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A Quantitative Study of the Coexistence of Peptides in Varicosities Within the Superficial Laminae of the Dorsal Horn of the Rat Spinal Cord

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While several peptides have been shown to coexist in perikarya within dorsal root ganglia of rat, coexistence of peptides has not been confirmed in axons associated with these neurons. In this study, the coexistence of substance P (SP) with somatostatin (SOM), calcitonin gene-related peptide (CGRP), dynorphin A 1-8 (DYN), neurotensin (NT), galanin (GAL), and 5-HT in varicosities was visualized using fluorescence immunohistochemistry. Densities of immunoreactive varicosities within laminae I and II of the dorsal horn of the rat spinal cord were quantified by computer-assisted image analysis. Decreases in densities of immunoreactive varicosities as a result of multiple unilateral dorsal rhizotomies were used to determine proportions of immunoreactive varicosities associated with primary afferent neurons. Three observations were made. (1) Dorsal rhizotomy depleted greater than one-third of the varicosities individually immunoreactive for SP, SOM, GAL, or DYN, confirming the association of these peptides with primary afferent neurons. (2) SP coexisted with CGRP, GAL, and DYN in varicosities within the dorsal horn of normal animals. (3) CGRP-, SP+CGRP-, and SP+GAL-immunoreactive varicosities were nearly depleted following dorsal rhizotomy. The depletion of these peptides, particularly in combination, indicates that they may be used as markers for varicosities of some primary afferent neurons within the superficial laminae of the dorsal horn of the rat spinal cord.

The majority of information concerning the coexistence of peptides in primary afferent neurons has come from studies of dorsal root and cranial nerve ganglia. Coexistence of 2 substances has been documented in rat perikarya (SP+CGRP: Wiesenfeld-Hallin et al., 1984; Lee et al., 1985a, b; Skofitsch and Jacobowitz, 1985b; SP+CCK-8: Dalsgaard et al., 1982; Tuchscherer and Seybold, 1985), and up to 4 substances have been shown to coexist in the guinea pig (SP+CCK+CGRP+DYN: Gibbins et al., 1987) and cat perikarya (SP+CCK: Gibson et al., 1984a; SP+CCK+SOM+VIP: Leah et al., 1985). While data concerning patterns of coexistence of peptides are useful in categorizing populations of primary afferent neurons, it is also of interest to determine the significance that corelease of these substances may have for synaptic transmission. Before pursuing physiological studies, it is important to confirm whether combinations of peptides that coexist in neuronal perikarya are also present in varicosities of primary afferent neurons.

One cannot assume that immunoreactivity present within a perikaryon will also be present within axonal varicosities associated with the neuron. An antigenic determinant visualized in a neuronal perikaryon by immunohistochemistry is likely to be contained in a precursor protein. In some neurons, the precursor molecule may be processed during axonal transport to products that no longer contain the recognizable amino acid sequence. For example, somatostatin-like immunoreactivity has been visualized within neurons of the reticular nucleus of the thalamus (Graybiel and Elde, 1983). The majority of the neurons within this nucleus project to the dorsal thalamus. However, somatostatin-immunoreactive varicosities are not found within this region (Graybiel and Elde, 1983).

Conversely, posttranslational processing during axonal transport may *create* a determinant used in antibody recognition. The density of dynorphin B-immunoreactive varicosities within the cat spinal cord is decreased following dorsal rhizotomy, suggesting a population of primary afferent neurons contains dynorphin (Basbaum et al., 1986). However, dynorphin-immunoreactive perikarya have not been visualized to date in cat dorsal root ganglia (Basbaum et al., 1986; M. G. Garry and V. S. Seybold, unpublished observations). Therefore, it is possible that *new* patterns of coexistence of peptides may emerge by studying varicosities associated with primary afferent neurons.

In order to determine which peptides may coexist in axonal processes of primary afferent neurons, we studied the coexistence of peptides in varicosities within terminal fields of these neurons in the spinal cord. Visualization of the coexistence of the 2 substances within varicosities was possible by immunofluorescence. The densities of substance P (SP)-, somatostatin (SOM)-, calcitonin gene-related peptide (CGRP)-, galanin (GAL)-, and dynorphin A 1-8 (DYN)-immunoreactive varicosities alone or in combination with SP, were quantified using computer-assisted image analysis. Spinal segment L4 was used in our study. This segment receives input from the sciatic and saphenous nerves whose primary afferent receptive fields are known to be largely somatic (Schmalbruch, 1986).

Within the dorsal horn of segment L4, laminae I and II were

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selected as areas for analysis because the peptides listed above have been localized to predominantly small-diameter perikarya within dorsal root or trigeminal ganglia of the rat (SP: Hökfelt et al., 1975; Tuchscherer and Seybold, 1985; CGRP: Rosenfeld et al., 1983; Gibson et al., 1984a; GAL: Ch'ng et al., 1985; Skofitsch and Jacobowitz, 1985a; DYN 1–8: Botticelli et al., 1981), and small-diameter primary afferent neurons have been shown to terminate within laminae I and II (Kumazawa and Perl, 1976, 1978; Light and Perl, 1979; Sugiura et al., 1986). Decreases in densities of immunoreactive varicosities as a result of multiple dorsal rhizotomies were used to determine the proportions of varicosities that were associated with primary afferent neurons.

In addition to the 5 peptides outlined above, the densities of neurotensin (NT)- and 5-HT-immunoreactive varicosities were also quantified. Since NT- and 5-HT-immunoreactive varicosities within the dorsal horn of the spinal cord are believed to be entirely from intrinsic (NT: Seybold and Elde, 1982; 5-HT: LaMotte and deLanerolle, 1983) or supraspinal sources (5-HT: Basbaum et al., 1978), their densities were indicators of nonspecific changes due to ischemia or changes of laminae volume as a result of the surgical procedure.

The results of this study indicate that some patterns of peptide coexistence occurring among varicosities within laminae I and II are specific for primary afferent neurons.

Materials and Methods

Ten adult male (200–300 gm) Sprague-Dawley rats were used in this study. The animals were generally housed in standard wire-mesh cages. Animals that had undergone unilateral dorsal rhizotomy were housed in solid-bottom plastic cages to minimize injury during their recovery. All animals were maintained under 12 hr light/12 hr dark conditions and were allowed access to food *ad libitum*.

Experimental animals. The animals were divided into 2 groups: a control group ($n = 4$) that received no surgical treatment and an experimental group ($n = 6$). The animals of the experimental group underwent surgery for multiple unilateral dorsal rhizotomies (dorsal roots of spinal levels L1–S1 were transected). The procedure used for the surgical technique has been described in detail elsewhere (Tuchscherer et al., 1987).

On the 10th postoperative day, all 6 animals of the experimental group were vascularly perfused (intraaortically) with Zamboni fixative (Steffanini et al., 1967). The animals of the control group were perfused using the same protocol on the 11th day. Spinal segment L4 was removed from each animal and was immersed in the same fixative overnight at 4°C. The tissue was then transferred to a 5% sucrose solution in 0.1M phosphate buffer, pH 7.2, at 4°C for storage. At the time of microtomy, all 10 L4 segments were embedded in one block. Five micrometer transverse spinal cord sections were cut using a Bright cryostat.

Immunofluorescence microscopy. All tissue sections were stained for simultaneous immunofluorescent localization of 2 substances (Erichsen et al., 1982; Wessendorf and Elde, 1985). Each section was stained for visualization of SP-immunoreactivity (SP-IR) and immunoreactivity of one other substance. The immunohistochemical staining protocol used has been described in detail (Tuchscherer et al., 1987). Table 1 summarizes the antisera used in the study and the working dilutions at which they were used. The mixture of secondary antibodies contained goat anti-rat IgG labeled with fluorescein isothiocyanate (FITC, Dako Chemical, 1/20 dilution) and goat anti-rabbit IgG labeled with lissamine rhodamine sulfanyl chloride (L-RSC, Dako Chemical, 1/10 dilution). The sections were viewed and photographed with an Olympus BH-2 RFL fluorescence microscope using epi-illumination. FITC and L-RSC were visualized independently using selective filter combinations as described previously (Tuchscherer et al., 1987).

Characterization of the antisera. Based on our experience, no one model has uniformally provided data on cross-reactivity of antisera that are consistent with the visualization of endogenous antigens immobilized within tissue sections. Therefore, we have used 2 approaches, in addition to observations made on tissue sections, to draw conclusions regarding known antigens that our antisera may be visualizing within the material being analyzed. Several of the antisera used in this study have been used by our laboratory for many years, and their characterizations have been previously described (see Table 1). Data in these studies indicated each antiserum was specific for its homologous antigen. Two of the antisera, rabbit anti-CGRP and rabbit anti-galanin, have not been previously characterized by us; thus, data on their cross-reactivities are reported here.

First, absorption controls were performed. When each diluted primary antiserum or antibody was treated for 6–8 hr with the homologous antigen (10 μM in diluted serum), no positive staining was observed in sections of the spinal cord. Next, the model system of Larsson (1981) was used to survey the cross-reactivity of the GAL and CGRP antisera against antigens known to be present in the dorsal horn of the spinal cord. The antigens included: bombesin, CGRP, cholecystokinin-octapeptide, dynorphin A (1–8), leucine-enkephalin, methionine-enkephalin, GAL, neurotensin, oxytocin, 5-HT conjugated to BSA (5-HT-BSA), SOM, substance K, SP, thyrotropin-releasing hormone, vasopressin, and vasoactive intestinal polypeptide. Cross-reactivity to 1 nmol of each heterologous peptide was determined. The primary antisera were used at a concentration of 1/1000, and immunostaining was visualized with the peroxidase antiperoxidase (PAP) approach (Sternberger et al., 1970). Using this model, the GAL antiserum was determined to recognize GAL, alone, while the CGRP antiserum exhibited cross-reactivity to GAL, 5-HT-BSA, SOM, substance K, or SP. The cross-reactivity of the antiserum to 5-HT-BSA is believed to be due to BSA, since the antiserum was generated against a CGRP-BSA conjugate and the antiserum visualized BSA, alone.

As an additional assessment of the cross-reactivity of the CGRP antiserum, heterologous absorption controls were carried out on rat spinal cord and trigeminal ganglia. The CGRP antiserum was used at 1/100, and individual aliquots of this dilution were preincubated with 10 μM concentrations of GAL, 5-HT-BSA, SOM, substance K, and SP. Immunostaining was visualized by immunofluorescence. Absorption with 10 μM substance K, SP, or 5-HT-BSA caused no diminution of the intensity or density of CGRP-immunoreactive varicosities in the superficial laminae of the dorsal horn of the spinal cord or immunoreactive perikarya in the ganglion. Absorption with 10 μM SOM caused an apparent decrease in intensity of staining but not density of immunoreactive varicosities.

The ability of the CGRP antiserum to detect peptides known to be in high concentration in selected areas of the brain was also assessed. The CGRP antiserum did not visualize SOM-containing perikarya in the periventricular region of the hypothalamus. These perikarya were intensely labeled with an antibody to SOM after lesion of their axons. In addition, the CGRP antiserum did not visualize SP-containing varicosities in the globus pallidus, where SP immunoreactivity is known to be high.

Based on the total of these observations, we conclude that the data obtained with the CGRP antiserum does not reflect significant cross-reactivity for 5-HT, SOM, substance K, or SP in tissue sections. However, we cannot exclude the possibility that some of the varicosities visualized with this antiserum reflect binding of antibodies to GAL.

While the cross-reactivities of the other antisera used in this study have been characterized in earlier reports (see Table 1), limited tests were included for GAL and CGRP, whose discovery has been made only recently. Using the model of Larsson (1981), none of the antisera cross-reacted with 1 nmol CGRP, and the 5-HT antiserum did not detect 1 nmol GAL. However, the SP, SOM, NT, and DYNA (1–8) antisera all detected 1 nmol GAL. The cross-reactivity of the SOM antiserum for GAL was estimated to be between 1–10%. The cross-reactivity of the NT antiserum was estimated to be less than 1%. When these antisera were preabsorbed with 10 μM GAL, there was no diminution in the density or intensity of SP- or DYN-immunoreactive varicosities in the superficial laminae of the dorsal horn; however, the amount of NT and SOM immunoreactive varicosities was decreased. These observations indicate that data reported for SP- and DYN-immunoreactive varicosities do not reflect significant cross-reactivity with endogenous GAL, but the data for NT- and SOM-immunoreactive varicosities may include detection of some GAL-immunoreactive varicosities. However, experimental results of the present study do not support this (see Discussion).

Tests have been performed on the secondary antisera to establish

Table 1. Specifications of antisera used

Antiserum	Source	Dilution	Reference
Rat anti-SP NCL/34HL	Sera Labs	1:200	Cuello et al., 1979
Rabbit anti-SOM R176-D	R. Elde	1:100	Sasek et al., 1984
Rabbit anti-CGRP RPN.1842 Lot 3	Amersham	1:100	This study[a]
Rabbit anti-NT Lot 8351022	Immunonuclear Corp	1:100	Seybold and Elde, 1982
Rabbit anti-DYN R2-3	E. Weber	1:330	Sasek et al., 1984
Rabbit anti-5-HT R196D	R. Elde	1:100	Maley and Elde, 1982
Rabbit anti-GAL RAS 7153N	Peninsula	1:100	This study[a]

[a] See Materials and Methods.

their immunological specificity and have been described previously (Tuchscherer et al., 1987). No cross-reactivity was observed.

Quantification methods. Six tissue sections from each animal were analyzed for immunoreactivity using one section for each of the mixtures of antisera. The tissue sections were chosen at random from a variety of distances through the segment. In preliminary tests of the data, no difference in density of SP-IR varicosities in normal animals was measured among the sections selected for quantification. Therefore, differences in densities of immunoreactive varicosities are not likely to reflect a sampling bias. In each tissue section, the region of the superficial laminae at the point deep to the dorsal root entry zone was selected for analysis. As some of the immunoreactivity exhibited medial to lateral differences in distribution, the dorsal root entry zone served as a landmark in all sections. One side of each section from control animals was analyzed. However, in sections from experimental animals, the sides contralateral and ipsilateral to the lesion were analyzed. The working magnification used throughout the study was 250× actual size. Significant differences in the number of varicosities quantified between control and experimental groups were determined using Student's *t* test.

Two areas of termination of primary afferent neurons in the dorsal horn of rat spinal segment L4 were studied: (1) laminae I and II-outer (LI/IIo) and (2) lamina II-inner (LIIi). These areas within the field of observation were identified by producing a dark-field image of each tissue section. The area of least density from the dorsolateral fasciculus (of Lissauer) to the laminae II/III border was then divided such that the dorsal half of the area was operationally defined as LI/IIo, and the ventral half as LIIi. These delineations approximate measurements based on ultrastructural analyses of laminae I and II in the rat (Ribeiro-da-Silva and Coimbra, 1982; Ribeiro-da-Silva et al., 1986). Images of immunoreactive varicosities were stored without moving the stage after the dark-field image was obtained.

The procedure used for acquiring images was modified from the procedure described previously (Tuchscherer et al., 1987). Briefly, 6 images of each tissue section were created using an International Imaging Systems model 75 image processor, which runs 575 software on a MASSCOMP 535 minicomputer. Images of FITC-labeled immunoreactive varicosities and L-RSC-labeled immunoreactive varicosities were created individually for LI/IIo and LIIi. The FITC-labeled varicosities were assigned the color green, and the L-RSC-labeled varicosities, red. Next, the green and red images for each area were superimposed. Varicosities that contained both the FITC-label and the L-RSC label appeared yellow. Finally, the numbers of green, red, and yellow varicosities within the 2 regions were quantified.

In contrast to the earlier study, which derived the density of immunoreactive varicosities from the total image area of varicosities, a custom computer program was used to quantify images of individual varicosities. The program allowed us to define the minimum image area to count as a varicosity. The average minimum size for all immunoreactive varicosities was empirically determined to be 10 pixels.

In addition to controls which estimated the degree of spurious overlap of immunoreactive varicosities described previously (Tuchscherer et al., 1987), several additional controls were developed for this study. Daily changes in microscope lamp alignment or output were monitored using fluorescent microspheres. Before each image-collection session, microspheres (0.5 μm diameter, approximating the size of small peptidergic varicosities; Seybold and Maley, 1984) that had been labeled with the secondary antibodies were viewed with the fluorescence microscope. Images of the fluorescence of the microspheres were produced in the same manner as the fluorescence within the tissue sections. The intensity of the fluorescence of the microspheres as recorded by the digitizing camera was measured. Any alterations in intensity from the previous session were interpreted as differences in illumination, and the lamp was realigned or changed. The silicone-intensified target video camera (DAGE-MTI model 66) used in this study was equipped with controls that allowed changes in gain and amount of contrast in the image delivered to the computer monitor. Preliminary studies were done to identify the optimum settings for the gain and contrast parameters on tissue sections stained with various antisera. After adjustments in microscope illumination of the specimens, these parameters remained constant throughout the study. These control steps decreased the likelihood that changes in densities of immunoreactive varicosities quantified on tissue sections were due to alterations in detection of varicosities by the camera.

To monitor nonspecific differences between tissue sections from different animals, the intensity of the background and the intensity of individual varicosities were measured on each image. The ranges for background staining and the ranges for the least intense to most intense varicosities were not significantly different between sections from control and experimental animals as determined by Student's *t* test. Finally, a restraint was added in order to eliminate experimenter bias in detection of varicosities. One of the interactive steps in the quantification process required the investigator to assign a minimum intensity level to detect varicosities. Values used in the detection step remained constant throughout the study.

Due to changes in volume caused by dorsal rhizotomy, a correction factor was applied to density data from experimental animals. This factor was derived from measurements of the depth of laminae I and II in each tissue section. The mean depth of laminae I and II in experimental animals was found to be 72% of the mean depth of laminae I and II in control animals. Therefore, in order to normalize the density determinations, each datum collected from sections of experimental animals was multiplied by 0.72.

Results

Densities of varicosities immunoreactive for each substance

A summary of the densities of total peptide- and 5-HT-immunoreactive (5-HT-IR) varicosities within the superficial laminae of the dorsal horn of control animals and those that had undergone unilateral dorsal rhizotomy (hereafter referred to as experimental animals) is shown in Figure 1. The results demonstrate that in control animals, CGRP-immunoreactive (CGRP-IR) varicosities occurred in the highest density within both areas analyzed (LI/IIo and LIIi) compared with the other substances studied. The data also indicate that varicosities immunoreactive for all of the peptides studied and 5-HT occurred at a greater or equal density in LI/IIo compared with LIIi, except NT-immunoreactive (NT-IR) varicosities.

Comparing varicosity densities in control animals (Fig. 1*A*) with values ipsilateral to dorsal rhizotomies in experimental animals, only the density of NT-IR varicosities was unchanged. Significant reductions in the densities of immunoreactive varicosities occurred for all of the other peptides and 5-HT. The depletion of CGRP-IR varicosities (Fig. 2) was almost complete

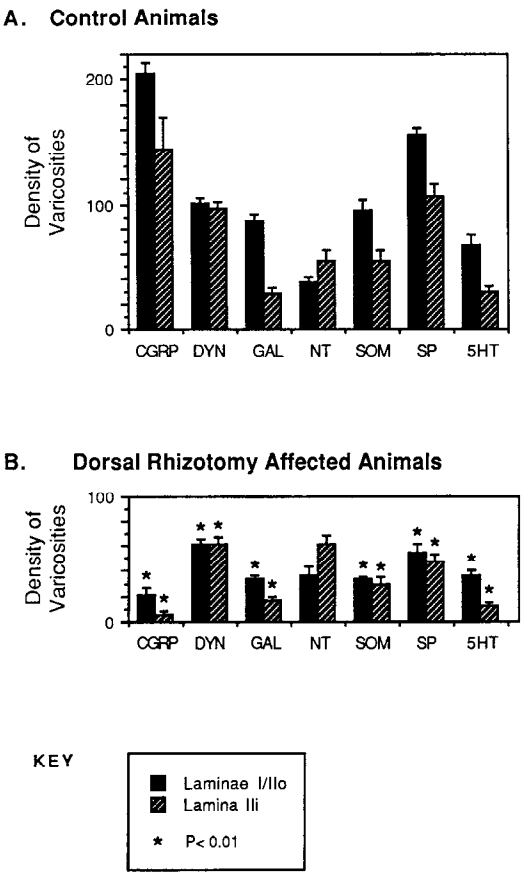

Figure 1. Histograms of densities of total immunoreactive varicosities within laminae I and II_{outer} (LI/IIo) and lamina II_{inner} (LIIi) of the dorsal horn of the spinal cord of the rat. The *asterisks* indicate reduction in density of a population of varicosities immunoreactive for one peptide that is significantly different from the value of the control group at $p < 0.01$ (Student's t test). Values represent densities of immunoreactive varicosities/6600 μm^2.

from both LI/IIo and LIIi. The density of SP-IR varicosities was decreased by approximately 70% in experimental animals, while the reductions for SOM (see Fig. 3) and GAL (see Fig. 7, *A, C*) were about 60%. Reductions in density of approximately 50 and 40% for 5-HT-IR and DYN-IR varicosities, respectively, were observed in experimental animals. For all substances, values for the side contralateral to the dorsal rhizotomies in experimental animals (data not shown) were not different from the values shown in Figure 1*A*.

Coexistence in control animals

Figure 4 summarizes the densities of varicosities exhibiting coexistence of peptide and 5-HT-immunoreactivities with SP-IR in control animals. With the possible exception of CGRP-IR varicosities, these values reflect varicosities that may arise from brain stem, spinal, and primary afferent sources. SP-IR did not coexist in varicosities containing 5-HT-IR (see Fig. 5), SOM-IR, or NT-IR within the areas analyzed. SP-IR coexisted with immunoreactivity for CGRP, GAL (see Fig. 7, *A, B*) or DYN (see Fig. 6) within varicosities.

The proportion of total peptide-immunoreactive varicosities containing SP-IR will be compared first. Approximately one-half of the density of total CGRP-IR varicosities in LI/IIo contained SP-IR, while only one-third of the density of total CGRP-IR varicosities in LIIi contained SP-IR. In both LI/IIo and LIIi, 45% of the density of total DYN-IR varicosities contained SP-IR. Forty percent of the density of the total varicosities immunoreactive for GAL in LI/IIo were immunoreactive for SP, and 34% of the density of total GAL-IR varicosities in LIIi contained SP-IR.

The proportion of the total SP-IR varicosities containing other peptide immunoreactivities can also be described. In LI/IIo, 50% of the density of total SP-IR varicosities were immunoreactive for CGRP, while only 25% of the density of the total SP-IR varicosities contained GAL-IR and 20% contained DYN-IR. In LIIi, one-third of the density of total SP-IR varicosities contained CGRP-IR or DYN-IR, while only one-fifth of the density of total SP-IR varicosities contained GAL-IR.

Coexistence in varicosities of primary afferent neuron origin

Figure 8 summarizes the calculated densities of coexistence of peptide- and 5-HT-immunoreactivities with SP-IR in varicosities of primary afferent origin. The results were derived from tissue sections of experimental animals by subtracting data collected from the side ipsilateral to the lesion from data collected from the contralateral side. SP-IR did not coexist with 5-HT-IR, SOM-IR, or DYN-IR in varicosities of primary afferent origin. Comparison of Figures 4 and 8 shows that all varicosities that cocontained SP-IR and CGRP-IR or GAL-IR were of primary afferent origin. Approximately 76% of the density of SP-IR varicosities of primary afferent origin contained CGRP-IR in LI/IIo. One-third of the density of SP-IR varicosities of primary afferent origin contained GAL-IR in LI/IIo. Approximately 50% of the densities of GAL-IR or CGRP-IR varicosities of primary afferent origin contained SP-IR in LI/IIo.

Discussion

This study employed computer-assisted image processing to quantify densities of CGRP-, DYN-, GAL-, NT-, SOM-, SP-, and 5-HT-IR varicosities in 2 distinct regions of the dorsal horn of the spinal cord in normal animals and animals that had undergone unilateral dorsal rhizotomy. The results of this study corroborate and extend the findings of other laboratories using immunohistochemical methods to compare relative densities of peptide immunoreactive varicosities in the dorsal horn of the spinal cord.

Antiserum cross-reactivity

In light of the data acquired with the antisera on tissue sections in the present study, it is possible to make additional comments concerning the cross-reactivity of antisera with *endogenous* antigens. It is not likely that the SOM or NT antisera detected GAL within our material since no coexistence of these substances with SP was detected, yet measurable densities of GAL+SP-IR varicosities were detected. It is also unlikely that the DYN antiserum detected GAL in our material since a population of DYN+SP-IR varicosities was not associated with primary afferent neurons. While our quantitative data on immunoreactive varicosities cannot exclude a cross-reactivity of CGRP with endogenous GAL, we do feel the data indicate that the DYN, SP, SOM, 5-HT, and NT antisera do not exhibit cross-reactivity with endogenous forms of the antigens against which the antisera were tested.

Density of immunoreactive varicosities in normal animals

In studies where densities for similar peptides were compared (Seybold and Elde, 1980; Gibson et al., 1981; Hunt et al., 1981),

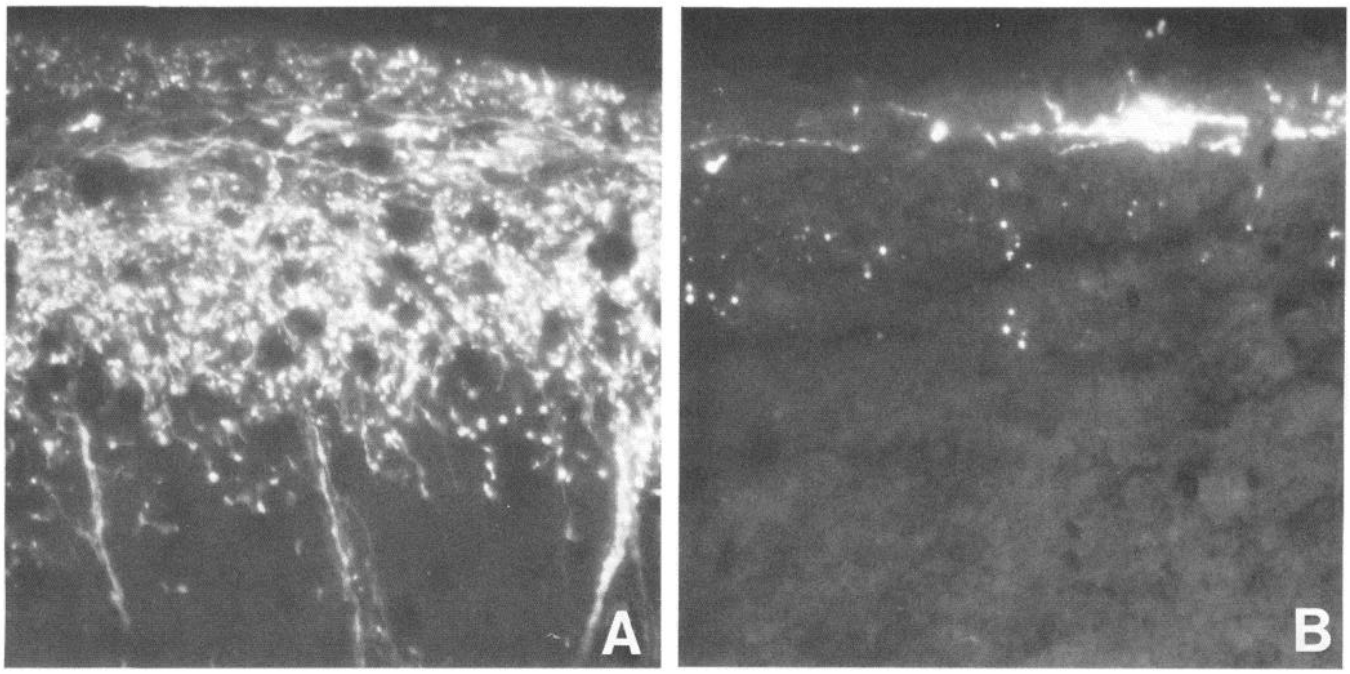

Figure 2. Photomicrographs illustrating the effect of dorsal rhizotomy on the density of CGRP-IR varicosities in the superficial laminae of the dorsal horn. *A,* Control animal; *B,* experimental animal. Magnification, ×1130. Note that deafferentation results in almost complete reduction of CGRP-IR elements from the superficial laminae of the dorsal horn of the spinal cord.

our results are in agreement with descriptions in which the density of SP-IR varicosities is high within both LI/IIo and LIIi, and the density of SOM-IR varicosities is considerably less than that of SP-IR. Our results also agree with descriptions in which the density of SOM-IR varicosities is greater than that of NT-IR varicosities in LI/IIo (Seybold and Elde, 1980). Our results also showed that of the peptides studied, only NT-IR varicosities existed at a higher density in LIIi than in LI/IIo. This finding is in agreement with previous reports (Hunt et al., 1981; Seybold and Elde, 1982) in which the superficial laminae of the dorsal horn were also studied separately.

Our study included quantification of densities of CGRP and GAL immunoreactivities. As these peptides have only recently been discovered (Rosenfeld et al., 1983; Tatemoto et al., 1983),

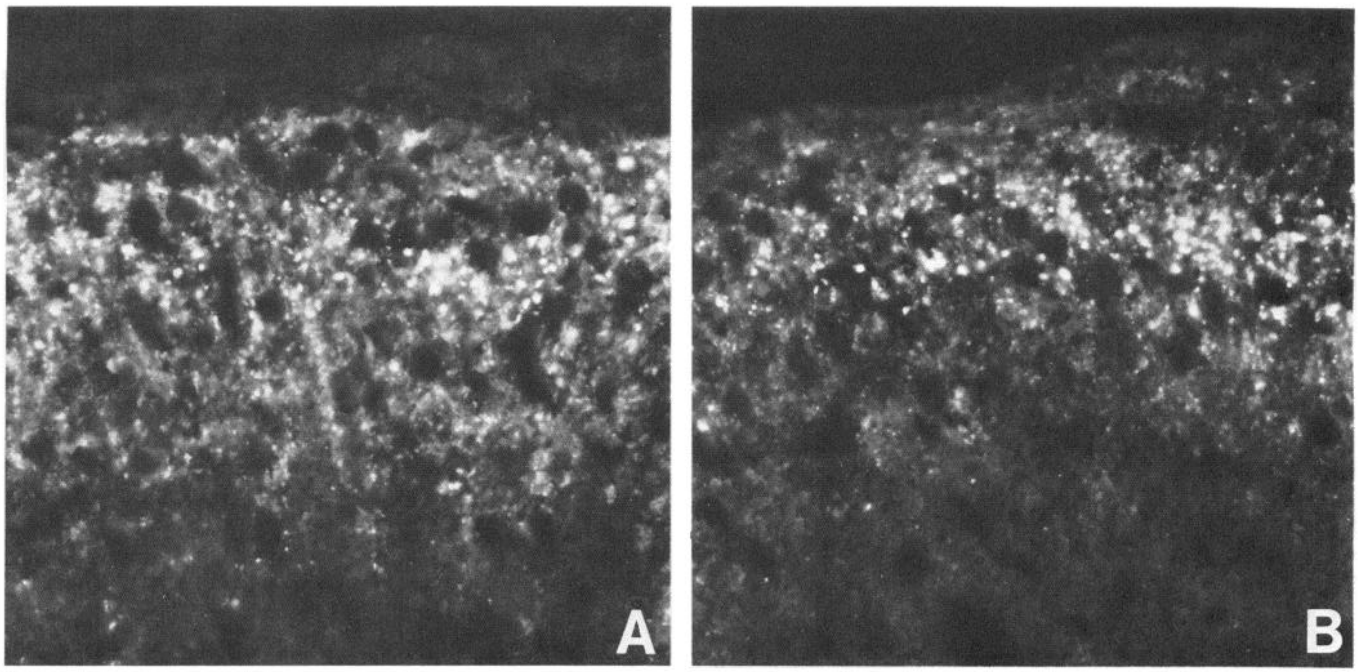

Figure 3. Photomicrographs illustrating the effect of dorsal rhizotomy on the density of SOM-IR varicosities in the superficial laminae of the dorsal horn. *A,* Control animal; *B,* experimental animal. Magnification, ×1130. Note that deafferentation results in a partial reduction of SOM-IR elements in the dorsal horn of the spinal cord.

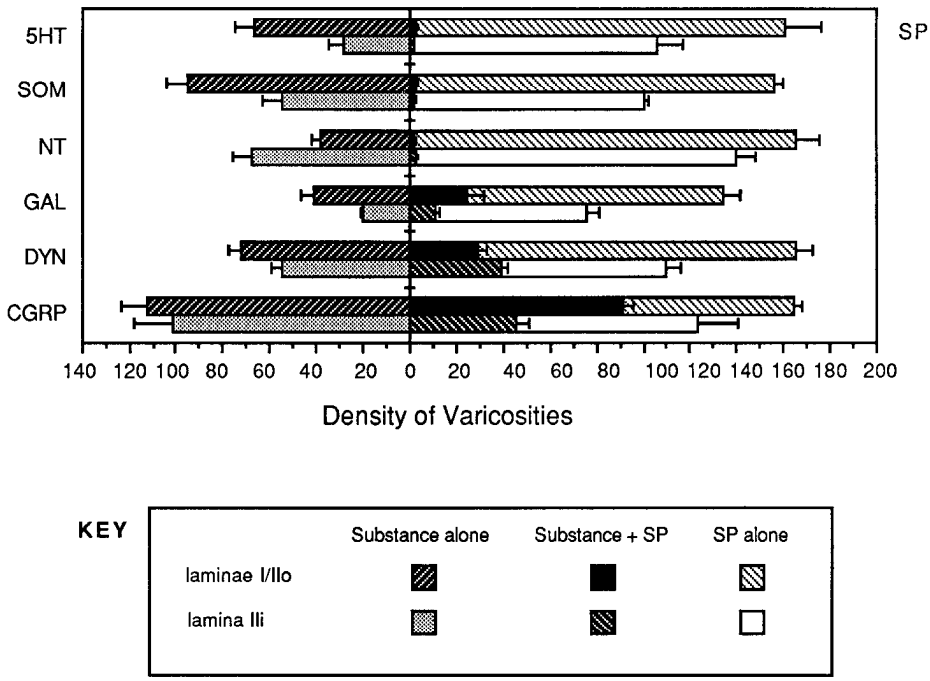

Figure 4. Histogram illustrating densities of varicosities immunoreactive for substances alone and densities of varicosities in which substances coexist with SP-IR in the superficial laminae of the dorsal horn. Bars to the *left* of the zero point reflect densities of varicosities containing one substance alone. Bars to the *right* of the zero are stacked. In cases where coexistence was observed, those values occur immediately to the right of the zero. Values represent densities of immunoreactive varicosities/6600 μm^2. See key and text for further details.

their relative densities have not been directly compared with densities of other substances in the dorsal horn of the spinal cord to date. Our results demonstrated that the density of CGRP-IR varicosities was greater than the density of any of the other substances we analyzed for both LI/IIo and LIIi.

Density of immunoreactive varicosities in experimental animals

Previous studies have reported reductions in the relative densities of many peptide-immunoreactive varicosities in the dorsal horn of the spinal cord as a result of dorsal rhizotomy (for review, see Salt and Hill, 1983; SP: Barber et al., 1979; Jessell et al., 1979; Seybold and Elde, 1980; SOM: Seybold and Elde, 1980; Stine et al., 1982; Schroder, 1984; CGRP: Gibson et al., 1984a, b). Our results corroborate these findings and add new information concerning changes in the relative densities of varicosities immunoreactive for several other peptides. Our results established that the densities of GCRP-, DYN-, GAL-, SOM-, SP-, and 5-HT-IR varicosities were significantly reduced ipsilateral to the lesion in animals that underwent unilateral dorsal rhizotomy. These data are consistent with observations of most of the substances within dorsal root ganglia (SP: Hökfelt et al., 1975; SOM: Hökfelt et al., 1976; CGRP: Rosenfeld et al., 1983; GAL: Skofitsch and Jacobowitz, 1985a; DYN: Sweetnam et al., 1982). However, we cannot conclude that the magnitude of the decrease in each instance is due solely to substances contained in primary afferent axons. The possibility exists that a portion of the decrease reflects a change secondary to the loss of primary afferent neuronal transmission in the spinal cord. In addition, our observation that significant densities of SP- and SOM-IR varicosities remained in LI/IIo and LIIi ipsilateral to the lesion in experimental animals complements studies showing intraspinal and/or bulbospinal elements immunoreactive for SP (Bowker et al., 1981; Gibson et al., 1981; Hunt et al., 1981; Johansson et al., 1981) and SOM (Dalsgaard et al., 1981; Schroder, 1984).

Some contradiction exists in the literature regarding the alteration of DYN-IR in the dorsal horn of the spinal cord following dorsal rhizotomy. Although DYN-IR has been localized in dorsal root ganglia of the rat (Botticelli et al., 1981) and mouse (Sweetnam et al., 1982), Botticelli and coworkers (1981) found that dorsal rhizotomy did not alter spinal levels of DYN-IR when measured by radioimmunoassay. Our results, however, showed that the density of DYN-IR varicosities was significantly reduced in both LI/IIo and LIIi in experimental animals compared with levels in normal animals. Differences in experimental approach may account for these divergent results. Immunohistochemical techniques may provide greater sensitivity and resolution for detecting changes in the distribution of immunoreactivity among discrete regions. Our observations regarding DYNA (1–8) parallel those of Basbaum and coworkers (1986). These investigators reported significant reductions in DYNB immunoreactivity from the dorsal horn of the sacral spinal cord of the cat following dorsal rhizotomy as detected by immunohistochemical techniques.

Our results also showed that the density of GAL-IR varicosities was significantly reduced ipsilateral to the lesion in both LI/IIo and LIIi and that the decrease in density of GAL-IR varicosities was greater in LI/IIo than in LIIi. These results extend the findings of an earlier report demonstrating that GAL-IR in the dorsal horn of the spinal cord was reduced following neonatal application of capsaicin (Ch'ng et al., 1985).

An unexpected result of dorsal rhizotomy was a decrease in the density of 5-HT-IR varicosities from the superficial laminae of the dorsal horn. We interpret this as a change secondary to deafferentation. Although there is one report of 5-HT immunoreactivity in primary afferent neurons (Kai-Kai and Keen, 1985), the validity of the data may be questioned since 5-hydroxytryptophan treatment was used to enhance 5-HT immunoreactivity. This substance may be nonspecifically taken up and decarboxylated by a catecholamine neuron. Thus, this approach may have labeled catecholamine-positive neurons,

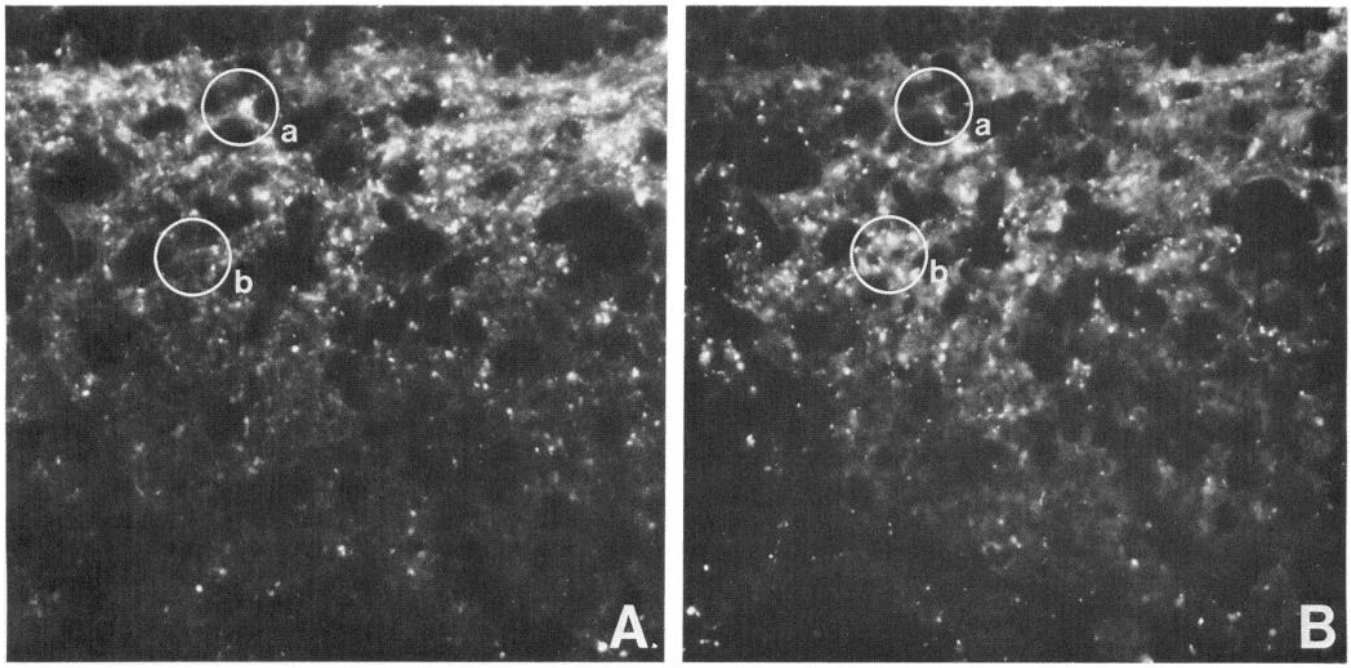

Figure 5. Photomicrographs illustrating that SP-IR and 5-HT-IR do not coexist in the superficial laminae of the dorsal horn. *A,* SP-IR; *B,* 5-HT-IR. *Circles* labeled *a* in both hotomicrographs indicate one field in which SP-IR varicosities do not contain 5-HT-IR. *Circles* labeled *b* in both photomicrographs indicate one field in which 5-HT-IR varicosities do not contain SP-IR. Magnification, ×1130.

which have been shown to exist in sensory ganglia on the basis of tyrosine hydroxylase immunoreactivity (Price and Mudge, 1983). Other reports have established that 5-HT-IR elements in the dorsal horn of the spinal cord arise primarily from bulbospinal pathways (Basbaum et al., 1978; Basbaum and Fields, 1979; Bowker et al., 1981) or from projections of intraspinal neurons that exist ventral to the central canal (LaMotte and deLanerolle, 1983). Our data are consistent with a recent report that SP does not coexist with 5-HT in varicosities within the superficial laminae of the dorsal horn (Wessendorf and Elde, 1987). It has been suggested that some 5-HT terminals modify primary afferent processing (see LaMotte, 1986, for review).

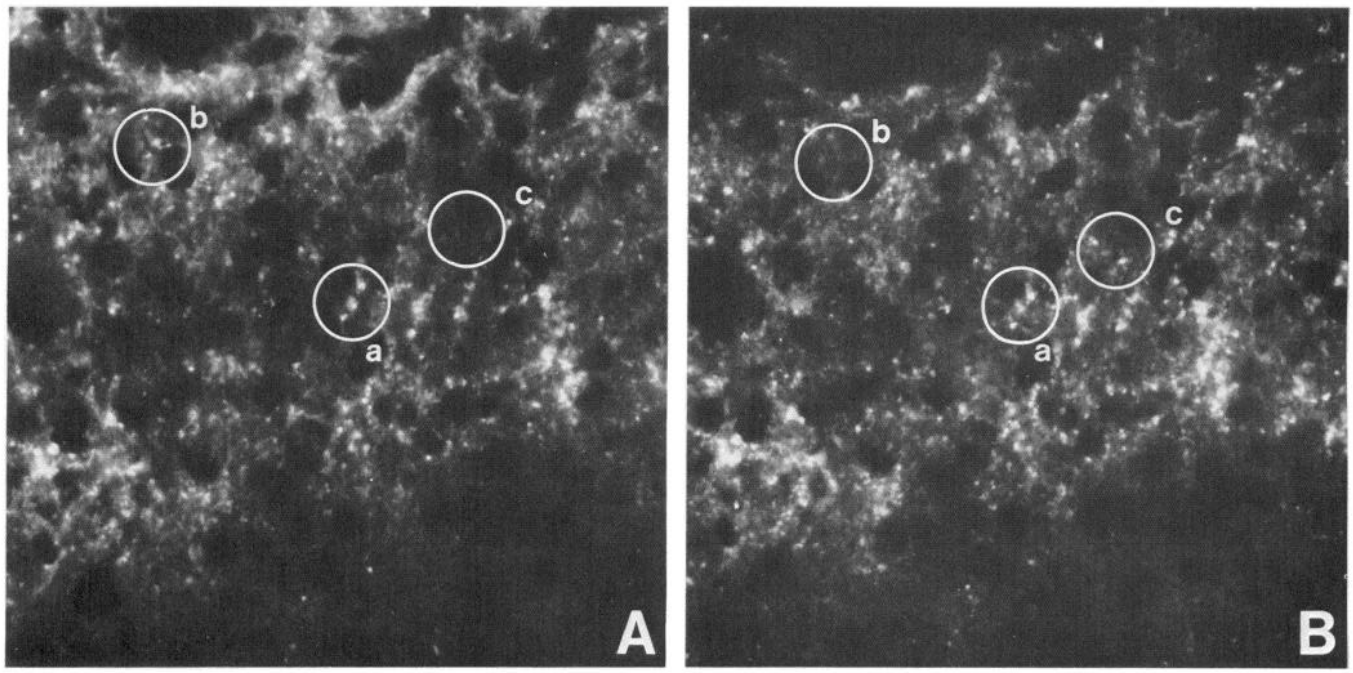

Figure 6. Photomicrographs illustrating that SP-IR and DYN-IR coexist in some varicosities within the superficial laminae of the dorsal horn. *A,* SP-IR; *B,* DYN-IR. *Circles* labeled *a* in both photomicrographs indicate one field in which SP-IR and DYN-IR coexist within varicosities. *Circles* labeled *b* indicate one field in which several SP-IR varicosities do not contain DYN-IR. *Circles* labeled *c* indicate one field in which several DYN-IR varicosities do not contain SP-IR. Magnification, ×1130.

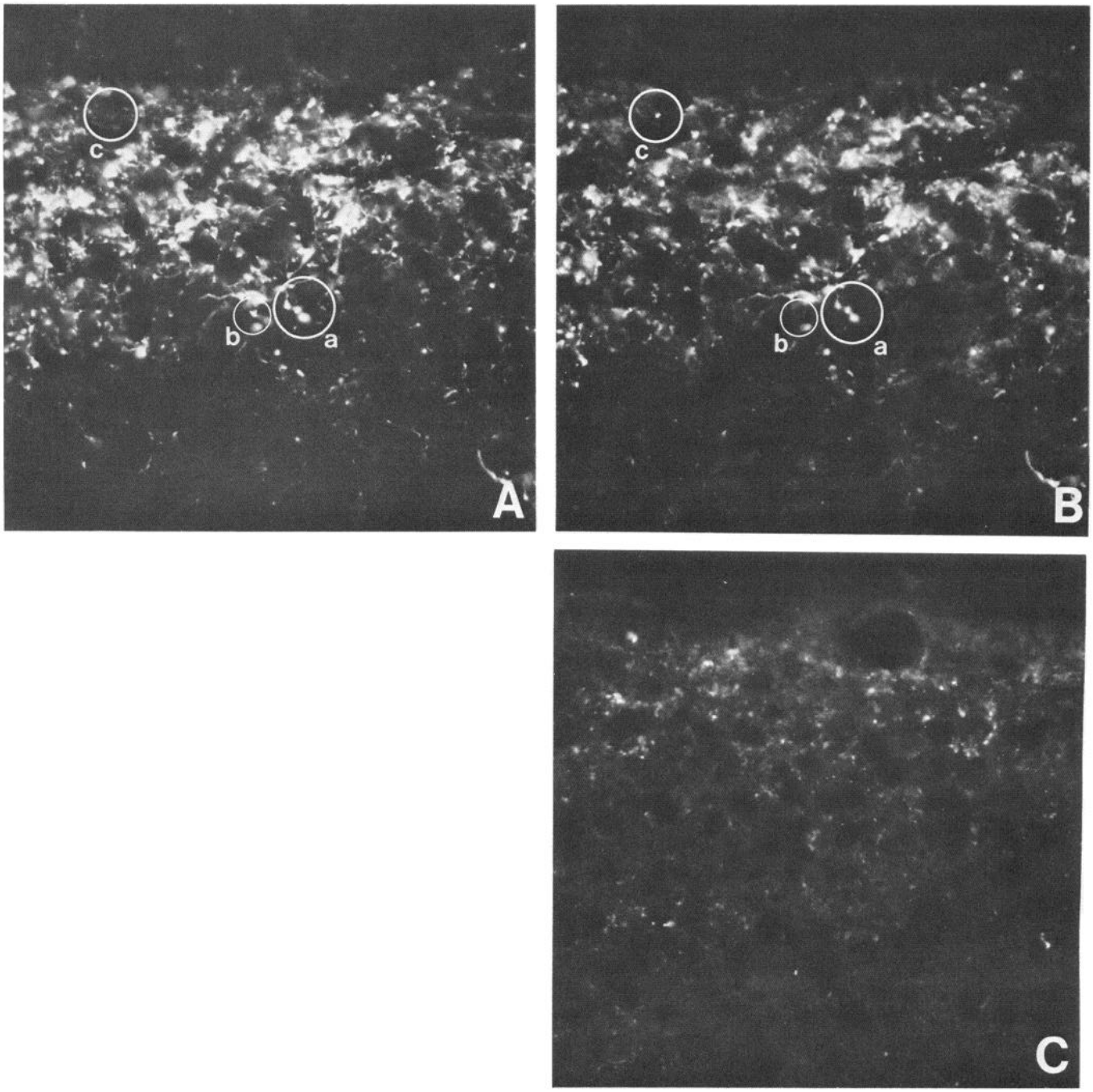

Figure 7. Photomicrographs illustrating coexistence of SP-IR and GAL-IR in some varicosities (*A* and *B*) and the effect of dorsal rhizotomy on the density of GAL-IR (*B* and *C*) within the superficial laminae of the dorsal horn. *A*, SP-IR of a control animal; *B*, GAL-IR within the same section as *A*; *C*, GAL-IR of an experimental animal. *Circles* labeled *a* indicate one field in which varicosities exhibit immunoreactivity for both SP and GAL. *Circles* labeled *b* indicate one field in which some varicosities are immunoreactive for SP but not GAL. *Circles* labeled *c* indicate one field in which some varicosities are immunoreactive for GAL but not for SP. In *C*, note that deafferentation results in a partial reduction in GAL-IR elements in the dorsal horn of the spinal cord. Magnification, ×1130.

Therefore, it is possible that loss of primary afferent neuron input causes a reduction in expression of 5-HT-IR within elements that contact primary afferent terminals.

Coexistence of peptides in varicosities within laminae I and II in normal and experimental animals

Our results confirm studies showing that SP-IR coexists with CGRP-IR in primary afferent neuron perikarya [Wiesenfeld-Hallin et al., 1984 (guinea pig); Gibson et al., 1984a (man); Lee et al., 1985a, b (rat)] and in peripheral processes of sensory neurons of guinea pigs (Gibbins et al., 1987). However, our report represents the first evidence of varicosities that contain both SP and CGRP (SP+CGRP), immunoreactivity in the superficial laminae of the dorsal horn of the rat. Furthermore, we have shown that the total density of SP+CGRP varicosities in LI/IIo and LIIi of the dorsal horn of the spinal cord arose from

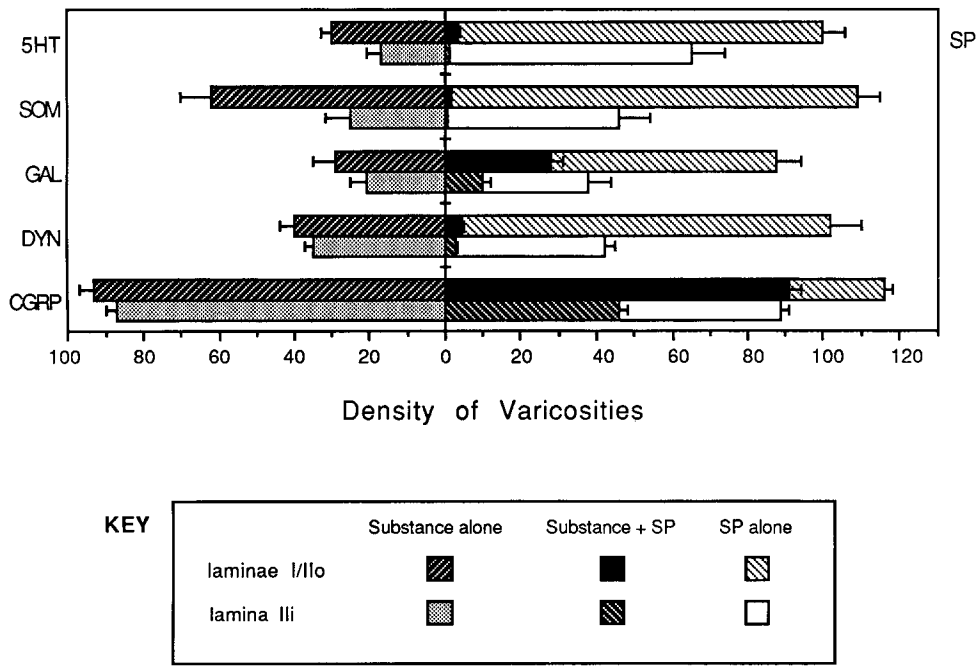

Figure 8. Histogram illustrating coexistence of peptides and 5-HT with substance P in primary afferent varicosities within the superficial laminae of the dorsal horn of the spinal cord. Bars to the *left* of the zero point reflect densities of varicosities containing one substance alone. Bars to the *right* of the zero are stacked. In cases where coexistence was observed, those values occur immediately to the right of the zero. Values represent densities of immunoreactive varicosities/6600 μm^2. See key and text for further information.

primary afferent neurons as dorsal rhizotomy induced complete reduction of these varicosities from the areas studied.

Although studies carried out in cat have demonstrated the coexistence of SOM and SP immunoreactivity in primary afferent perikarya (Leah et al., 1985), our studies determined that SP and SOM immunoreactivity do not coexist in varicosities of the dorsal horn of the spinal cord of rat. This was observed in both control and experimental animals, and confirms earlier studies in the rat (Hökfelt et al., 1976; Tuchscherer and Seybold, 1985), which demonstrated separate populations of perikarya immunoreactive for SP and SOM within dorsal root ganglia.

The present study demonstrated 2 previously unreported instances of coexistence of peptide immunoreactivities. First, we have shown that SP and GAL (SP+GAL) immunoreactivities coexisted in varicosities within LI/IIo and LIIi of the dorsal horn of the spinal cord. Further, as was the case for SP+CGRP varicosities, the population of SP+GAL varicosities was found to be almost entirely of primary afferent origin. The SP+GAL population of primary afferent varicosities was considerably smaller than the SP+CGRP population of primary afferent varicosities. Second, we have shown that SP and DYN (SP+DYN) immunoreactivities also coexisted in varicosities of LI/IIo and LIIi of the dorsal horn of the spinal cord. In this case, however, dorsal rhizotomy had no effect on the density of SP+DYN varicosities in either area studied. Although a portion of the *total* density of DYN-IR varicosities was reduced by dorsal rhizotomy—and, therefore, some DYN-IR varicosities are likely to be of primary afferent origin—the population of SP+DYN varicosities more likely arises from either descending or intraspinal systems of input. The sources of the SP+DYN elements have not been addressed in the present study, though previous studies have shown that DYN-IR perikarya (Miller and Seybold, 1987) and SP-IR perikarya (Hökfelt et al., 1975) are present within the superficial laminae of the dorsal horn of the rat.

Primary afferent neuron markers

Recently, several laboratories have begun to approach the complexity of the primary afferent system from an "organizational" point of view by suggesting that the variety of peptides localized in small-diameter perikarya may serve as markers for specific populations of functionally described primary afferent neurons. Vasoactive intestinal polypeptide and DYN, for example, have been localized preferentially to levels of the spinal cord that receive visceral afferents (Basbaum and Glazer, 1983; Honda et al., 1983; Gibson et al., 1984b; Kuo et al., 1985). It has been suggested that these peptides may be markers for primary afferent neurons associated with viscera. Similarly, individual patterns of coexistence of peptides in primary afferent perikarya and peripheral processes of these neurons have been observed within specific peripheral targets (Gibbins et al., 1987). In this study, we have presented data suggesting that patterns of peptide immunoreactivity observed within the spinal cord may also serve as markers of primary afferent neuron processes. We have shown that CGRP alone, SP+CGRP, and SP+GAL varicosities are almost entirely of primary afferent origin. Therefore, these substances in combination may be used as markers of primary afferent neurons in morphological studies that address circuitry in the superficial laminae of the dorsal horn of the spinal cord.

Conclusions

In summary, the major conclusions to be drawn from this work are the following: (1) Significant proportions of the densities of SP, SOM, GAL, CGRP, and DYN varicosities in LI/IIo and LIIi of the dorsal horn of the rat spinal cord are derived from primary afferent neuron sources; (2) SP+CGRP, SP+GAL, and SP+DYN coexist in varicosities within the spinal cord; and (3) densities of CGRP, SP+CGRP, and SP+GAL varicosities in LI/IIo and LIIi are derived almost entirely from primary afferent neuron sources, and may be considered markers of primary afferent axons in the areas outlined.

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