

## Serotonergic, peptidergic and GABAergic innervation of the ventrolateral and dorsolateral motor nuclei in the cat S1/S2 segments: an immunofluorescence study

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### Abstract

Indirect single- and double-staining immunofluorescence techniques were used to study the serotonergic, peptidergic and GABAergic innervation of the ventrolateral (Onuf's nucleus) and dorsolateral (innervating intrinsic foot sole muscles) nuclei, located in the S1/S2 segments of the cat spinal cord. The relative density of 5-hydroxytryptamine-, thyrotropin-releasing hormone-, substance P- and  $\gamma$ -aminobutyric acid-immunoreactive axonal varicosities was similar in both nuclei. The highest relative density was recorded for varicosities immunoreactive to  $\gamma$ -aminobutyric acid, while those immunoreactive to 5-hydroxytryptamine or thyrotropin-releasing hormone yielded the lowest values. The density of enkephalin-immunoreactive varicosities was higher in the ventrolateral than in the dorsolateral nucleus. Calcitonin gene-related peptide-like immunoreactivity could be seen in neurons of the ventrolateral and dorsolateral nuclei. Occasionally, calcitonin gene-related peptide-immunoreactive axonal fibers were also encountered in these nuclei. Virtually all thyrotropin-releasing hormone-immunoreactive varicosities in the ventrolateral and dorsolateral nuclei also contained 5-hydroxytryptamine-like immunoreactivity, while a somewhat smaller number of them were co-localized with substance P. About 5–10% of the 5-hydroxytryptamine-immunoreactive varicosities were devoid of peptide-like immunoreactivity, and the number of 5-hydroxytryptamine-immunoreactive varicosities lacking thyrotropin-releasing hormone-like immunoreactivity was higher in the dorsolateral than in the ventrolateral nucleus. Finally, the free fraction of substance P-immunoreactive varicosities, i.e., those lacking both 5-hydroxytryptamine and thyrotropin-releasing hormone, was about 39% in the ventrolateral and 26% in the dorsolateral nucleus. Spinal cord transection at the lower thoracic level induced a depletion of 5-hydroxytryptamine and thyrotropin-releasing hormone-immunoreactive fibers from the ventrolateral and dorsolateral nuclei, indicating an exclusive supraspinal origin for these fibers. A reduction in substance P-like immunoreactivity following spinal cord transection alone or spinal cord transection combined with unilateral dorsal rhizotomy was also detected in both nuclei, suggesting a dual origin for substance P-immunoreactive fibers, i.e., both supra- and intraspinal. The decrease in number of substance P-immunoreactive fibers was however smaller than expected from the analysis of the fraction of substance P-immunoreactive fibers co-localized with 5-hydroxytryptamine, indicating thus that the experimental lesions may have triggered a sprouting of substance P-immunoreactive axons originating from spinal cord sources. The distribution of  $\gamma$ -aminobutyric acid in the ventrolateral and dorsolateral nuclei was not affected by the different lesion paradigms. It is therefore assumed that these inputs are intrinsic to the spinal cord. Finally, both in the ventrolateral and the dorsolateral nucleus a small but statistically significant increase of axonal fibers immunoreactive to enkephalin was seen in response to the experimental lesions.

**Keywords:** Motoneuron; Immunohistochemistry; Pelvic muscle; Plantar muscle; Neuropeptide; Coexistence; 5-HT;  $\gamma$ -Aminobutyric acid; Dorsal rhizotomy; Spinal cord transection; Spinal cord

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## 1. Introduction

The ventrolateral nucleus (VLN; see, e.g., Romanes, 1951; Ueyama et al., 1984; Thor et al., 1989), considered to be the feline homologue to Onuf's nucleus in man (Onuf, 1900), is located in the ventral horn at the S1/S2 spinal segment levels. VLN motoneurons innervate striated perineal muscles, such as the sphincter urethrae, the sphincter ani externus and the ischiocavernosii muscles (e.g., Sato et al., 1978; Kuzuhara et al., 1980; Thor et al., 1989). Dorsolateral to the VLN is the somatic motor nucleus (dorsolateral nucleus; DLN) whose motoneurons innervate the intrinsic muscles of the foot sole (e.g., Romanes, 1951; Egger et al., 1980; Ulfhake and Kellerth, 1983).

The predisposition of VLN motoneurons to certain degenerative diseases differs from that seen in other somatic motor nuclei, such as the DLN, and seems related to the selective vulnerability of autonomic spinal cord centers, such as the sacral parasympathetic nucleus. VLN motoneurons are thus selectively spared in amyotrophic lateral sclerosis (see e.g., Mannen et al., 1977, 1982; Sung, 1982) and acute poliomyelitis (Iwata and Hirano, 1978; Sung, 1982), but extensively affected by degenerative changes in for example Shy-Drager disease (Sung et al., 1979; Mannen et al., 1982). These findings have led to the assumption that the VLN takes up an intermediate position between autonomic and somatic nuclei. Another interesting characteristic of the VLN, not seen in the DLN, is the sexual dimorphism in terms of motoneuron size and number reported in the rat (Jordan et al., 1982; McKenna and Nadelhaft, 1986).

The innervation of the VLN by serotonin (5-hydroxytryptamine, 5-HT), a neurotransmitter known to facilitate the excitation of spinal cord motoneurons (White, 1985a,b; for references see also Arvidsson et al., 1990), has been shown in several species (e.g., Micevych et al., 1986; Tashiro et al., 1989a,b; Rajaofetra et al., 1992). The neuropeptides substance P (SP) and thyrotropin-releasing hormone (TRH) coexist with 5-HT in axonal fibers in the ventral horn of the spinal cord (e.g., Hökfelt et al., 1978; Johansson et al., 1981; Gilbert et al., 1982; Appel et al., 1987; Staines et al., 1988; Tashiro and Ruda, 1988; Arvidsson et al., 1990; Wessendorf et al., 1990), but innervation by TRH has, to our knowledge, only been described in the VLN of primates (Lechan et al., 1984; Rajaofetra et al., 1992). There are reports on the SP input to the VLN in cat (e.g., Erdman et al., 1984; Tashiro et al., 1989a,b) and rat (e.g., Katagiri et al., 1986; Micevych et al., 1986), but more detailed information about the origin of the observed SP-immunoreactive (IR) fibers is not available. A dense network of enkephalin (ENK)-IR fibers is also present in the VLN (e.g., Glazer and Basbaum, 1980; Hunt et al., 1981; Romagnano and Hamill, 1985). The density of ENK-IR fibers is higher here than in other lumbosacral

motor nuclei, resembling the innervation pattern of the sacral parasympathetic nucleus (e.g., de Groat et al., 1983; Romagnano and Hamill, 1985; Katagiri et al., 1986). This may indicate a parallel organization of the afferent input to the VLN and the sacral parasympathetic nucleus, in order to coordinate somatic and parasympathetic reflex circuitries.  $\gamma$ -Aminobutyric acid (GABA), one of the major inhibitory neurotransmitters in the central nervous system, has been shown to play an important role in spinal cord circuitries (Davidoff, 1972) and is also responsible for an extensive input to the VLN. In this nucleus, GABA-IR axonal boutons account for nearly one third of the total membrane covering of axonal boutons impinging on cell bodies and dendrites (Ramírez-León and Ulfhake, 1993).

The aim of this study was to provide a quantitative survey of the serotonergic, peptidergic and GABAergic innervation of two nearby located sacral motor nuclei, the VLN and DLN, and also to try to establish the origin(s) of these inputs.

## 2. Materials and methods

### 2.1. Surgical procedures and tissue collection

Adult female cats divided into two groups were used throughout the study: (1) five cats not submitted to previous experimental procedures constituted a reference group for immunohistochemical analysis of the normal S1/S2 spinal segments; (2) four cats underwent surgical interventions 44 days prior to the immunohistochemical studies. Spinal cord transection was performed in two cats at the Th12-L1 border, while the remaining two cats underwent spinal cord transection in combination with unilateral dorsal rhizotomy below the transection level. All surgery was performed under deep anesthesia, and the surgical procedures have been described in detail elsewhere (Arvidsson et al., 1990). The use of cats and the performed experiments for the purpose of this study were approved by the Local Ethical Committee (i.e., Stockholms Norra Djurförsöksetiska Nämnd; projects A226/88 and N27/90).

The procedures for fixation and tissue preparation did not differ between the animal groups. Briefly, the cats were anesthetized with pentobarbitone-sodium (35–40 mg/kg body wt.) i.p. and xylazine chloride (0.4 mg/kg) s.c. and perfused through the descending aorta with calcium-free Tyrode's solution at 35°C, followed by a cold (4°C) fixative mixture containing 4% paraformaldehyde, 0.2% picric acid (Zamboni and De Martino, 1967) and 0.5% glutaraldehyde (Willingham, 1983) dissolved in 0.1 M phosphate-buffered saline (PBS). The S1/S2 spinal cord segments were rapidly dissected out and immersed in fresh fixative for 90 min at 4°C and then transferred to a 10% sucrose buffer solution containing 0.02% Bacitracin (Sigma) and 0.01% sodium

azide (Merck, Darmstadt, Germany). The tissue was cut in transverse slices of about 1 mm thickness and treated with 1% sodium borohydride (Willingham, 1983; Kosaka et al., 1986; Tsuruo et al., 1987) for 30 min.

## 2.2. Immunofluorescence procedure

The indirect immunofluorescence technique of Coons and collaborators (see Coons, 1958) was used, and the double-staining method (Fraser, 1969; Erichsen et al., 1982; Wessendorf and Elde, 1985) was employed to establish coexistence between two compounds. The tissue was cut transversely in 8–14  $\mu\text{m}$  thick sections in a cryostat (Dittes, Heidelberg, Germany) and mounted on glass slides dipped in chrome alum-gelatine. After rehydration in 0.1 M PBS the sections were incubated with primary antisera (see Table 1) diluted in PBS containing 0.3% Triton X-100 (Hartman et al., 1972). All incubations with primary antisera were performed at 4°C for 16–48 h. After repeated rinses in PBS the sections were transferred to a humid chamber where single-stained sections were incubated for 30 min at 37°C with fluorescein isothiocyanate (FITC)- or tetramethylrhodamine isothiocyanate isomere R (TRITC; rhodamine)-conjugated secondary antibodies (see Table 1). Double-stained sections were incubated with a mixture containing the appropriate FITC- and TRITC conjugated secondary antisera (see Table 1). All secondary antisera were diluted in PBS containing 0.3% Triton X-100 (Hartman et al., 1972). The sections were rinsed in PBS and mounted in a mixture of glycerol and PBS (dilution 3:1) that also contained the anti-fading agent *p*-phenylenediamine (0.1%; Johnson and Nogueira Araujo, 1981; Platt and Michael, 1983).

A Nikon Mikrophot-FX microscope equipped for FITC (filter-cube B-2A; 510 nm dichroic mirror, 450–490 nm excitation filter, 520–560 nm bandpass filter, and 550 nm extra shortpass filter) and rhodamine (filter cube G-1B; 580 nm dichroic mirror, 546/10 nm excitation filter, and 610 nm longpass filter) fluorescence was used to examine the sections. Photographs were

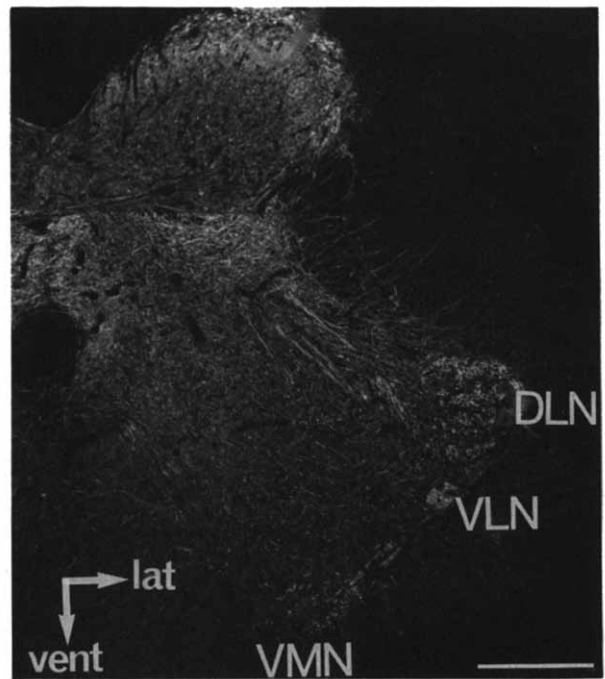


Fig. 1. Immunofluorescence micrograph (montage) of a 14- $\mu\text{m}$  thick transverse section through the S1/S2 segments of the cat spinal cord after incubation with a mouse monoclonal antibody against microtubule-associated protein 2 (MAP2; Sigma; dilution 1:1000). A FITC-conjugated sheep anti-mouse antibody (Amersham, UK; dilution 1:10) was used as secondary antiserum. Since MAP2-LI stains cell bodies and dendrites, the location of the three motor nuclei encountered at this spinal level (VMN, ventromedial nucleus; VLN, ventrolateral nucleus; DLN, dorsolateral nucleus) can be visualized. Scale bar = 400  $\mu\text{m}$ .

taken on black and white Tri-X, 400 ASA (Kodak, Rochester, NY, USA) film.

After the photographic documentation of immunostaining, a number of sections were counterstained with cresyl-violet in order to confirm the cytoarchitectonic location of the sacral motor nuclei subjected to analysis here, i.e., the ventrolateral (VLN) and dorsolateral (DLN) nuclei.

Table 1  
Antisera used in this study

Antigen	Primary antibody		Raised in	Reference	Secondary antibody	
	Specification	Dilution			TRITC <sup>a</sup>	FITC <sup>a</sup>
5-HT	SER P-102-2, 102-3	1:400	Guinea pig	Verhofstad, unpublished	CaGoAGp 1:10	
TRH	4319	1:1600	Rabbit	Visser et al., 1978	DaSwARb 1:10	BoGoARb 1:40
SP	NCI/34 (monoclonal)	1:20	Rat	Cuello et al., 1979		DaRbARt 1:10
ENK	Met-ENK; K336	1:400	Rabbit	Schultzberg et al., 1978		BoGoARb 1:40
GABA	1324	1:2000	Rabbit	Séguéla et al., 1984		BoGoARb 1:40
CGRP	Lot No. 000114	1:400	Rabbit	Peninsula labs		BoGoARb 1:40

<sup>a</sup>Abbreviations: Bo, Boehringer Mannheim Scandinavia (Stockholm, Sweden); Ca, Cappel (Eastchester, PA, USA); Da, Dakopatts (Copenhagen, Denmark); A, anti; Go, goat; GP, guinea pig; Rb, rabbit; Rt, rat; Sw, swine.

### 2.3. Immunohistochemical controls

The antisera used in this study and combinations thereof have been characterized previously and the selectivity in staining for 5-HT (tested as described by Verhofstad et al., 1983), TRH- (Visser et al., 1978), SP- (Cuello et al., 1979), ENK- (Schultzberg et al., 1978) and GABA-like (Séguéla et al., 1984) immunoreactivity (-LI) has been shown. Preabsorption of each antisera with an excess of the respective antigen resulted in complete absence of immunostaining and cross-reactivity tests (according to Wessendorf and Elde, 1985) between primary

antisera, between primary and secondary antisera and between secondary antisera have also been performed previously (5-HT, TRH and SP, see Arvidsson et al., 1990; ENK, see Arvidsson et al., 1992).

### 2.4. Quantification methods

Single-stained sections were randomly selected to quantify the density of axon terminals immunoreactive to 5-HT, TRH, SP, ENK and GABA in the VLN and DLN of the S1/S2 spinal segments. Double-stained sec-

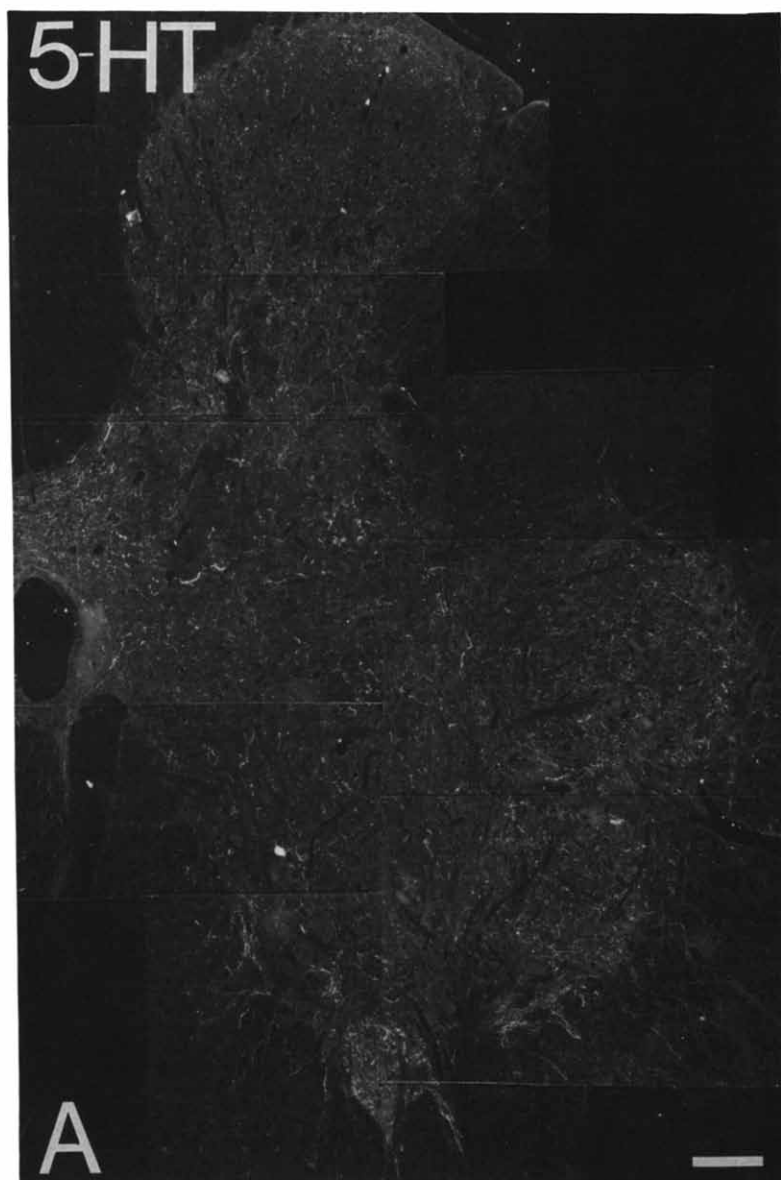


Fig. 2. Immunofluorescence micrographs (montage) of adjacent 14  $\mu\text{m}$ -thick transverse sections through the S1/S2 segments of the cat spinal cord after incubation with antiserum against (A) 5-HT, (B) TRH and (C) SP, respectively. Note the similar distribution of IR fibers in the VLN and DLN. Scale bar = 200  $\mu\text{m}$ .

tions for the combinations 5-HT/TRH, 5-HT/SP and TRH/SP were also selected in order to estimate the degree of coexistence between these compounds. The selected material came from both animal groups, i.e., the normal reference group and the cats subjected to different lesion paradigms. Areas within the VLN and DLN were photographed on color slides (Kodak, Ektachrome 400 ASA, Rochester, NY, USA) in order to enable quantification according to the procedure described by Arvidsson et al. (1990). Photographs were taken in three focal planes through each selected section. The color diapositives were then projected on a screen

and a central field measuring  $78 \times 78 \mu\text{m}$  was chosen to represent the area for the counting of immunoreactive profiles. With a section thickness of  $8 \mu\text{m}$ , the studied volume of the tissue at each location was approximately  $49 \mu\text{m}^3$ . For each of the signal substances subjected to analysis, a transparent film covering the projected diapositive image was used to mark out the location of immunoreactive axonal terminals. When co-localization between two compounds was to be established, landmarks were chosen so that the film could be superimposed on the projection of the same area when the second immunoreactive substance was displayed.

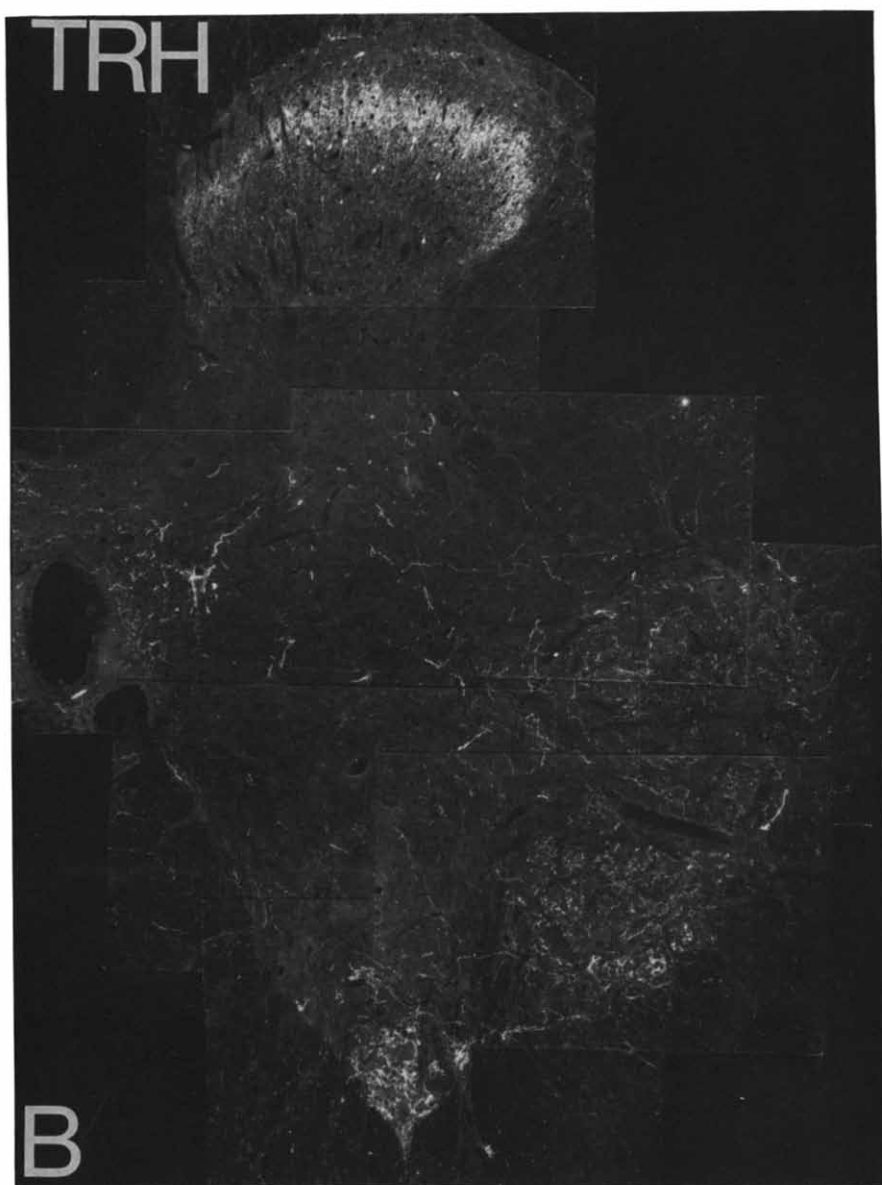


Fig. 2. (continued).

Comparisons between data samples were made using two-tailed Student's *t*-test and differences were considered as statistically significant at  $P < 0.05$ .

### 3. Results

Fig. 1 shows the cat spinal cord at the S1/S2 level after incubation with an antibody against microtubule-associated protein 2 (MAP2; for further details, see figure legend). In this micrograph, the location of the ventromedial (VMN), ventrolateral (VLN) and dorsolateral (DLN) sacral motor nuclei, can be seen.

#### 3.1. Innervation pattern of the VLN and DLN

5-Hydroxytryptamine (5-HT)-immunoreactive (IR) axonal fibers and varicosities could be seen over the entire ventral horn of the S1/S2 spinal segments (Fig. 2A). There were no differences in relative density of 5-HT-IR varicosities between the VLN and the DLN (Figs. 2A, 3A).

The distribution of thyrotropin-releasing hormone (TRH)-like immunoreactivity (LI) in the S1/S2 ventral horn resembled that of 5-HT-LI (c.f. Figs. 2A,B). The relative density of TRH-IR varicosities recorded both in

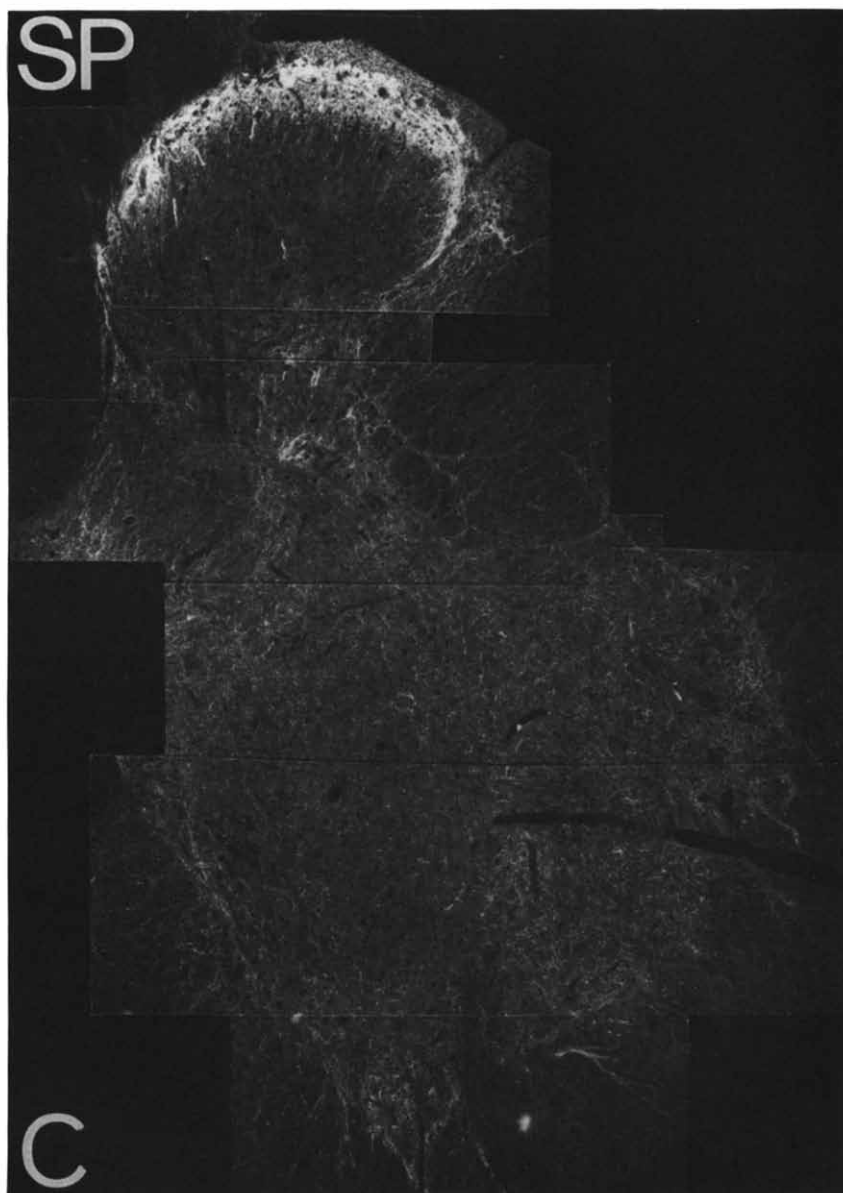


Fig. 2. (continued).

the VLN and DLN did not differ significantly from that of 5-HT (Fig. 3A).

The distribution of substance P (SP)-IR axonal fibers in the ventral horn (Fig. 2C) was also similar to that of 5-HT and TRH. No clear differences between the SP input to the VLN and DLN could be detected (Fig. 3A). The relative density of SP-IR varicosities in the studied

nuclei exceeded however that of varicosities IR to both 5-HT and TRH by 30–33% ( $P < 0.01$ , VLN;  $P < 0.05$ , DLN; see Fig. 3A).

Enkephalin (ENK)-IR axonal fibers could be seen throughout the ventral horn at the S1/S2 spinal segments, but were particularly numerous in the VLN, where they formed a dense network of fibers (Figs. 4A,C). A difference in the relative density of ENK-IR varicosities could therefore be noticed between the VLN and DLN ( $P < 0.001$ , Figs. 3A, 4A–C). Furthermore, the relative density of ENK-IR varicosities in the VLN was 66% higher than that of varicosities IR to SP (Fig. 3A;  $P < 0.01$ ) and almost twice that of varicosities IR to 5-HT or TRH (Fig. 3A;  $P < 0.001$ ).

A dense network of GABA-IR axonal fibers could be seen throughout the ventral horn at the S1/S2 spinal levels (not shown). The relative density of GABA-IR varicosities in the VLN and DLN was higher than for any other of the substances subjected to quantitative analysis. Thus, the density of the GABAergic innervation was 5–6 times that of 5-HT or TRH, almost 4 times that of SP and 2–3 times that of ENK (Fig. 3A). No differences between the GABA input to the VLN and DLN could however be detected (Fig. 3A).

Calcitonin gene-related peptide (CGRP)-LI could be seen in neurons of the VLN and DLN (Fig. 5A). Occasionally, CGRP-IR axonal fibers were also encountered in these nuclei (Figs. 5B,C).

### 3.2. Coexistence of 5-HT-, SP- and TRH-LI

Virtually all TRH-IR varicosities in the VLN and DLN (96–97%) also contained 5-HT (Figs. 3B, 6A,B), indicating an almost complete coexistence of TRH with 5-HT. In comparison, the number of 5-HT-IR varicosities also IR to TRH was higher ( $P < 0.01$ ; Fig. 3B) in the VLN (96%) than in the DLN (84%).

The fraction of SP-IR varicosities also containing 5-HT amounted to 61% in the VLN and 74% in the DLN

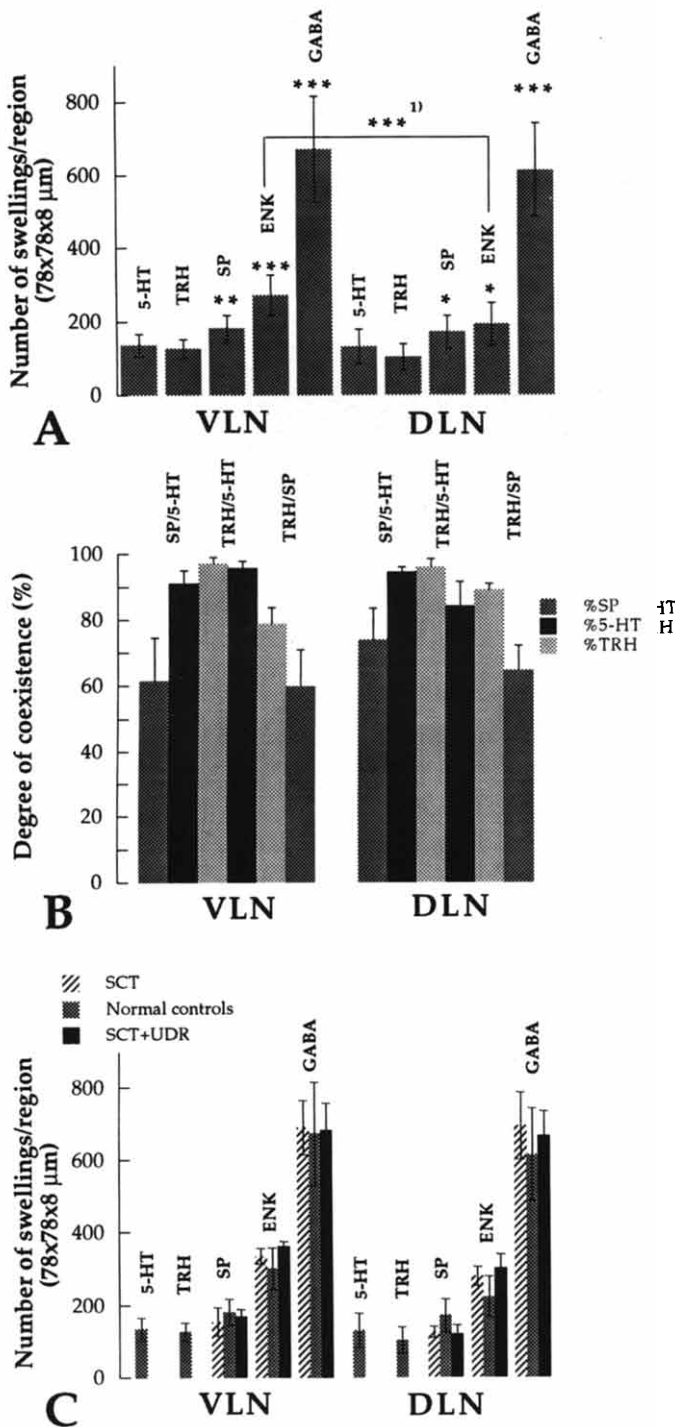


Fig. 3. Histograms illustrating the quantitative results of the study. Mean values and S.D. have been indicated. (A) shows the density of 5-HT-, TRH-, SP-, ENK- and GABA-IR axonal varicosities in the VLN and DLN, respectively. Asterisks indicate statistically significant differences in density between 5-HT-IR axonal varicosities and varicosities IR to the other signal substances for each motor nucleus ( $*P < 0.05$ ;  $**P < 0.01$ ;  $***P < 0.001$ ). Note also the difference in density of ENK-IR varicosities<sup>(1)</sup> between the VLN and DLN. (B) shows the degree of coexistence between SP/5-HT-, TRH/5-HT- and TRH/SP-IR varicosities in the VLN and DLN, respectively, as calculated from double-labelled sections. (C) illustrates changes in density of 5-HT-, TRH-, SP-, ENK-, and GABA-IR varicosities in the VLN and DLN, respectively, following either chronic spinal cord transection (SCT) or SCT in combination with unilateral dorsal rhizotomy (UDR). No lesion values are included for 5-HT or TRH since virtually all 5-HT- and TRH-IR varicosities disappeared from the VLN and DLN following both lesion paradigms.

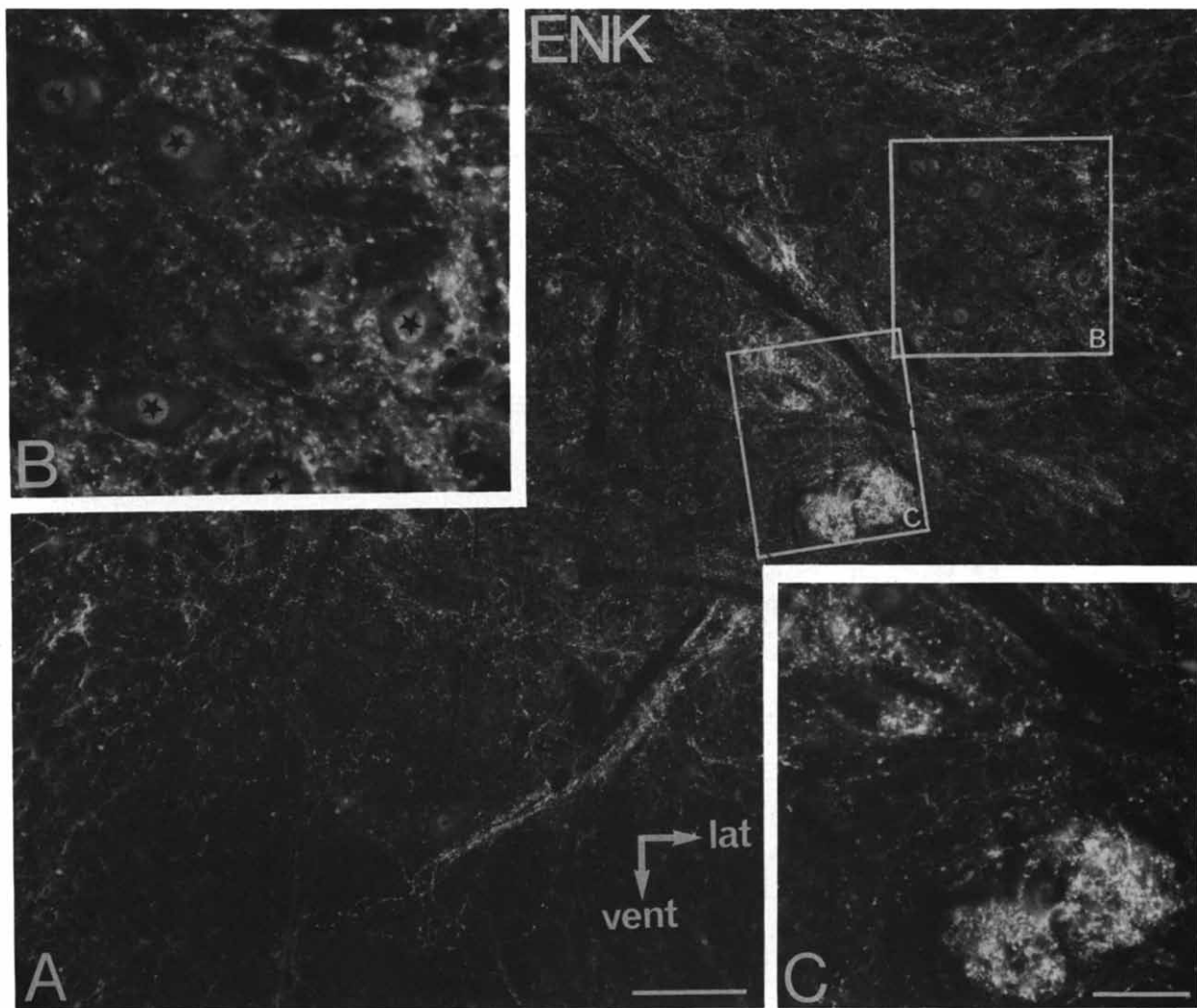


Fig. 4. (A) Immunofluorescence micrograph from the ventral horn at the S1/S2 spinal segment level in cat after incubation with ENK antiserum. (B,C) Micrographs showing the DLN (B) and VLN (C), i.e., the framed regions in (A), at higher magnification. Stars in (B) indicate neuronal cell bodies. Note also the dense network of ENK-IR fibers present in the VLN. Scale bars: A, 200  $\mu$ m; C (also valid for B), 80  $\mu$ m.

(Figs. 3B, 7A–C). The difference between the two nuclei was however not statistically significant. In addition, 91% of the 5-HT-IR varicosities in the VLN and 94% of those in the DLN were also IR to SP (Figs. 3B, 7A–C). Because of the high coexistence values obtained in the VLN and DLN for the combinations 5-HT/TRH and 5-HT/SP, a high degree of coexistence between TRH and SP was also expected, and verified by analysis of sections double-labelled against these substances. Thus, 60–65% of the SP-IR varicosities also contained TRH-LI (Fig. 3B), while 79–89% of the TRH-IR varicosities were IR to SP (Fig. 3B).

Taken together, virtually all TRH-IR varicosities in the VLN and DLN seemed also to contain 5-HT, while

a somewhat smaller number of them also contained SP. The 'free fraction' of 5-HT-IR varicosities, i.e., those containing neither TRH nor SP, amounted to less than 5–10%, and the number of 5-HT-IR varicosities devoid of TRH was higher in the DLN than in the VLN. Finally, the 'free fraction' of SP-IR varicosities, i.e., those lacking 5-HT and/or TRH, was about 39% in the VLN and 26% in the DLN.

### 3.3. Lesion effects

Virtually all 5-HT- and TRH-LI disappeared from the S1/S2 ventral horn following spinal cord transection (SCT; Figs. 3C, 8A). A decrease in the number of SP-IR



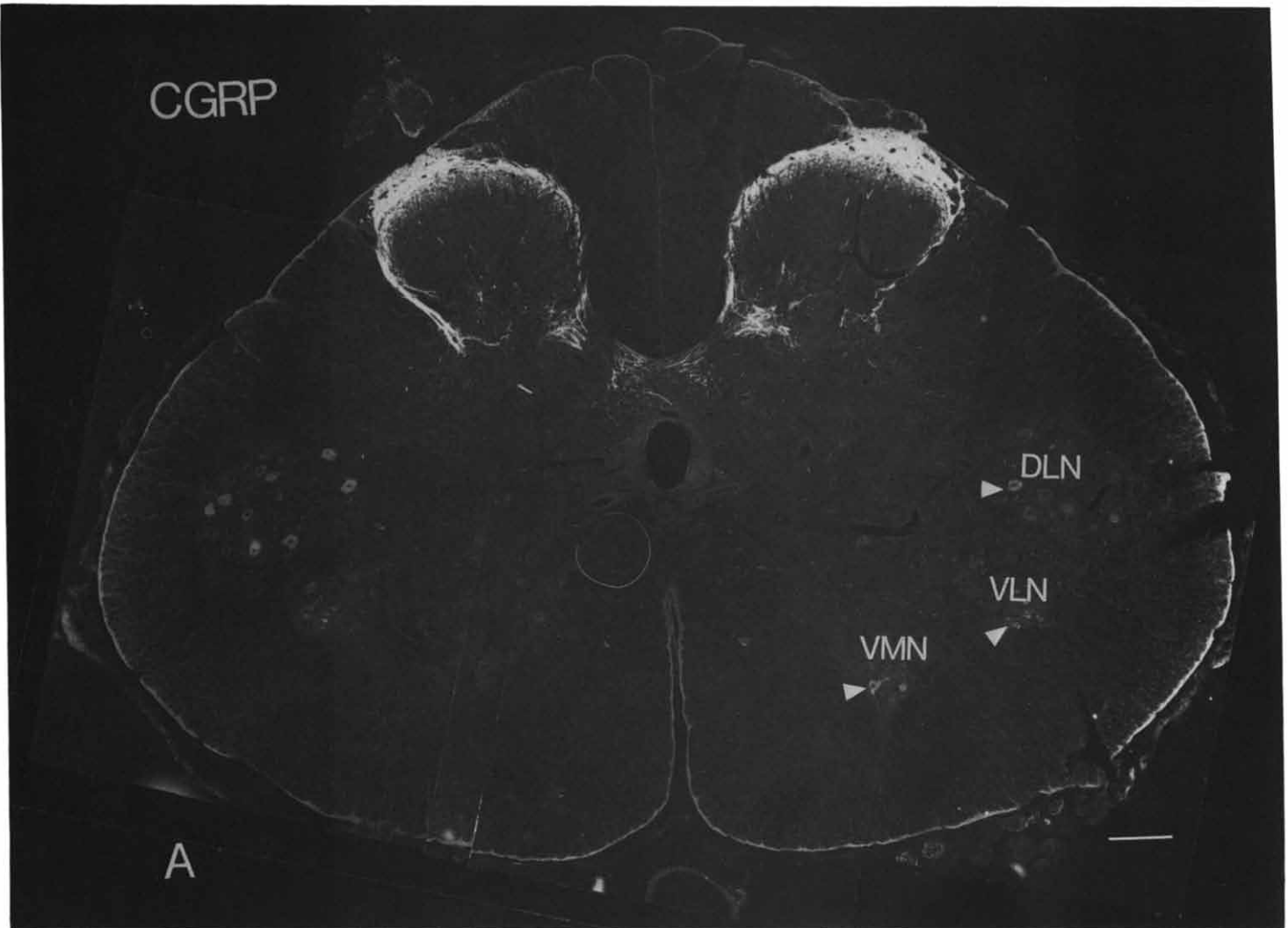


Fig. 5. (A) Immunofluorescence micrograph (montage) of a 14  $\mu\text{m}$ -thick transverse section through the S1/S2 segments of the cat spinal cord after incubation with CGRP antiserum. CGRP-IR neurons (arrowheads) can be seen in the VMN, VLN and DLN. (B) Immunofluorescence micrograph showing CGRP-IR neurons and fibers (arrows) in the VLN and DLN. The VLN is seen at higher magnification in (C). Scale bars: A, 200  $\mu\text{m}$ ; B, 150  $\mu\text{m}$ ; C, 50  $\mu\text{m}$ .

varicosities was seen as a result of SCT alone (Figs. 3C, 8B). A similar effect was also obtained by SCT in combination with unilateral dorsal rhizotomy (UDR; Fig. 3C). The decrease in SP-LI following both lesion paradigms was however more extensive in the DLN (27.2–30%) than in the VLN (7–15%), which is consistent with the findings (see above) that SP-IR fibers in the DLN derive to a larger degree from the descending 5-HT system, while the VLN receives relatively more SP-IR fibers originating from spinal cord sources.

The number of ENK-IR varicosities increased both in the VLN and DLN following either SCT alone or SCT + UDR (Figs. 3C, 9). Interestingly, the small increase in the number of ENK-IR varicosities was more

evident in the DLN ( $P < 0.001$ ) than in the VLN ( $P < 0.05$ ) after both lesion types (Fig. 3C).

Finally, neither SCT alone nor combined SCT+UDR induced any change in the GABAergic innervation of the VLN or DLN (Figs. 3C, 10A,B), suggesting therefore an exclusive intraspinal origin for these inputs.

#### 4. Discussion

##### 4.1. The descending 5-HT system and peptide coexistence

The quantitative analysis performed here did not reveal any major difference in the density of the serotonergic innervation between the DLN and the

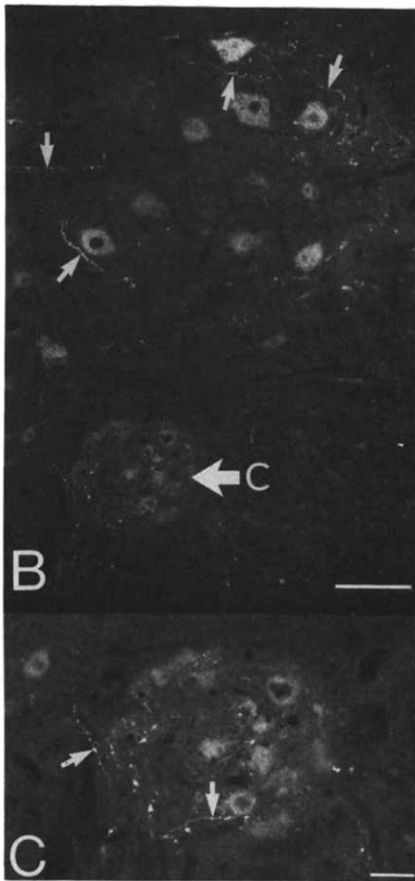


Fig. 5 (continued).

VLN. 5-HT-IR fibers disappeared from both nuclei following spinal cord transection (SCT), indicating an exclusive supraspinal origin. This is in agreement with studies of the acute response to SCT in the VLN of cat (Tashiro et al., 1989b) and rat (Micevych et al., 1986), as well as the chronic response to SCT in the L7 spinal segment of the cat (Arvidsson et al., 1990). Since virtually all TRH-IR fibers in the VLN and DLN were found to contain 5-HT, it was not surprising that the lesion experiments also revealed a strictly supraspinal origin for TRH-IR fibers. According to other studies, most if not all TRH-LI in the motor nuclei of the ventral horn is confined to axonal fibers of the descending 5-HT system (e.g., Johansson et al., 1981; Arvidsson et al., 1990) that originates from cell bodies in the raphé nuclei of the medulla oblongata (Dahlström and Fuxe, 1964a,b).

Substance P is also present in neurons of the bulbospinal 5-HT system (e.g., Hökfelt et al., 1978; Johansson et al., 1981; Gilbert et al., 1982; Tashiro and Ruda, 1988; Arvidsson et al., 1990). The density of SP-IR varicosities was quite similar in the VLN and DLN, and a large number of SP-IR fibers, 61% in the VLN and 74% in the DLN, also contained 5-HT and/or TRH, being thus with all probability of supraspinal origin. A substantial number of SP-IR fibers was however still present in the VLN and DLN following chronic SCT. Since SCT in combination with unilateral dorsal

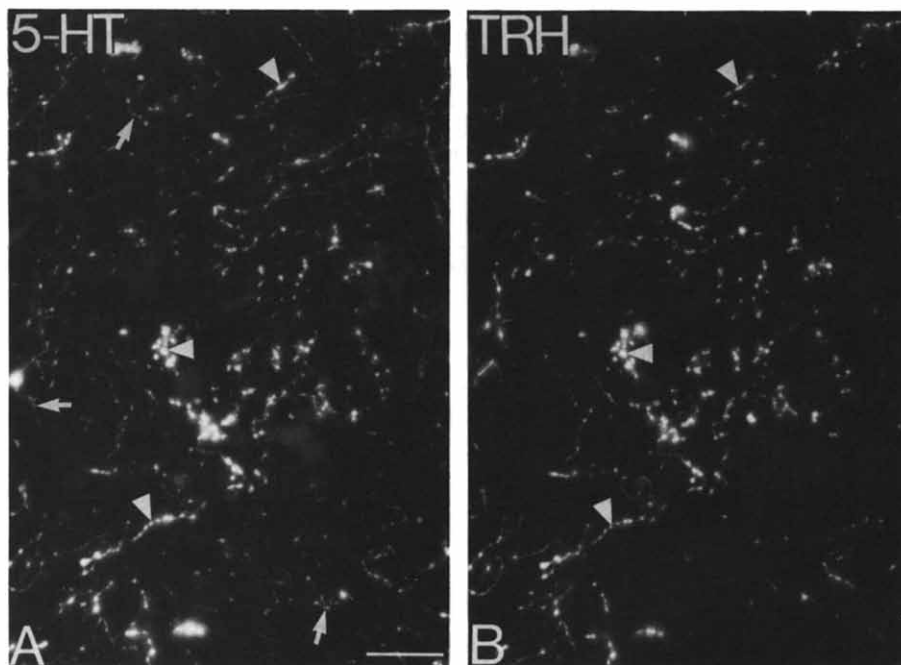


Fig. 6. Immunofluorescence micrographs from the VLN after double labelling with (A) 5-HT and (B) TRH antisera. Virtually all TRH-IR varicosities and fibers were also IR to 5-HT (arrowheads). Arrows point at 5-HT-IR varicosities and fibers lacking TRH-LI. Scale bar = 40  $\mu$ m.

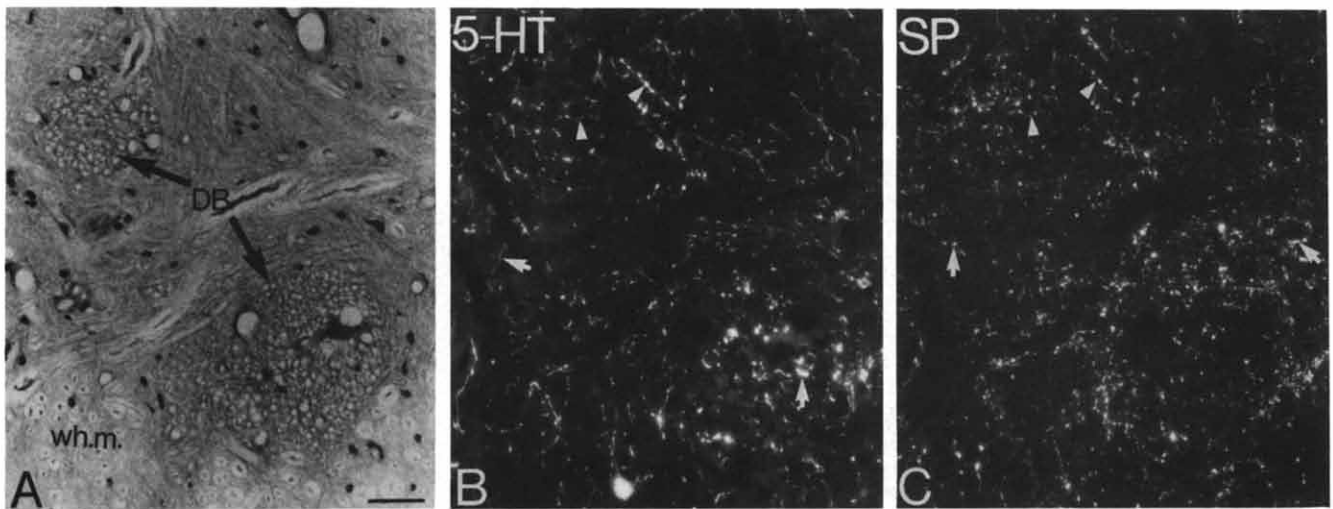


Fig. 7. (A) Light micrograph of a cresyl-violet stained section showing the VLN. Note the high occurrence of bundled dendrites (DB, dendritic bundles; wh.m., white matter). (B,C) Immunofluorescence micrographs of the same section, that were taken prior to counterstaining with cresyl-violet. Double labelling with (B) 5-HT and (C) SP antiserum can be seen. Arrowheads indicate examples of fibers where 5-HT- and SP-LI coexist. Arrows point at 5-HT-IR varicosities and fibers lacking SP-LI (B), and fibers and varicosities only IR to SP (C), respectively. Scale bar = 40  $\mu\text{m}$ .

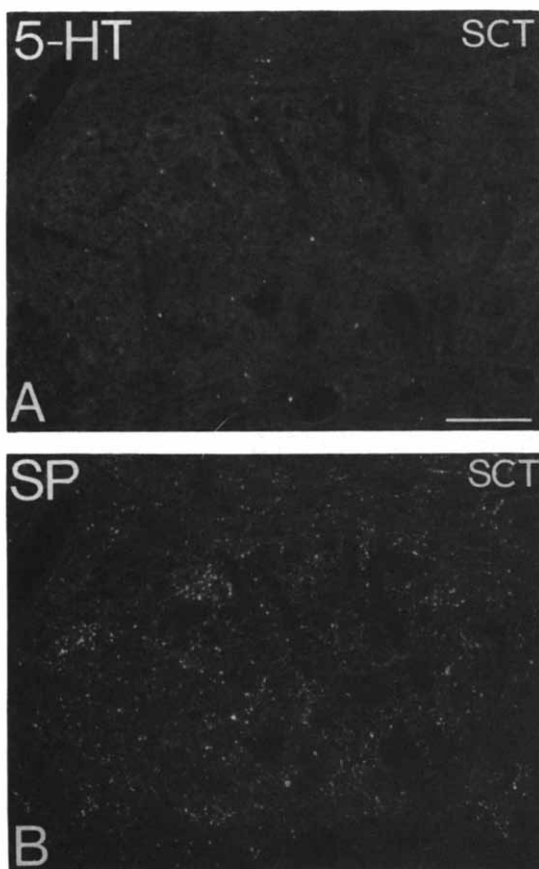


Fig. 8. Immunofluorescence micrographs from the ventral horn in the S1/S2 segments of the cat spinal cord after double labelling with (A) 5-HT and (B) SP antisera, as seen 44 days after spinal cord transection (SCT). Practically all 5-HT-LI has disappeared, while many SP-IR fibers are still present, both in the DLN and VLN. Scale bar = 100  $\mu\text{m}$ .

rhizotomy (UDR) gave similar results as SCT alone we suggest that a fraction of the SP input to both the VLN and DLN derives from neurons intrinsic to the spinal cord (Hököfelt et al., 1975; Ljungdahl et al., 1978; Tessler et al., 1980, 1981). Similar conclusions have been drawn by Tashiro and co-workers (1989b) who reported a severe depletion of axons double-labelled with 5-HT and SP 10–11 days following SCT in the cat VLN, but no detectable density changes of axons single-labelled with SP. The reduction in the number of SP-IR varicosities following SCT or SCT+UDR in this study was however much smaller than what would be expected from the degree of coexistence of SP with descending 5-HT-IR fibers. It can therefore be assumed that part of the SP-LI detected after the lesions derives from a compensatory proliferation of SP-IR axonal fibers originating from spinal sources. This would be consistent with the findings of Tessler et al. (1980, 1981) who demonstrated a subsequent partial recovery of SP-LI in the dorsal horn following UDR, due to axonal sprouting mediated by spinal neurons. Finally, this study showed that the fraction of SP-IR boutons devoid of 5-HT-LI was larger in the VLN, indicating a relatively more extensive segmental SP input to this nucleus when compared to the DLN.

In an ultrastructural study of the VLN (Ramírez-León et al., in press) we report that signal substances that do not share the same origin(s) also seem to differ from each other with respect to their spatial distribution within the dendritic arborizations of VLN motoneurons. TRH-IR axonal boutons are thus preferentially located in apposition to medium-to-large dendrites, i.e., on more proximal parts of the VLN dendritic trees,



Fig. 9. Immunofluorescence micrograph (montage) of a 14  $\mu\text{m}$  thick transverse section through the S1/S2 segments of the cat spinal cord after incubation with ENK antiserum, as seen 44 days after SCT in combination with UDR. The dense network of ENK-IR fibers in the VLN is virtually unaffected by the lesions. Scale bar = 200  $\mu\text{m}$ .

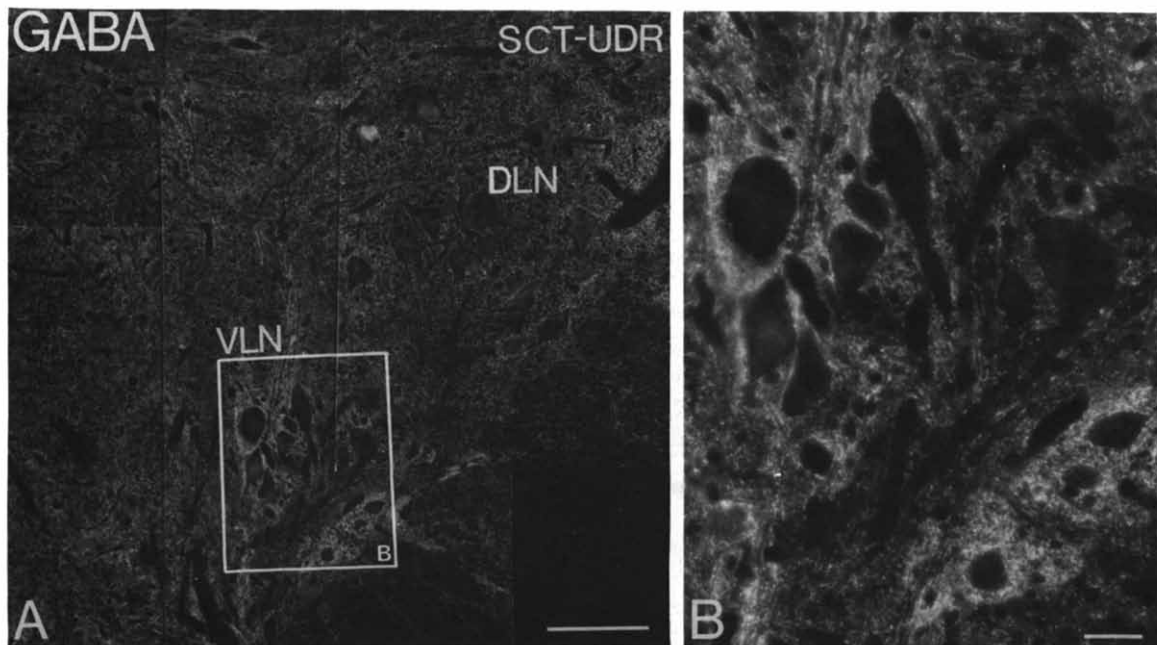


Fig. 10. (A) Immunofluorescence micrograph from the ventral horn in the S1/S2 segments of the cat spinal cord after incubation with GABA anti-serum, as seen 44 days following SCT in combination with UDR. The GABA-IR fiber plexuses in the VLN and DLN are unaffected. The VLN region, framed in (A), is shown at higher magnification in (B). Note the dense innervation of GABA-IR fibers surrounding cell bodies. Scale bars: A, 200  $\mu$ m; B, 40  $\mu$ m.

while SP-IR boutons can be found in synaptic contact with VLN dendrites of all calibre sizes. From the results obtained here it can therefore be assumed that SP-IR boutons in contact with medium-to-large dendrites are preferentially of supraspinal origin, while those impinging on fine-calibre distal branches derive more likely from neurons intrinsic to the spinal cord.

5-HT is known to enhance synaptic excitation of motoneurons (e.g., White, 1985a,b; for references see also Arvidsson et al., 1990). Considering the data obtained in this study, the 5-HT input to VLN and DLN motoneurons seems to be of a similar order of magnitude. In a previous study (Arvidsson et al., 1990) we noted that the 5-HT input to motoneurons innervating weight-bearing proximal muscles of the hind limb seemed to be more extensive than that to motoneurons innervating distal hind limb muscles; a corresponding difference is however not present between the motor nuclei examined here. TRH that is frequently co-localized in 5-HT-IR axon terminals is also known to increase motoneuron excitability (White, 1985a,b). This action is probably mediated through influence on potassium and/or calcium conductances (Takahashi, 1985; Rekling, 1990; Bayliss et al., 1992; Fisher and Nistri, 1993) via a specific postsynaptic TRH receptor (Sharif and Burt, 1983; see also Wu et al., 1992). In this study there was a small but statistically significant difference between the VLN and DLN in the number of 5-HT-IR

varicosities that also contained TRH-LI, indicating the possibility of a more extensive 5-HT/TRH co-transmission in the VLN. Several studies have shown evidence that also SP enhances motoneuron excitability (see e.g., White, 1985a,b) and this mechanism is believed to be indirectly mediated through a presynaptic receptor (Mitchell and Fleetwood-Walker, 1981). The function of SP in local spinal pathways, that represent 26–39% of the SP input to the VLN and DLN, has not been clarified. It should be noted here that the spinal SP input to the VLN is larger than to the DLN, and may thus be related to the SP input to the sacral parasympathetic nucleus (see e.g., Katagiri et al., 1988), in order to co-ordinate the activity of this autonomic nucleus and the VLN (see below).

#### 4.2. Enkephalinergic innervation

Although high coexistence figures have been reported between 5-HT- and ENK-LI in cell bodies of the raphe nuclei (e.g., Glazer et al., 1981; Hunt and Lovick, 1982; Léger et al., 1986; Millhorn et al., 1989), a corresponding coexistence is rather infrequent in axonal fibers of the spinal cord ventral horn (Wessendorf et al., 1990; Arvidsson et al., 1992). According to several studies in the VLN of cat and rat, no decline in the distribution of ENK-LI can be observed 1–2 weeks (Konishi et al., 1985; Micevych et al., 1986; Tashiro et al., 1989b) or > 1

month (Erdman et al., 1984; Romagnano et al., 1987) following SCT, indicating that the main source of origin for the ENK input to this nucleus is spinal. In the present study, a small increase in the number of ENK-IR varicosities was detected both in the VLN and DLN following SCT or SCT+UDR, suggesting thus the possibility of a lesion-induced proliferation of ENK-IR fibers intrinsic to the spinal cord.

Several investigators have previously shown that the dense innervation by peptides such as ENK (e.g., Glazer and Basbaum, 1980; Hunt et al., 1981; Romagnano and Hamill, 1985; Katagiri et al., 1986), somatostatin (e.g., Schröder, 1984; Katagiri et al., 1986) and neuropeptide Y (e.g., Hunt et al., 1981; Gibson et al., 1984; Katagiri et al., 1986) is a characteristic feature of the VLN, not present in other lumbosacral motor nuclei. Of the signal substances subjected here to quantitative analysis, only ENK disclosed a difference in innervation density between the VLN and DLN. The dense innervation of the VLN by ENK-IR fibers is rather similar to the innervation pattern seen in the sacral parasympathetic nucleus (e.g., de Groat et al., 1983; Romagnano and Hamill, 1985; Katagiri et al., 1986; Gibson et al., 1988) that supplies the smooth muscles of the bladder and rectum. Interestingly, the ENK-IR fibers of the sacral parasympathetic nucleus are also believed to derive from spinal sources (Romagnano et al., 1987). Similarities in the afferent input between this nucleus and the VLN, as in the case of neuropeptide Y, somatostatin and ENK, may thus be a mean to coordinate the activity pattern of somatic and visceral structures involved in complex functions such as micturition and defecation. Furthermore, ENK-IR neurons originating from the sacral parasympathetic nucleus (Glazer and Basbaum, 1980; Hunt et al., 1981; Romagnano and Hamill, 1985) may also be among the contributors to the ENK input to the VLN.

#### 4.3. GABAergic innervation

GABA was found here to represent the largest input to the VLN and DLN. In both nuclei, the highest relative density of all studied signal substances was that recorded for GABA-IR varicosities. GABAergic interneurons are widely distributed throughout the spinal cord (Barber et al., 1982), where they are believed to mediate both pre- and postsynaptic inhibition (for review see Rudomin, 1990). Since the spinal cord levels of glutamic acid decarboxylase (the synthesizing enzyme for GABA) are not affected by SCT (Tappaz et al., 1976) it has been assumed that a large part of the GABAergic input to the ventral horn is of spinal origin. This seems also to be the case in the VLN and DLN, since neither SCT nor combined SCT+UDR seemed to alter the GABAergic innervation of these nuclei. It is of interest in this context to indicate that both GABA and

ENK, two inputs presumed here to be of mainly spinal origin, also share a rather similar distribution within the dendritic arborizations of the VLN motoneurons (Ramirez-León et al., in press).

#### 5. Concluding remarks

Whether the VLN is a purely somatic, purely autonomic or mixed somatic and autonomic nucleus has been an issue subjected to debate for years. Onuf (1900) originally classified this nucleus in man as somatic, and experimental animal studies have later shown that VLN motoneurons innervate striated pelvic muscles and external sphincters (e.g., Sato et al., 1978; Kuzuhara et al., 1980; Schröder, 1980; McKenna and Nadelhaft, 1986; Thor et al., 1989). C-type axon terminals (Conradi, 1969; McLaughlin, 1972) that have been associated with  $\alpha$ -motoneurons but not with  $\gamma$ -motoneurons or Renshaw cells (Conradi, 1969; Lagerbäck and Ronnevi, 1982) have furthermore been found apposing VLN motoneurons both in cat (Pullen, 1988) and human (Pullen et al., 1992), which is in favour of a somatic nature. VLN motoneurons have however also been shown to be strongly related with the sacral parasympathetic nucleus (e.g., Rexed, 1954; Nadelhaft et al., 1980; Mawe et al., 1986) and they share some properties with autonomic spinal centers, such as the selective vulnerability to certain autonomic diseases (e.g., Sung et al., 1979; Mannen et al., 1982) and resistance to other motoneuron diseases (see Introduction). Some investigators have therefore assumed that the VLN is in fact a somatic extension of the sacral parasympathetic nucleus (e.g., Laruelle, 1937; Rexed, 1954; Holstege et al., 1986).

In this study the DLN innervating the intrinsic muscles of the foot sole (e.g., Romanes 1951; Egger et al., 1980; Ulfhake and Kellerth, 1983) was selected as an example of a purely 'somatic' nucleus, and its innervation pattern was compared with that of the VLN. The majority of the studied inputs, i.e., both the supraspinal ones (5-HT, TRH, SP) as well as those intrinsic to the spinal cord (SP, GABA) did not disclose large differences between the VLN and DLN. Only ENK, one of the peptides known to densely innervate both the VLN and the sacral parasympathetic nucleus (e.g., de Groat et al., 1983; Romagnano and Hamill, 1985; Katagiri et al., 1986; Gibson et al., 1988), showed clear differences between the VLN and DLN. In addition, there were also minor differences between the VLN and DLN in the number of 5-HT-IR varicosities devoid of TRH-LI, as well as the fraction of SP-LI deriving from spinal sources. Interestingly, both direct (ENK) and indirect (SP) signs of a compensatory increase of axonal fibers could be detected in response to lesions. Thus, both the VLN and DLN showed signs of plasticity. With respect to synaptic input, the VLN motoneurons resemble therefore both the purely somatic motoneurons of the

nearby located DLN as well as the preganglionic neurons of the sacral parasympathetic nucleus.

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