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Possible Target Neurons of 5-Hydroxytryptamine Fibers in the Lamprey Spinal Cord: Immunohistochemistry Combined With Intracellular Staining With Lucifer Yellow

PAUL A.M. VAN DONGEN, TOMAS HÖKFELT, STEN GRILLNER, ALBERT A.J. VERHOFSTAD, AND HARRY W.M. STEINBUSCH

Departments of Histology (P.A.M.V.D., T.H.) and Physiology III (S.G.), Karolinska Institute, Stockholm, Sweden, Department of Anatomy and Embryology, Catholic University, Nijmegen, The Netherlands (A.A.J.V.), and Department of Pharmacology, Free University, Amsterdam, The Netherlands (H.W.M.S.)

ABSTRACT

Intracellular recordings were made from 76 neurons belonging to various cell types in the lamprey spinal cord, and these neurons were subsequently stained with Lucifer yellow. Sections were made of spinal cords containing Lucifer-yellow-filled neurons, and in the same sections 5-hydroxytrytamine (5-HT)-containing neurons and fibers were made visible with immunohistochemical methods. Motoneurons and lateral cells appeared to send part of their dendrites into a dense ventromedial 5-HT plexus, and these dendrites were adjacent to 5-HT varicosities. No or few 5-HT varicosities have been found adjacent to cell bodies or dendrites of sensory dorsal cells, giant interneurons, and edge cells. The combined application of intracellular staining and immunohistochemistry appeared to be suited to screen for possible transmitter-identified contacts on morphologically identified neurons.

Key words: 5-hydroxytryptamine, Lucifer yellow, intracellular staining, lamprey, spinal cord, identified neurons

In the lamprey spinal cord numerous 5-hydroxytryptamine-(5-HT)-containing neurons and fibers are present (Baumgarten, '72; Van Dongen et al., '85). Many longitudinally oriented 5-HT fibers are seen in the various "axon columns," into which the dendrites of the spinal neurons extend, and a dense ventromedial 5-HT plexus has been found. As a basis for future electrophysiological studies aiming at understanding the action of 5-HT in the lamprey spinal cord, it is of interest to know the target neurons of the 5-HT fibers, as well as the type of contacts involved. The combined application of intracellular staining and immunohistochemistry (Kawata et al., '83; Reaves et al., '83; Hoffert et al., '83) is a suitable approach to investigate this problem. Intracellular staining with horseradish peroxidase (HRP; Snow et al., '76) or with Lucifer yellow (LY; Stewart, '78) make virtually all processes of neurons visible (Takato and Goldring, '79). However, for a combination with immunofluorescence, LY is more appropriate. In this study, intracellular recordings were made from various neurons in the lamprey spinal cord, and LY was injected intracellularly by ionophoresis. Here we report data on the relationship between 5-HT fibers and sensory dorsal neurons, motoneurons, giant interneurons, lateral cells, and edge cells (cf. Rovainen, '79).

MATERIALS AND METHODS

Animals

Eleven silver lampreys (*Ichtyomyzon unicuspis*, 0.15–0.32 m) were used, obtained from Iowa (USA). They were kept in aerated aquaria at a temperature of 5–10°C.

Preparation

The preparation was mainly performed according to Cohen and Wallén ('80). Pieces (0.06 m) of the spinal cord were dissected from the region of the dorsal fins (n = 5), or the region between the last gill opening and the dorsal fins

Address reprint requests to: T. Hökfelt, Dept. of Histology, Karolinska Institute, P.O. Box 60400, S-104 01 Stockholm, Sweden.

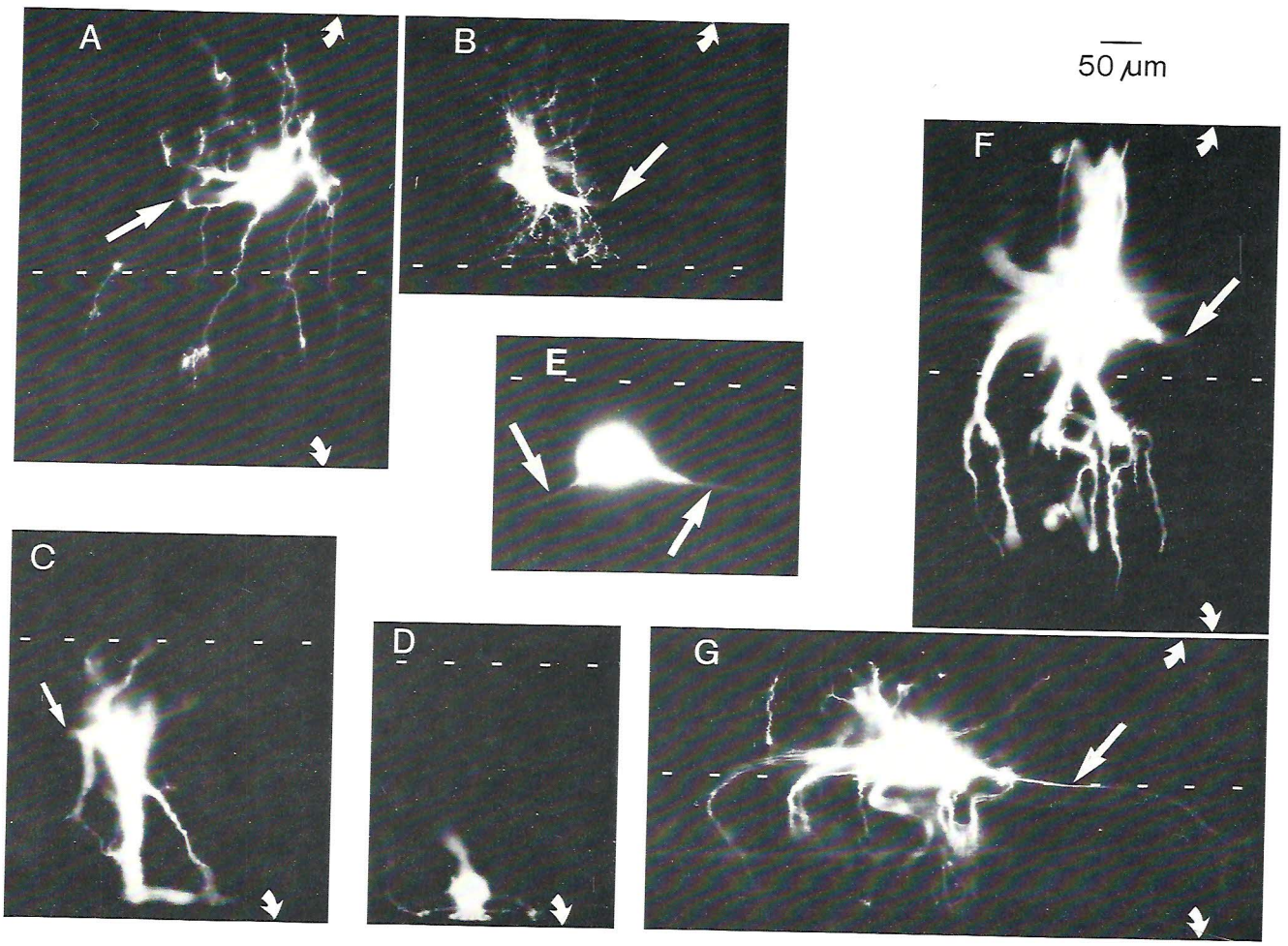

Fig. 1. Micrographs of various types of LY-filled neurons in whole-mount preparations of the spinal cord (horizontal cords); the edge of the spinal cords is indicated by a curved arrow, the midline by a stippled line, and the axon (if visible in the micrograph) by a straight arrow (all micrographs of this figure at the same magnification). Only a part of the neuron could be in focus due to the thickness of the spinal cord (200 μm). A, B. Motoneurons. C. Lateral cell. D. Edge cell. E. Sensory dorsal cell. F, G. Giant interneurons.

(n = 6), and placed into the experiment chamber. Under microscopic control, the perimeningeal tissue and the meninx primitiva dorsal to the cord were removed, the ventral roots were cut, and the spinal cord was removed from the notochord. The spinal cord was placed ventral side up, stretched to its in situ length, and fixed with miniature "staples" which were placed over the cord. The ventral perimeningeal tissue and meninx primitiva were also removed. Neuronal cell bodies were clearly visible with the dissection microscope in the transilluminated spinal cord, which aided the microelectrode impalement. Spinal cords dissected this way were used for 2 days.

Recordings

Intracellular recordings were made with glass micropipettes filled with 5% Lucifer yellow CH (LY, Sigma) in 0.1 M LiCl. Occasionally, a part of the spinal cord was damaged during the dissection; no recordings were made from damaged segments. When stable recordings were obtained from neurons with resting potentials below at least −40 mV, and action potential amplitudes of at least 45 mV, the cells were filled with LY by ionophoresis with a negative current of 2.5–4.0 nA for 30 minutes. Within 3 hours after completion of the dye injection, a piece of spinal cord (three to ten segments) containing the LY-filled neurons was removed from the experiment chamber and fixed (see below).

Histochemistry

The histochemical preparation was performed mainly according to Van Dongen et al. ('85). A piece of spinal cord with LY-filled neurons was put on a small piece of sylgard gel, stretched with miniature needles to its in situ length, and fixed. Wholemounts of these spinal cords with LY-filled neurons were inspected with a Zeiss fluorescence microscope (see below). The LY-filled cells were classified (see below), and pictures were taken at different focal depths. Horizontal serial cryostat sections (14 μm) were made, processed for 5-HT immunofluorescence (Steinbusch et al. '78) with tetramethyl-rhodamine-isothiocyanate (TRITC)-conjugated second antibodies (cf. Coons, '58; Hökfelt et al., '73, '75, '83), and inspected with the microscopic equipment described earlier (Van Dongen et al., '85). For LY, the follow-

TABLE 1. Classification of Lucifer-Yellow-Filled Neurons, According to the Criteria Mentioned in the Methods-Section

	Nos. of neurons
Classified	39 (34)
Sensory dorsal cells	4 (4)
Motoneuronlike cells	18 (16)
Giant interneurons	3 (2)
Lateral cells	4 (4)
Edge cells	7 (7)
5-HT cells	3 (1)
Unclassified	37 (29)
Small cell body	6 (6)
Medium cell body	31 (23)
Axon identified	16 (14)
Axon not identified	21 (15)
Ipsilateral dendrites	34 (27)
Bilateral dendrites	3 (2)
Total	76 (63)

The nos. between parentheses indicate the nos. of LY-filled neurons with intact 5-HT immunostaining.

ing Zeiss filters were used: excitation filter KP 500, and emission filter LP 520 and sometimes also either BP 546 or KP 560 to reduce the TRITC fluorescence; and for TRITC: excitation filter BP 546 and sometimes also LP 455 to reduce LY fluorescence, and emission filter LP 590 (Hökfelt et al., '83). Single or sequentially exposed black-and-white (Tri-X, Agfa-Gevaert) pictures were made with filter settings for TRITC (5-HT) and LY. In one series, which included 13 LY-filled neurons, no 5-HT immunoreactive elements could be made visible (probably due to the fixation); however, the distribution of dendrites and the general shape of these neurons were similar to those obtained in sections where 5-HT immunoreactivity was observed. Complete series of photonegatives or color slides were taken of some neurons. These were projected, and the LY-filled contours of the sections were drawn superimposed upon each other. Thereby, projections on a horizontal plane of neurons and all their processes were obtained ("reconstructions").

Classification

The LY-filled neurons were classified according to the following criteria (cf. Rovainen, '79; Figs. 1, 10).

Sensory dorsal cells. Sensory dorsal cells are large neurons located in the medial columns (cf. Rovainen, '67; Martin and Wickelgren, '71). These cells are bipolar, having a rostrally and a caudally directed ipsilateral axon, and often a few short, dendritelike processes (Boman, Christensen and Grillner, unpublished).

Motoneurons. Motoneurons are medium-sized neurons located in the lateral gray column with an axon that can be followed into the ventral root. Such neurons have been found to innervate muscle fibers (Teräväinen and Rovainen '71; Wallén et al., '85), but it cannot be entirely excluded that some of these neurons innervate the viscera (Johnels, '56). Neurons whose axons were seen to turn toward the ventral roots have been included, even if the axons could not be traced out into the roots.

Giant interneurons. Giant interneurons are very large neurons located in the caudal two-thirds of the spinal cord (Rovainen '67, '74). They have large dendrites that extend over the whole mediolateral width of the spinal cord ipsi- and contralateral to the cell body. They also have a prominent axon that ascends, gradually turning to the side of the spinal cord contralateral to the cell body. These axons could be followed to the most rostral part of the piece of spinal cord used in this study.

Edge cells. Edge cells are medium-sized neurons located in the lateral column, situated near the lateral margin of the spinal cord (Rovainen, '74). The cells have processes extending to the most lateral margin of the spinal cord, where these processes form nestlike ramifications (Grillner et al., '84). Edge cells have ipsilateral and sometimes also contralateral dendrites. Most edge cells have ascending axons.

Lateral cells. Lateral cells are neurons located in the rostral part of the spinal cord (rostral to the cloaca) with a large elongated cell body that extends into the lateral axon column (Rovainen, '74). Lateral cells have ipsilateral dendrites. Only cells having descending axons are regarded as "lateral cells" (cf. Rovainen et al., '73).

5-HT cells. 5-HT cells are small (generally less than 15 μm) midline neurons located ventral to the central canal (Baumgarten, '72; Van Dongen et al., '85).

Unclassified neurons. A heterogeneous group of neurons (41%) did not fulfill the above-mentioned criteria (cf. Table 1).

RESULTS

Lucifer-yellow-stained neurons

Of 76 well-filled neurons, 39 could be classified in one of the categories described above (Table 1). With the use of excitation filter KP 500 and emission filter LP 520, the red TRITC (5-HT) fluorescent cell bodies and fibers and the yellow LY-filled neurons were simultaneously visible. This is best shown on color prints, but it is possible to demonstrate this with three black-and-white prints (see below). To establish the presence of 5-HT varicosities adjacent to the LY-filled neurons, all sections were scanned using a 400× magnification in order to find 5-HT fibers and LY-filled elements with a sharp image in the same focal depth. If the distance between a 5-HT varicosity and a LY-filled cell body or dendrite was 1 μm or less, it was called an "adjacent varicosity."

Motoneurons **(n = 18).** Generally, 5-HT varicosities were found to be located at a distance from motoneuronal cell bodies (Fig. 2A), which always was larger than 2 μm. All 18 motoneurons extended several (ten to 15) small (about 1.4 μm in diameter) ventromedial dendritic branches into the very dense 5-HT plexus (Figs. 2B, 9). Several 5-HT varicosities were seen adjacent to motoneuronal dendrites in this region, which was not the case in other regions. In our sample, 16 motoneurons had a dendritic tree that extended over less than 200 μm in a rostrocaudal direction (Fig. 1B), while the dendritic tree of two of these neurons extended over some 500 μm (Fig. 1A). These two groups might correspond to motoneurons that supply the ventral myotomes on the one hand, and the dorsal myotomes (Wallén et al., '85) or the dorsal fin muscles on the other hand (Rovainen and Birnberger '71). Neurons of both groups had dendrites which extended into the ventromedial 5-HT plexus. The medial dendrites of four motoneurons extended into the region of the 5-HT cell bodies; the distance between these dendrites and the 5-HT cell bodies could be less than 2 μm (Fig. 3).

Lateral cells **(n = 4).** Usually, 5-HT varicosities remained remote from the somata of the lateral cells: the distance was rarely less than 2 μm and always more than 1 μm (Fig. 4A). Three out of the four lateral cells were seen to send one or two dendrites from the cell body toward the

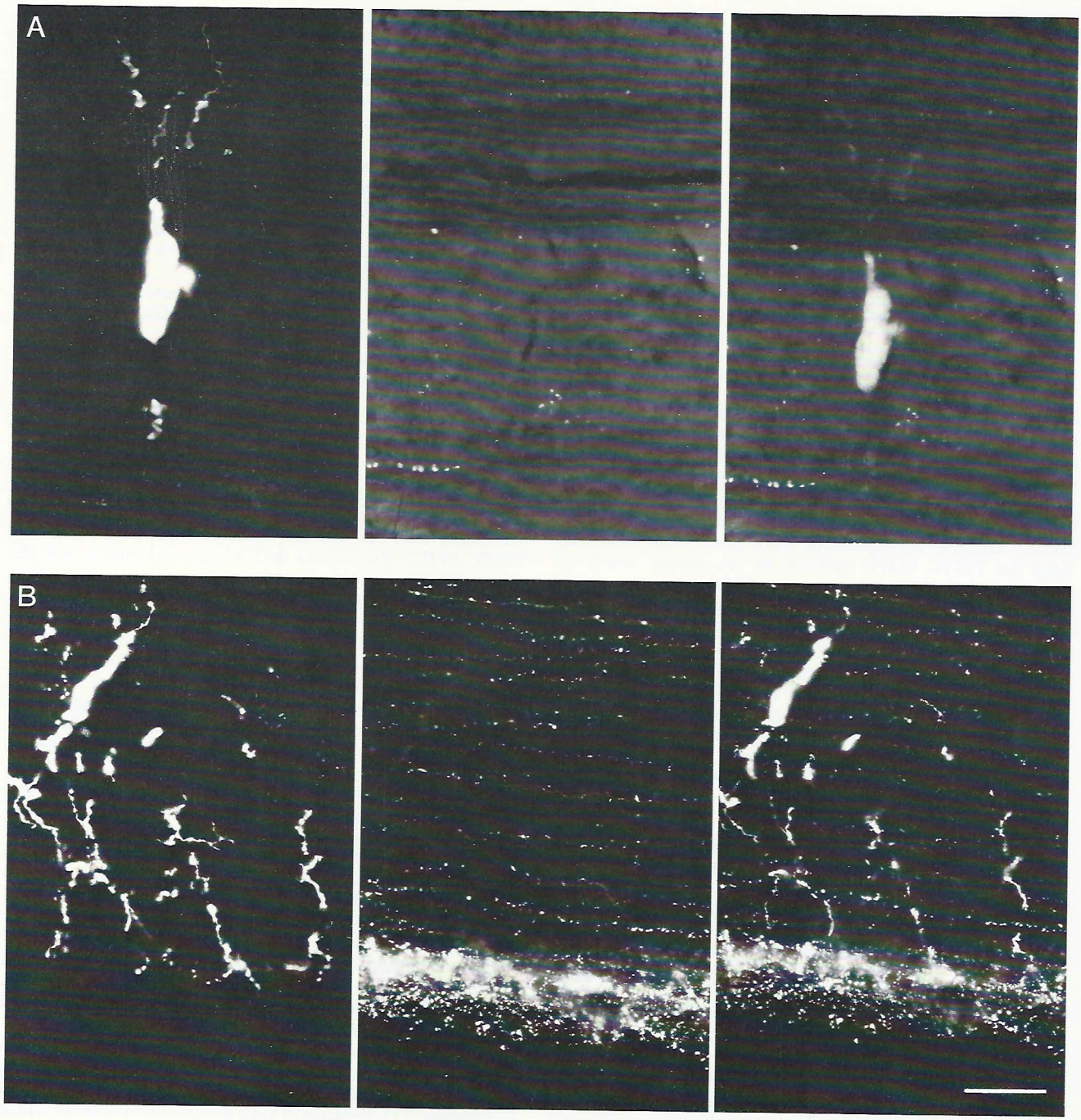

Fig. 2. Motoneurons in horizontal sections. A. Cell body in the intermediate plane. B. Ventral dendrite in the Müller-fiber plane; note the ventromedial dendrites in the 5-HT plexus. In Figures 3–7, the following protocol applies. Left: LY (exc. filter KP 500, emiss. filter LP 520 plus KP 560). Middle: 5-HT (exc. filter BP 546 often plus LP 455, emiss. filter LP 590). Right: double exposure showing both LY and 5-HT (all micrographs of the Figures 2–8 are at the same magnification). Bar = 50 μm.

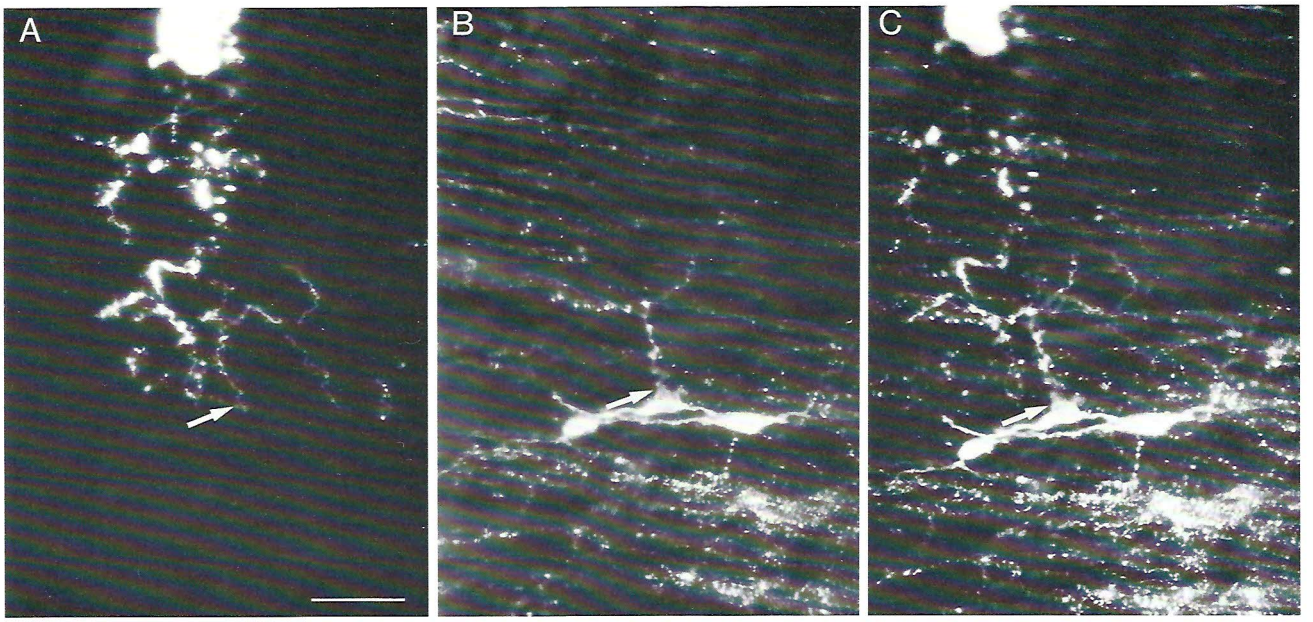

Fig. 3. Medial dendrites of a motoneuron in the region of the 5-HT cell bodies; a dendrite is found (arrow) at a distance less than 2 μm from a 5-HT cell body. A. LY. B. 5-HT. C. Double exposure showing both LY and 5-HT. Bar = 50 μm.

midline, entering the 5-HT plexus with a few fine branches (Figs. 4B, 9) adjacent to 5-HT varicosities.

Sensory dorsal cells **(n = 4).** 5-HT varicosities were usually remote from the somata of the dorsal cells, and the distance always remained more than 1 μm (Fig. 5A). The axons of the dorsal cells followed a longitudinal course in the lateral part of the medial column in the dorsal plane, where the density of 5-HT fibers was high, and they were often accompanied by 5-HT fibers, but no adjacent (distance < 1 μm) 5-HT varicosities have been found (Fig. 5B).

Giant interneurons **(n = 3).** 5-HT varicosities adjacent to the cell bodies or the dendrites of giant interneurons were rare (Fig. 6A). A single case of 5-HT varicosities at a distance of about 1 μm or less from a lateral dendrite of a giant interneuron is shown in Figure 6B. The ventral dendrites of giant interneurons did not penetrate into the 5-HT plexus, but remained dorsal and lateral to this plexus.

Edge cells **(n = 5).** The cell bodies of the edge cells described in this study were all located at the very lateral margin of the spinal cord. Usually, 5-HT varicose fibers passed at a distance of 10, 20, or more μm from the edge cells' somata; 5-HT varicosities adjacent to these somata appeared to be rare or absent (cf. Fig. 7A). A varicosity possibly adjacent to a ventromedial dendrite has been found in a single case. Some of the edge cells had ventromedial dendrites, but these did not penetrate into the 5-HT plexus, but rather remained dorsal to it (Fig. 7B).

5-HT cells **(n = 3).** Small cell bodies in the midline column of the spinal cord could sometimes be seen in the living preparation and impaled. These small cells were only recorded from for a short period of time (< 15 minutes). 5-HT-immunoreactive material was present in these LY-filled cells (Fig. 8A,B). Although a limited proportion of their axonal tree was filled, it could be seen that they were multipolar.

Unclassified neurons **(n = 37).** A large heterogeneous population of unclassified neurons was found (Table 1). This was expected since the majority of the lamprey spinal cord neurons has not yet been classified (cf. Rovainen '79; Fig. 10). Most of these unclassified cells had a medium-sized cell body and ipsilateral dendrites. In many cases (N = 21) no axon was found. Of the 37 unclassifed neurons, seven extended their dendrites into the ventromedial 5-HT plexus. No further attention will be paid here to these unclassified neurons.

DISCUSSION

Comments on the technique

The combined application of intracellular staining and immunohistochemistry in serial sections enabled the light microscopical detection of transmitter-identified varicosities adjacent to cell bodies or dendrites of LY-filled neurons (cf. Kawata et al., '83; Reaves et al., '83; Hoffert et al., '83). With light microscopical methods, however, no definite conclusions can be drawn with regard to synaptic connections between 5-HT-containing nerve endings and LY-filled dendrites. Moreover, although LY "stains" fine branches of neuronal dendrites (see also Takato and Goldring, '79), the dendritic tree may not be completely filled out by LY, and thus the distance between dendrite and 5-HT terminal may be overestimated. Furthermore, there is evidence from studies on the rat that 5-HT in cortical areas may exert its action at nonsynaptic release sites (Descarries et al., '75). By this method only adjacent varicosities could be identified oriented toward the LY-filled element in the same plane as the sections. That is, in the present 14-μm horizon-