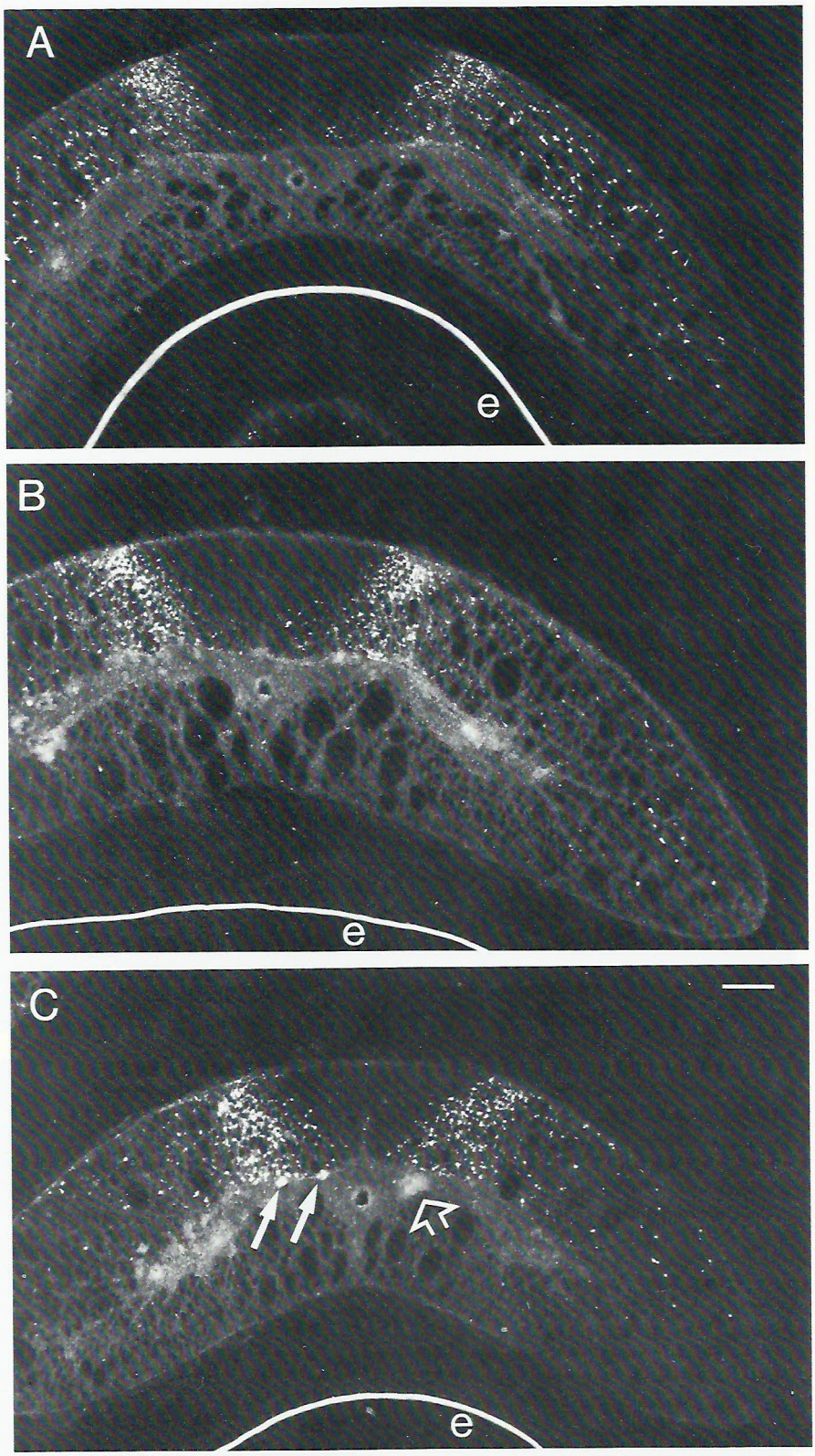

Fig. 13. NPY-immunoreactive material in transverse sections of the spinal cord of *Ichth.* e, elastica of the notochord. A. Gill region. B. Intermediate region. C. Caudal region, containing NPY cell bodies (closed arrows) and an autofluorescent dorsal cell (open arrow). The dorsolateral and lateral columns in the gill region contain more NPY fibers than in the other regions. Bar = 50 μm.

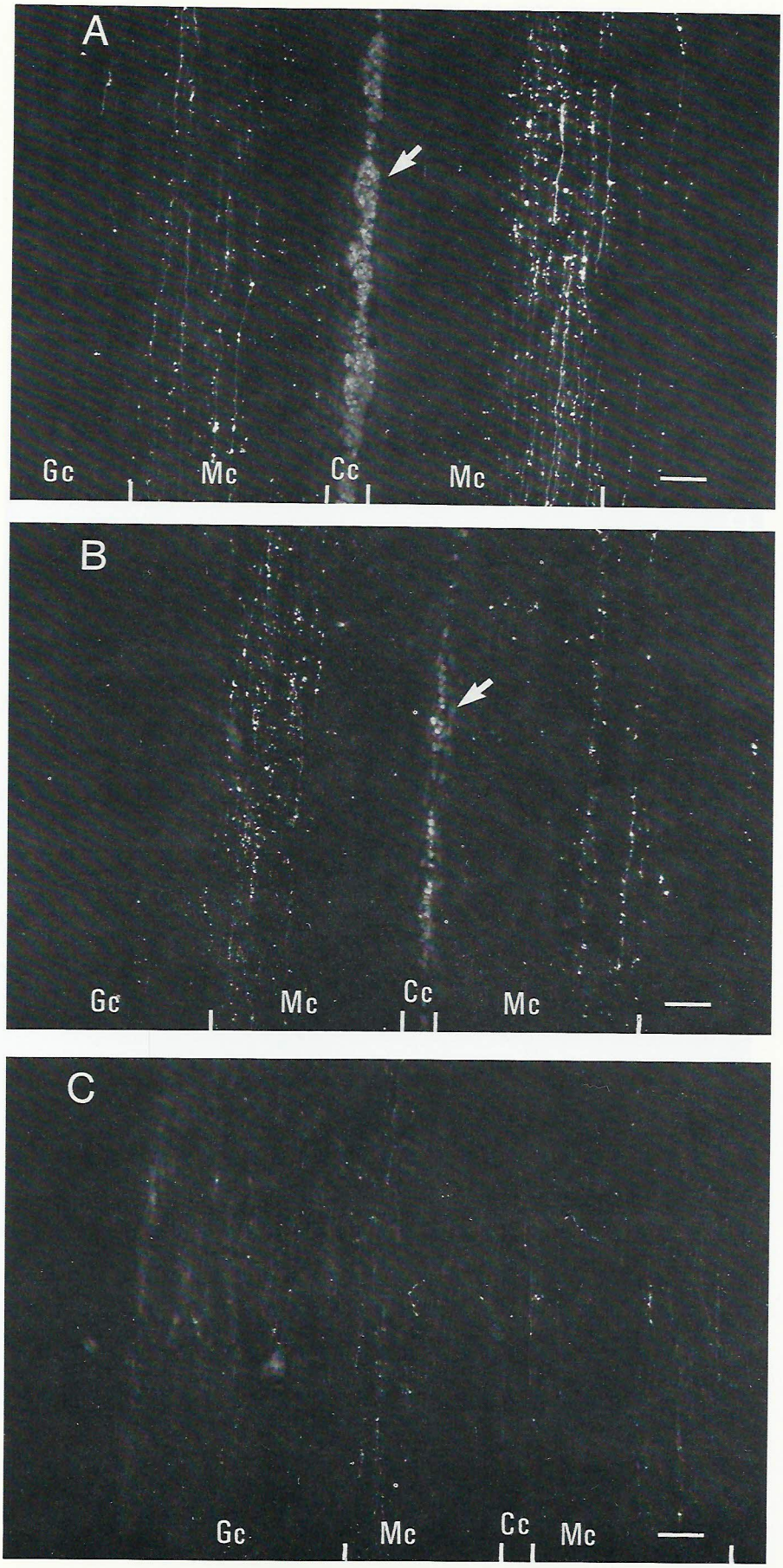

Figure 14

spinal 5-HT innervation and an increase in the supraspinal contribution has been found in the series lamprey, stingray and rat. In this respect, the lamprey is the most primitive vertebrate which has been investigated.

5-HT in spinal ganglia and spinal nerves

Earlier findings have been confirmed in this study with regard to the presence of large and small neurons in the lamprey spinal ganglia (Freud, 1878; Johnels, '56); many of the small neurons now appear to contain 5-HT. In earlier studies several small neurons were described in the branches of dorsal and ventral roots, and in the caudolateral nerve, which were regarded to be "sympathetic" or "autonomic" or "vegetative neurons," and their axons were reported to follow the blood vessels (Freud, 1878; Tretjakoff, '27; Johnels, '56). Many small, 5-HT-containing cell bodies have been found in various nerves, but 5-HT axons only rarely followed the blood vessels around the spinal cord. In the isolated spinal cord the 5-HT fibers in the proximal stumps of the dorsal and ventral roots and in the medial columns of the dorsal plane had a normal appearance 4 weeks after dorsal or ventral root transections; 5-HT fibers did not enter the scar tissue of the transection after the same amount of time. Since it is reasonable to assume that transected 5-HT fibers distal to the cell body have degenerated after this period of time (see above), it appears likely that spinal 5-HT cells send their fibers into the dorsal and ventral roots. Whether or not the small 5-HT neurons in the spinal ganglia extend their axons into the spinal cord is not clear. Other authors have described fibers leaving the lamprey spinal cord via the dorsal roots (Johnels, '56). The dorsal roots of mammals (cat) are reported to contain 5-HT fibers, and these fibers might leave rather than enter the spinal cord (Di Carlo, '83).

Tachykinins in the lamprey spinal cord

Evidence has been presented that substance P is a neurotransmitter (cf. Pernow, '83), and it has been speculated that other tachykinins also can be used as neurotransmitters. With antibodies to substance P we could detect substance-P-like immunoreactive material in fibers of the lamprey spinal cord. The peptide detected by this antibody in the lamprey spinal cord is probably not substance P (Van Dongen et al., '85b), and studies to characterize it are in progress. Although TK cell bodies could so far be detected in only a few animals, it is likely that they are a constant feature since TK fibers always were found in the isolated spinal cord. For reasons mentioned above it can be assumed that the TK cells provide a local spinal TK innervation. The lamprey dorsal roots contain fine TK fibers, which at least partly originate in the spinal cord.

Comparision with other vertebrates

In the spinal cord of the Atlantic stingray fibers containing substance-P-like immunoreactive material have been found (Ritchie and Leonard, '83): part of these fibers probably arise from cell bodies in the dorsal root ganglia, and part of them are probably of intraspinal origin, while no descending TK fibers have been found. The amphibian Rohon-Beard cells are probably a homologue of the lamprey sensory dorsal cells (Rovainen, '79) and have been found to contain substance-P-immunoreactive material (Clarke et al., '84). However, no TK-immunoreactive material has been found in the lamprey sensory dorsal cells, and moreover, the TK fibers in the lamprey spinal cord have a different shape and location than the axons of the sensory dorsal cells. The spinal cord of mammals contains substance P cell bodies and afferent substance P fibers originating in the dorsal root ganglia and in the brainstem (cf. Pernow, '83). So in contrast to mammals, the spinal TK innervation in the lamprey and the Atlantic stingray is segmental and primitive.

5-HT and tachykinin coexistence

Only a small proportion (about 4%) of the presently detected TK fibers also contained 5-HT. Since 5-HT fibers could be demonstrated reliably, it seems likely that most of these TK fibers do not contain 5-HT. It is, however, uncertain whether or not a larger proportion of the 5-HT fibers than those presently detected also contain TK, since the detection of TK fibers is less reproducible. It is our impression that several TK fibers escape detection by the presently available methods. In any case, coexistence between 5-HT and TK-peptides is not restricted to mammals (Chan-Palay et al., '78; Hökfelt et al., '78; Johansson et al., '81), but is also found in vertebrates as primitive as the lamprey.

NPY

NPY could be a neurotransmitter, since evidence has been presented that it is present in neurons, is released by discharging neurons, and has a postsynaptic action (cf. Everitt et al., '84; Emson and De Quidt, '84). Both cell bodies and fibers containing NPY-like immunoreactive material were found in the lamprey spinal cord, but not in the spinal ganglia. Data are not presently available on whether or not this peptide is NPY or a cross-reacting peptide perhaps belonging to the pancreatic polypeptide family (see Lin and Chance, '74; Kimmel et al., '75; Tatemoto, '82). After spinal transections and isolations, NPY fibers (and neurons) have been found in all spinal parts, but accumulation of NPY was found immediately rostral to the transection. Therefore, it is concluded that the NPY neurons provide the local spinal NPY innervation, but primarily innervate the parts of the spinal cord caudal to the cell bodies.

CONCLUSIONS

The present study provides evidence for the existence of extensive local 5-HT neuron systems in the lamprey spinal cord and in some peripheral tissues, as well as of more limited systems containing a TK-like and a NPY-like peptide (cf. Fig. 15). In some cases, the TK may coexist with 5-HT. Overall the number of neurons containing putative transmitter peptides found in the spinal cord of the lamprey

Fig. 14. NPY-immunoreactive fibers in horizontal sections through the dorsal plane of *Lamp.* A. Just rostral to transection, showing an increase in NPY fluorescence. B. Caudal part of the isolated cord, showing some increase in NPY fluorescence. C. Just caudal to the transection, showing a reduction in the NPY fluorescence. The pictures are taken with emission filter LP 520, making autofluorescent degeneration material (arrows) visible in A and B in the midline; this is the top of the autofluorescent triangle shown in Figure 7A. For the meanings of the abbreviations see the legends to Figure 2. Bar = 50 μm.

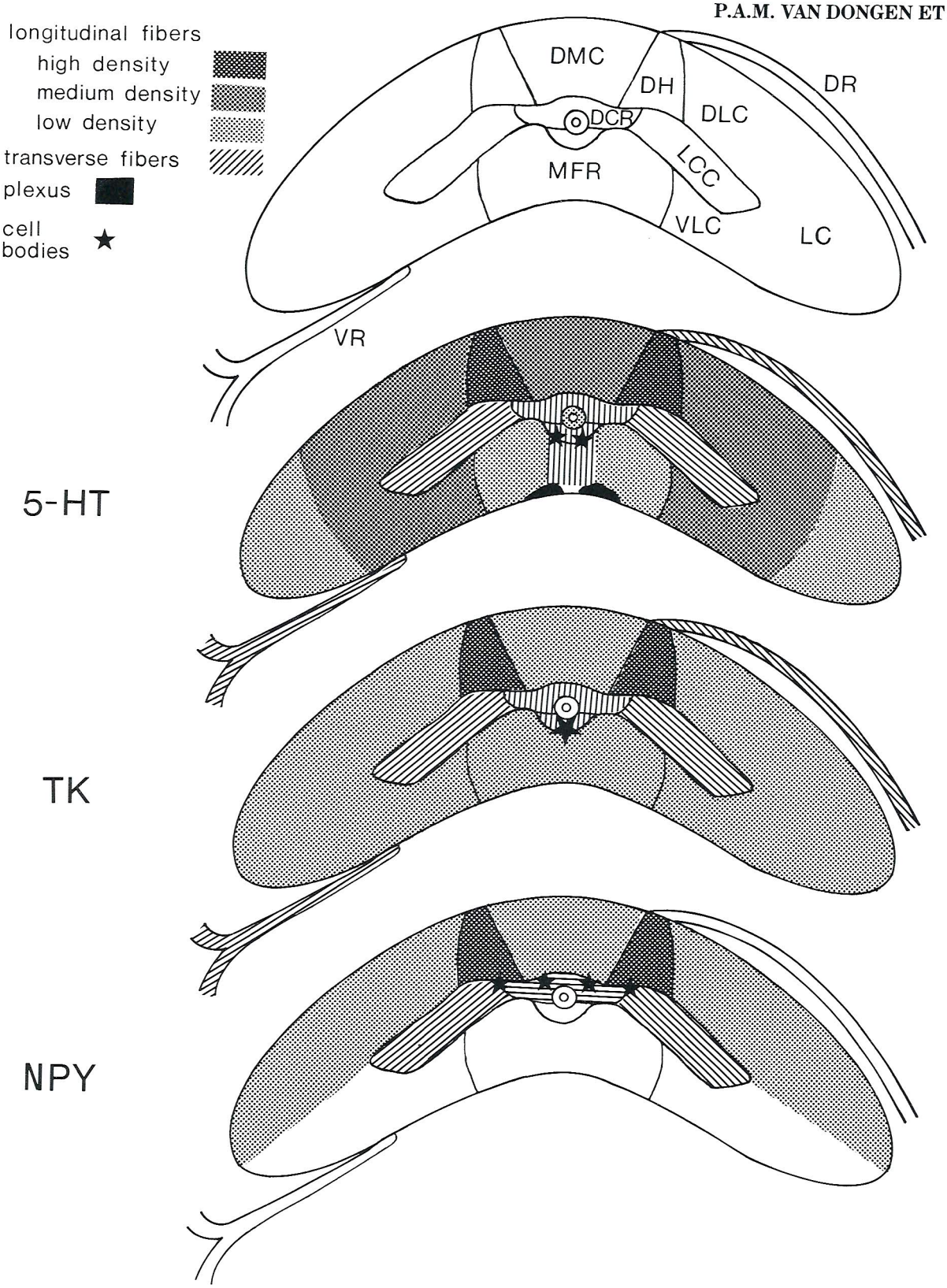

Fig. 15. Schematic survey of the innervation of the lamprey spinal cord by 5-HT, TK, and NPY; the shape and the proportions of the parts are indicated as they are in the spinal cord in situ. The predominant orientation of transverse fibers is indicated by the direction of the lines. DCR, dorsal cell region; DH, dorsal horn; DLC, dorsolateral column, DMC, dorsomedial column; DR, dorsal root; LC, lateral column; LCC, lateral column of cell bodies; MFR, Müllller fiber region; VLC, ventrolateral column; VR, ventral root.

is small as compared to, for example, the rat (see Hökfelt et al., '80). Whether the number of neurons is smaller or whether peptides present in the lamprey do not cross-react with antisera raised to mammalian peptides and thus escape detection remains to be elucidated.

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NOTE ADDED IN PROOF

Harris-Warrick et al. have found a certain reduction in 5-HT fiber content caudal to a spinal transection (15°C, 2 weeks), which they interpret to imply the presence of a descending 5-HT system in the lamprey spinal cord (Neurosci., in press). This is at variance with the findings reported here, and those of additional control experiments with lampreys kept at 14°C for four weeks after the spinal transection. The reason for this discrepancy is unclear.