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CALCITONIN GENE-RELATED PEPTIDE IMMUNOREACTIVITY IN THE SPINAL CORD OF MAN AND OF EIGHT OTHER SPECIES[1]

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Abstract

Calcitonin gene-related peptide (CGRP) immunoreactivity was found throughout the entire spinal cord of man, marmoset, horse, pig, cat, guinea pig, mouse, rat, and frog. CGRP-immunoreactive fibers were most concentrated in the dorsal horn. In the ventral horn of some species large immunoreactive cells, tentatively characterized as motoneurons, were present. Pretreatment of rats with colchicine enhanced staining of these large cells but did not reveal CGRP-immunoreactive cell bodies in the dorsal horn. In the dorsal root ganglia, CGRP immunoreactivity was observed in most of the small and some of the intermediate sized cells. Substance P immunoreactivity, where present, was co-localized with CGRP to a proportion of the small cells. In the cat the ratio of substance P-immunoreactive to CGRP-immunoreactive ganglion cells was 1:2.7 ($p < 0.001$).

The concentration of CGRP-immunoreactive material in tissue extracts was determined by radioimmunoassay. In the dorsal horn of the rat spinal cord the levels of peptide were found to range from 225.7 ± 30.0 pmol/gm of wet weight in the cervical region to 340.6 ± 74.6 pmol/gm in the sacral spinal cord. In the rat ventral spinal cord, levels of 15.7 ± 2.7 to 35.1 ± 10.6 pmol/gm were found. The concentration in dorsal root ganglia of the lumbar region was 225.4 ± 46.9 pmol/gm.

Gel permeation chromatography of this extractable CGRP-like immunoreactivity revealed three distinct immunoreactive peaks, one eluting at the position of synthetic CGRP and the others, of smaller size, eluting later.

In cats and rats, rhizotomy induced a marked loss of CGRP-immunoreactive fibers from the dorsal horn of the spinal cord. In the cat, unilateral lumbosacral dorsal rhizotomy resulted in a significant ($p < 0.05$) reduction of extractable CGRP from the ipsilateral lumbar dorsal horn (5.6 ± 1.2 pmol/gm of wet weight) compared to the contralateral side (105.0 ± 36.0 pmol/gm of wet weight).

We conclude that the major origin of CGRP in the dorsal spinal cord is extrinsic, from afferent fibers which are probably derived from cells in the dorsal root ganglia. The selective distribution of CGRP throughout sensory, motor, and autonomic areas of the spinal cord suggests many putative roles for this novel peptide.

The advent of recombinant DNA technology has allowed the prediction, by mRNA analysis, of the existence of previously unknown peptide sequences. In this way, Amara and others (Amara et al., 1982; Rosenfeld et al., 1983) recently suggested and subsequently demonstrated the existence of a calcitonin gene-related peptide (CGRP) which is synthesized in neural tissue. Using antibodies raised against a synthetic amino acid sequence corresponding to a portion of the predicted C-terminal of CGRP, this newly discovered peptide has been localized by immunocytochemistry to different areas of the central and peripheral nervous system. The distribution of CGRP in the nervous system suggests several roles in autonomic, sensory, and motor activities (Rosenfeld et al., 1983).

The purpose of the present study was to carry out a systematic investigation of the distribution and origin of CGRP immunoreactivity in the spinal cord of several mammalian and non-mammalian species. The techniques employed were immunocytochemistry, for the precise localization of the peptide, and radioimmunoassay, for the measurement and chemical characterization of the immunoreactive material.

To determine the origin of CGRP immunoreactivity in the spinal cord, the effects of surgical manipulation and pharmacological agents on the distribution of CGRP immunoreactivity were investigated. Since substance P has a similar and abundant distribution in the dorsal horn of the spinal cord (Barber et al., 1979; Gibson et al., 1981), a comparative study of the

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distribution of both peptides and their possible coexistence in the same primary sensory neuron of the dorsal root ganglion was carried out.

Materials and Methods

Experimental procedures

All animals undergoing surgery were anesthetized with either sodium pentobarbitone (Sagatal) (35 mg/kg of body weight, i.p.) or halothane (oxygen/nitrous oxide mixture).

Unilateral rhizotomy

The dorsal roots (L_2 to S_4) of four cats and dorsal roots (C_5 to C_8) of three rats were cut unilaterally as described previously (Gibson et al., 1984b, c). The cats and rats were killed 4 and 2 weeks after the operations, respectively. The success of the operations was judged by the way in which the animal held the limb when at rest and by the absence of motor deficiencies in the hind legs of cats and forelegs of the rats during feeding and grooming. After death, the operation site was inspected to verify that all rootlets had been cut.

Intraspinal colchicine application

Colchicine (2 to 10 μl, 10 mg/ml) dissolved in sterile saline was injected directly into the exposed thoracic spinal cord of rats ($n = 5$). The animals were killed 24 to 48 hr later.

Immunocytochemistry

Tissue processing

Five mice, 15 rats (7 normal, 5 colchicine treated, and 3 following cervical rhizotomy), 6 guinea pigs, 4 marmosets, and 6 cats were anesthetized with an overdose of sodium pentabarbitone (Euthanal) and fixed for immunocytochemistry by intracardial perfusion with a solution of *p*-benzoquinone (BDH Chemicals Ltd., Essex, United Kingdom, specially purified), 0.4% in 0.01M phosphate-buffered saline (PBS) (pH 7.2, 200 ml/100 gm of body weight). Entire spinal cords were removed by laminectomy and were placed in a solution of the same fixative for 1 to 7 hr. Dorsal root ganglia were dissected and further fixed by immersion for 0.5 to 1 hr.

The spinal cords obtained from eight adult and three neonate humans (24 hr post mortem), three horses (4 hr post mortem), four pigs, and four frogs and the lumbosacral spinal cords of the four rhizotomized cats were cut into 1- to 2-cm slices and fixed by immersion in *p*-benzoquinone solution for 5 to 16 hr. Dorsal root ganglia were fixed whole in the same solution for 3 to 6 hr. The samples were washed and stored in PBS containing sucrose (7% w/v) and sodium azide (0.01% w/v) at 4°C.

Immunostaining

Distribution and origin. Cryostat sections (20 μm) were mounted on poly-L-lysine-coated slides (Huang et al., 1983) and air-dried for 2 hr before incubation with primary antisera directed to either CGRP (diluted 1/4000), substance P (diluted 1/8000), or vasoactive intestinal polypeptide (diluted 1/8000) used as a control for successful immunostaining after colchicine (see below). The characteristics of the CGRP antisera used are shown in Table I (for substance P, see Gibson et al., 1984a, and for vasoactive intestinal polypeptide, see Gibson et al., 1984b). Sections were immunostained using a slightly modified (Gibson et al., 1981) peroxidase-antiperoxidase (PAP) method (Sternberger, 1979). The site of peroxidase attachment was demonstrated by development with either diaminobenzidine (brown) or 4-chloro-1-naphthol (blue/black) as the chromophore.

Antisera: Specificity. To ensure method specificity, either some sections were incubated with nonimmune rabbit serum or one incubation step in the PAP method was omitted. The specificity of the antiserum to CGRP was verified by lack of staining in adjacent sections incubated with antiserum preabsorbed with synthetic CGRP in the dilution range 0.01 to 0.1nmol/ml of diluted antiserum. Further dilutions of the peptide showed return of immunostaining, demonstrating that the very small amounts of antigen in the tissue were competing with the small amounts of added peptide.

Characterization of immunostaining of putative motoneurons. Sections from cervical and lumbar enlargements of the rat spinal cord were stained for CGRP and the location of positively stained large cells in the ventral horn was compared with adjacent serial sections stained for Nissl substance using cresyl violet.

TABLE I
Characteristics of CGRP antisera

	Immunocytochemistry	Radioimmunoassay
Donor species	Rabbit	Rabbit
Hapten	CGRP (rat) (24-37)Tyr[23]	CGRP (rat) (24-37) Tyr 23
Carrier	Human α-globulin	Human α-globulin
Coupling agent	Diazobenzidine	Diazobenzidine
Antibody dilution	1/4,000	1/120,000
Region specificity	C-terminal	C-terminal
Radiolabel	NA[a]	[^{125}I]CGRP
Iodination	NA	Chloramine T method
Assay sensitivity (fmol/assay tube)	NA	2.0
Assay standard	NA	Synthetic CGRP[b]
No cross-reaction with peptides[c]	Substance P 1-9, Somatostatin-14, calcitonin, bombesin	Substance P Calcitonin

[a] NA, not applicable.

[b] Synthetic CGRP was obtained from Peninsula laboratories, Inc., Belmont, CA.

[c] For immunocytochemistry the specificity of the CGRP antiserum was verified by lack of staining in adjacent sections incubated with antiserum preabsorbed with synthetic CGRP. In further control sections which had been incubated with a range of related and nonrelated peptides (10 nmol of antigen/ml of diluted antiserum), positive staining was unaffected.

Substance P- and CGRP-immunoreactive cells in cat and rat dorsal root ganglia

The peptide, substance P, is present in approximately 20% of small sized dorsal root ganglion cells (Hökfelt et al., 1980). For this reason we tried to establish (*1*) the ratio of substance P- to CGRP-immunoreactive ganglion cells and (*2*) whether substance P was co-located with CGRP.

i. Ratio of substance P- to CGRP-immunoreactive ganglion cells. A semi-quantitative analysis of the number of substance P- and CGRP-positive dorsal root ganglion cells in the lumbar region of six cats was made. The cell count was taken from 6 to 8 (20 μm) pairs of sections (one stained for substance P, the other for CGRP)/ganglion.

iia. Co-localization of substance P and CGRP: Serial sections. Cryostat sections (20 μm) were cut serially. The first section was picked up normally on the slide and the next serial section was flipped over onto the slide. Thus, the adjacent faces (mirror images) of paired sections were exposed. These were incubated, one for substance P and the other for CGRP. The resultant preparations were either drawn using a camera lucida attachment to the microscope or photographed, and like fields were superimposed.

iib. Co-localization of substance P and CGRP: Elution (adapted from Nakane, 1968). Sections (20 μm) were immunostained with antisera to substance P as primary antiserum. The slides were processed according to the PAP method and developed in 4-chloro-1-naphthol solution. The slides were coverslipped and photographed. Primary antiserum was eluted from the sections by immersion in a glycine/hydrochloric acid buffer (pH 2.2) leaving the blue end product of the reaction *in situ*. Control sections showed elution of immune reagents used for the localization of substance P to be complete after 4.5 hr. The second antibody, to CGRP, was then applied to the sections and was processed as above except that the final development stage was with diaminobenzidine. The sections were rephotographed and images of the same cells were matched.

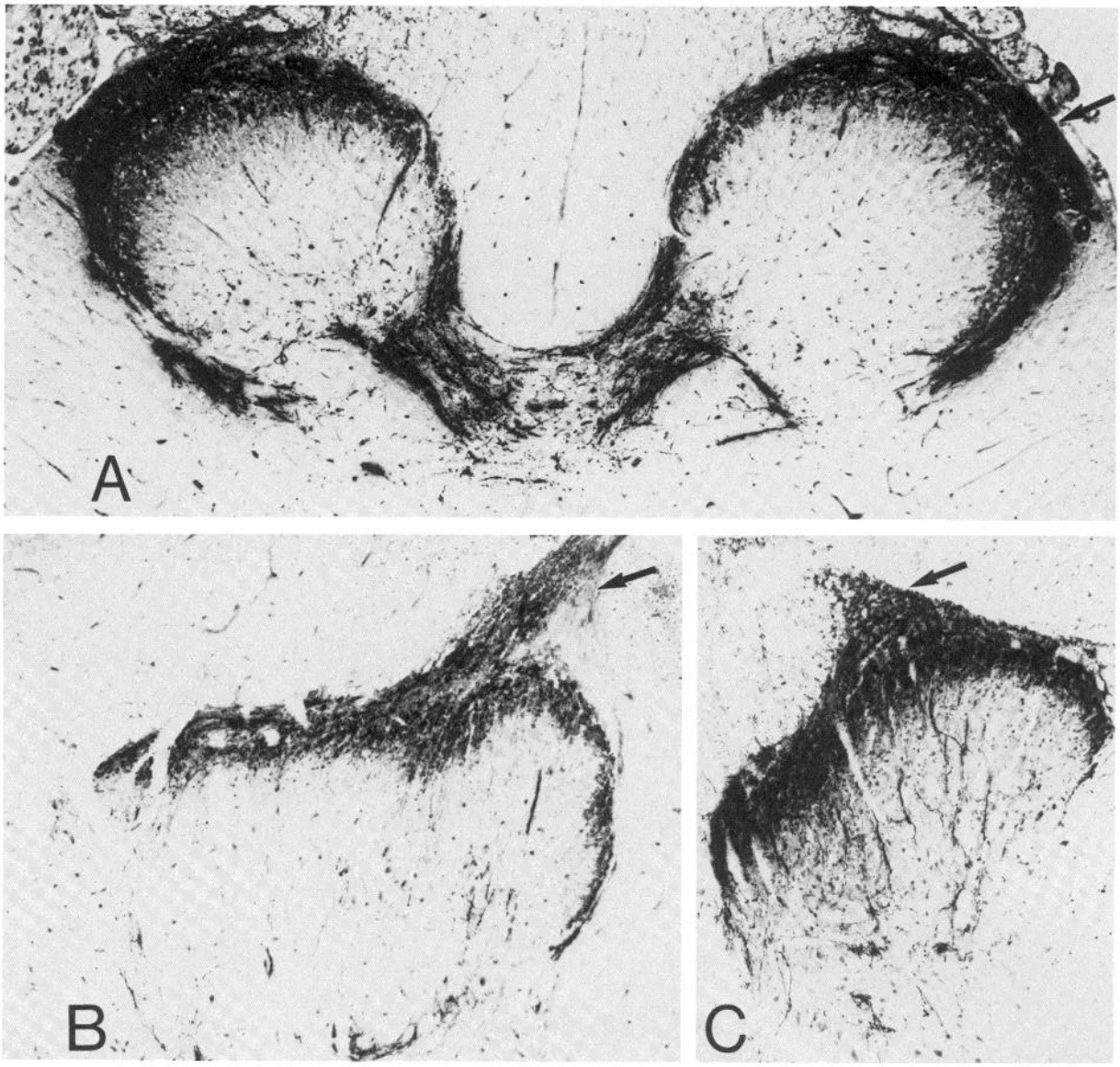

Figure 1. CGRP-immunoreactive fibers and terminals in the dorsal horn of (*A*) the lower lumbar level of the cat spinal cord (magnification × 45), (*B*) the mid-lumbar level of the pig spinal cord (magnification × 60), and (*C*) the thoracic region of the rat (magnification × 150). The immunoreactivity is intense in laminae I to III and in Lissauer's tract (*arrows*).

Radioimmunoassay

Tissue processing

Fresh tissue from the cervical, thoracic, lumbar, and sacral levels of the spinal cord was collected from five rats and four cats. From each region, the spinal cord was sampled as whole slices or divided into dorsal and ventral halves. Pooled samples of dorsal root ganglia (n = 5) from each level of the spinal cord from five rats were assayed. Pooled samples (two to four) of cat (n = 4) lumbar and sacral dorsal root ganglia were assayed for both CGRP and substance P.

In the rhizotomized cats (n = 3) the spinal cord was first divided through the midline and then into dorsal and ventral regions. The samples were placed immediately into boiling acetic acid (0.5 M 10 ml/gm tissue, 100°C) (Bryant and Bloom, 1982) for 10 min. The acid extract was then stored at −20°C prior to assay.

Assay

Aliquots (10 μl) of tissue extracts were assayed in phosphate buffer (0.06 M, pH 7.4) containing bovine serum albumin (3%) and EDTA (0.01 M). The aliquots were incubated for 4 to 5 days with iodinated synthetic CGRP as the tracer. Full details of the antisera are summarized in Table I. Extracts of cat dorsal root ganglia were assayed also for substance P using a previously described specific and sensitive system (Bloom and Long, 1982). "Bound" and "free" fractions were separated and counted with a gamma scintillation counter. All results are expressed in picomoles per gram of wet weight ± SEM. Substance P assay has been published previously (McGregor and Bloom, 1983).

Gel permeation chromatography

Characterization of CGRP-immunoreactive material was performed by gel permeation chromatography on a Sephadex G50 Superfine column (60 cm × 1 cm) eluted with phosphate buffer (0.06 M PO_4, 0.01 EDTA, pH 7.4, sodium salts) containing a 0.3% bovine serum albumin and 0.2 M sodium chloride at a flow rate of 3 ml/hr. Dextran blue, horse heart cytochrome C, and $Na^{125}I$ were used as molecular weight markers. Fractions of 0.6 ml were collected and 10-μl aliquots were assayed for CGRP-like immunoreactivity.

Results

Distribution in normal animals

Numerous fibers were immunostained with CGRP antiserum throughout the entire length of the spinal cord in all species studied. The CGRP-containing nerve fibers were mainly dis-

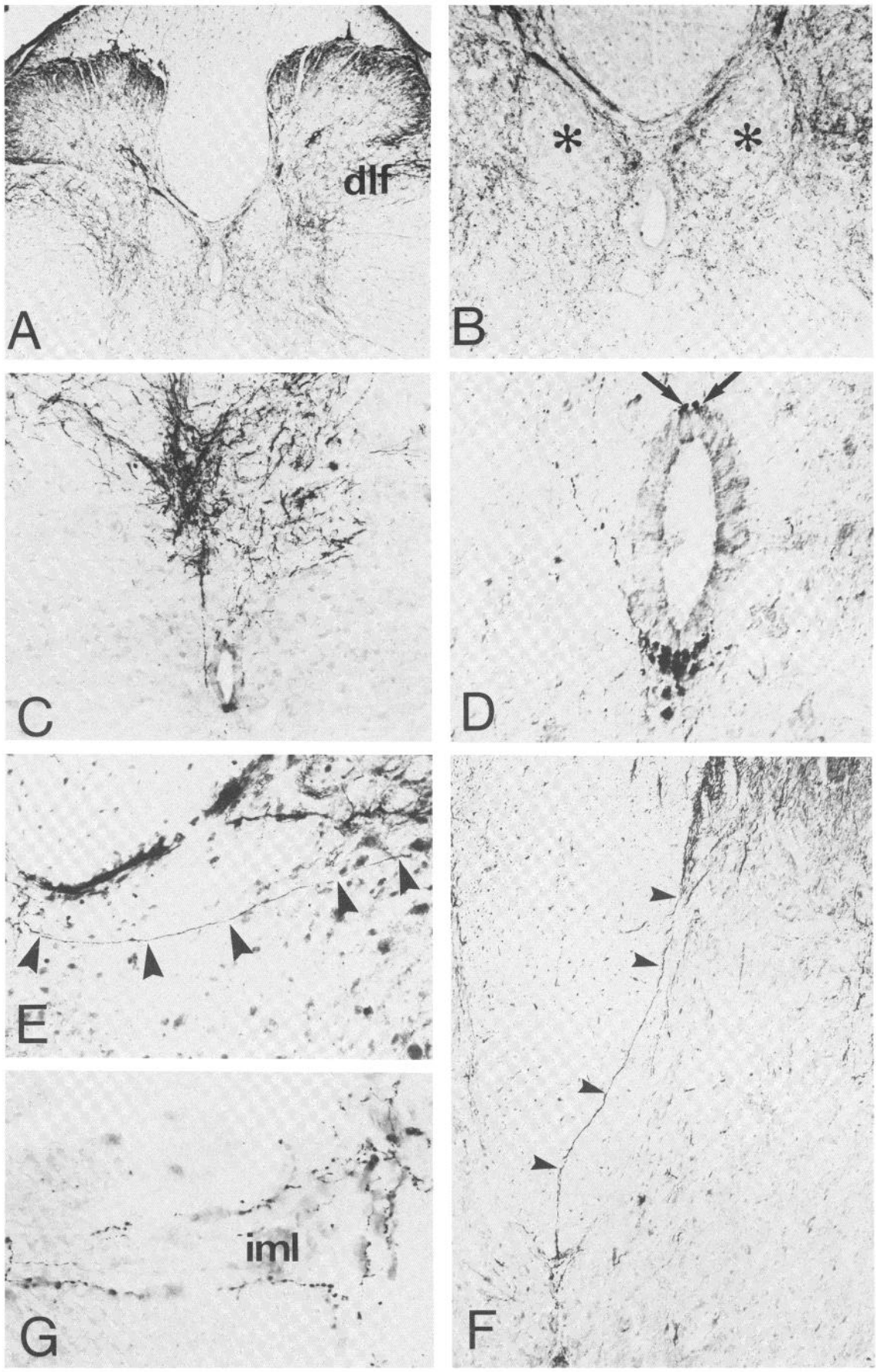

Figure 2

tributed within the sensory areas of the spinal cord and to a lesser extent in sympathetic and parasympathetic centers (Figs. 1 to 3). In addition, large cells in the ventral spinal cord, probably motoneurons, that were immunostained with anti-CGRP were seen in all species studied except the guinea pig (Fig. 3).

Nerve fibers containing CGRP were also found in dorsal and ventral roots (Fig. 4*A*). In the dorsal root ganglia of the marmoset, horse, pig, cat, rat, and mouse, many immunoreactive cells and fibers were seen (Fig. 4).

In the spinal cord the nerve fibers were most concentrated in laminae I and II and Lissauer's tract, where they formed a mesh. Lamina V was seen to contain abundant CGRP-immunoreactive fibers forming bundles which in some cases connected the medial portion of lamina V with the dorsal extent of lamina X (Fig. 2*E*). Fibers "running" between lamina II and lamina X were occasionally found (Fig. 2*F*).

Smaller nerve fibers with punctate immunostaining were scattered throughout most of the remaining gray matter of the spinal cord, including the ventral horn. In the white matter intense CGRP immunostaining was also observed in the dorsolateral funiculus where, in many cases, nerve fibers ran for relatively long distances in the dorsomedial plane toward (or away from) the nucleus proprius and lamina V (Fig. 2*A*). Scattered fiber lengths were also noted in the fasciculi proprii at the various levels of the spinal cord (Fig. 6*C*).

The relative intensity and areas of immunoreactivity were similar at cervical, thoracic, and lumbar levels of the spinal cord, with the exception of the thoracic segments where fibers were found in close association with cells of the intermediolateral cell columns (Fig. 2*G*) and in the sacral segments where the number of CGRP-immunoreactive fibers was greater. Throughout the species studied (mouse, rat, guinea pig, cat, marmoset, pig, horse, man, and frog) the distribution of CGRP-positive nerve fibers in the spinal cord was consistent and few significant species variations were apparent.

In the rat there were two areas of the spinal cord where the CGRP immunoreactivity was distinctive. (*a*) In lamina X, in the area around the central canal, there were two aggregates of fibers, the smaller one dorsal and the larger one ventral to the central canal, appearing as discrete bundles (Fig. 2, *C* and *D*) each of which, in parasagittal section, is seen to consist of 5 to 20 fibers. (*b* The parasympathetic nucleus of the sacral cord contained, as well as numerous CGRP-immunoreactive nerve fibers, CGRP-immunoreactive cell bodies (Fig. 3*D*). The latter were bipolar with large cell somata. The intensity of the immunostaining was found to be particularly high in the pig, marmoset, and cat, especially in laminae I and II and in Lissauer's tract of man, marmoset, horse, pig, cat, mouse, rat, and frog (Fig. 1). In the ventral spinal cord (laminae VI to X), large cells immunoreactive for CGRP were tentatively identified as motoneurons because of their co-location with cells in serial sections stained with cresyl violet for Nissl substance. The appearance of the reaction product in the cells stained for CGRP was granular (Fig. 3, *A*, *C*, and *D*).

In the dorsal root ganglia, CGRP immunoreactivity was localized to both fibers and cells. The intensity of immunostaining and relative number of CGRP-positive fibers and cell bodies demonstrated this peptide to be very abundant in the dorsal root ganglia, more so than any peptide yet localized there (Fig. 4, *B* to *F*). Fibers were numerous and were seen running in all directions, some in close proximity to the ganglion cells and others in pericellular networks (Fig. 4, *D* to *F*). Occasionally a fiber was seen emerging directly from the cell body (Fig. 4, *E* and *F*). CGRP-positive ganglia represented approximately half of the total number of ganglion cells (Fig. 4, *B* and *C*). In the rat, CGRP was most abundant in the small-sized cells (20-μm diameter), and these were intensely stained (Fig. 4*E*). A subpopulation of less intensely stained CGRP-immunoreactive cells of an intermediate size (50-μm diameter) was also present. In some instances the reaction product in these cells appeared only as small discrete deposits. The large ganglion cells were not immunoreactive (Fig. 4, *D* to *F*).

Substance P- and CGRP-immunoreactive cells in dorsal root ganglia

Ratio of substance P- to CGRP-immunoreactive cells

The results of cell counts from cat lumbar dorsal root ganglia are summarized in Table II. A one-way analysis of variance showed no difference between the cats studied for quotients of substance P and CGRP cells in the dorsal root ganglia. The mean quotient for each cat was compared to the value 1 using the t statistic, and each was significantly greater ($p < 0.001$). The mean ratio for all animals studied was 1:2.7 substance P-:CGRP-immunoreactive cells. All analyses on quotients were done using log-transformed data.

Co-localization of CGRP and substance P

Serial sections. In the cat, substance P-containing cells were few in number and ranged from strongly to weakly immunoreactive. Cell counts per section revealed that CGRP-immunoreactive cells were 2 to 5 times more abundant than substance P-containing cells (Fig. 5, *A* and *B*). In every field screened, substance P-containing cells could always be identified with CGRP-positive cells in the serial section (Fig. 5, *A* and *B*). In addition, substance P- and CGRP-containing fibers were occasionally observed around the same cell. However, CGRP-immunoreactive cells were often seen singly, not co-located with substance P-immunoreactive cells.

Elution method. Using the method of elution described by Nakane (1968), substance P was also found to coexist with CGRP-containing ganglion cells of small size in the cat and rat, whereas numerous cells of both small and intermediate diameter were stained only by CGRP antisera.

Radioimmunoassay

High concentrations of CGRP immunoreactivity were found throughout the rat and cat spinal cord with the greatest amount of peptide concentrated in the dorsal region. An increase in CGRP-immunoreactive material was noted in lumbosacral segments. In the cat, the quantity of CGRP in the dorsal horn was significantly greater in the lumbosacral than in the cervical and

Figure 2. *A*, Low power photomicrograph of CGRP-immunoreactive fibers in the guinea pig spinal cord at the mid-thoracic level. In the dorsolateral funiculus (*dlf*) there are numerous CGRP-immunoreactive fibers. Magnification × 60. *B*, High power photomicrograph to show the distribution pattern of immunoreactive fibers around the central canal of the guinea pig spinal cord. Note the absence of a bundle of fibers (shown in *C* and *D*) ventral to the central canal and the scarcity of immunostained fibers in Clarke's column (*asterisks*). Magnification × 105. *C*, CGRP-immunoreactive fibers around the central canal in the sacral spinal cord of the rat. Fibers are abundant dorsal to the central canal, and bundles of immunoreactive fibers are obvious directly dorsal and ventral to the canal. Magnification × 105. *D*, High power photomicrograph of the central canal region in the rat spinal cord. The distribution of CGRP-immunoreactive fibers in bundles dorsal (*arrows*) and ventral to the canal is distinctive. Magnification × 245. *E*, A single CGRP-immunoreactive fiber (*arrowheads*) spanning lamina V and lamina X in the mid-lumbar spinal cord of the mouse. Magnification × 210. *F*, A single CGRP-immunoreactive fiber (*arrowheads*) spanning lamina II and lamina X in the lumbar spinal cord of the rat. Magnification × 120. *G*, Accumulation of CGRP-immunoreactive fibers around the cells of the intermediolateral column (iml) in the thoracic spinal cord of the rat. Magnification × 210.

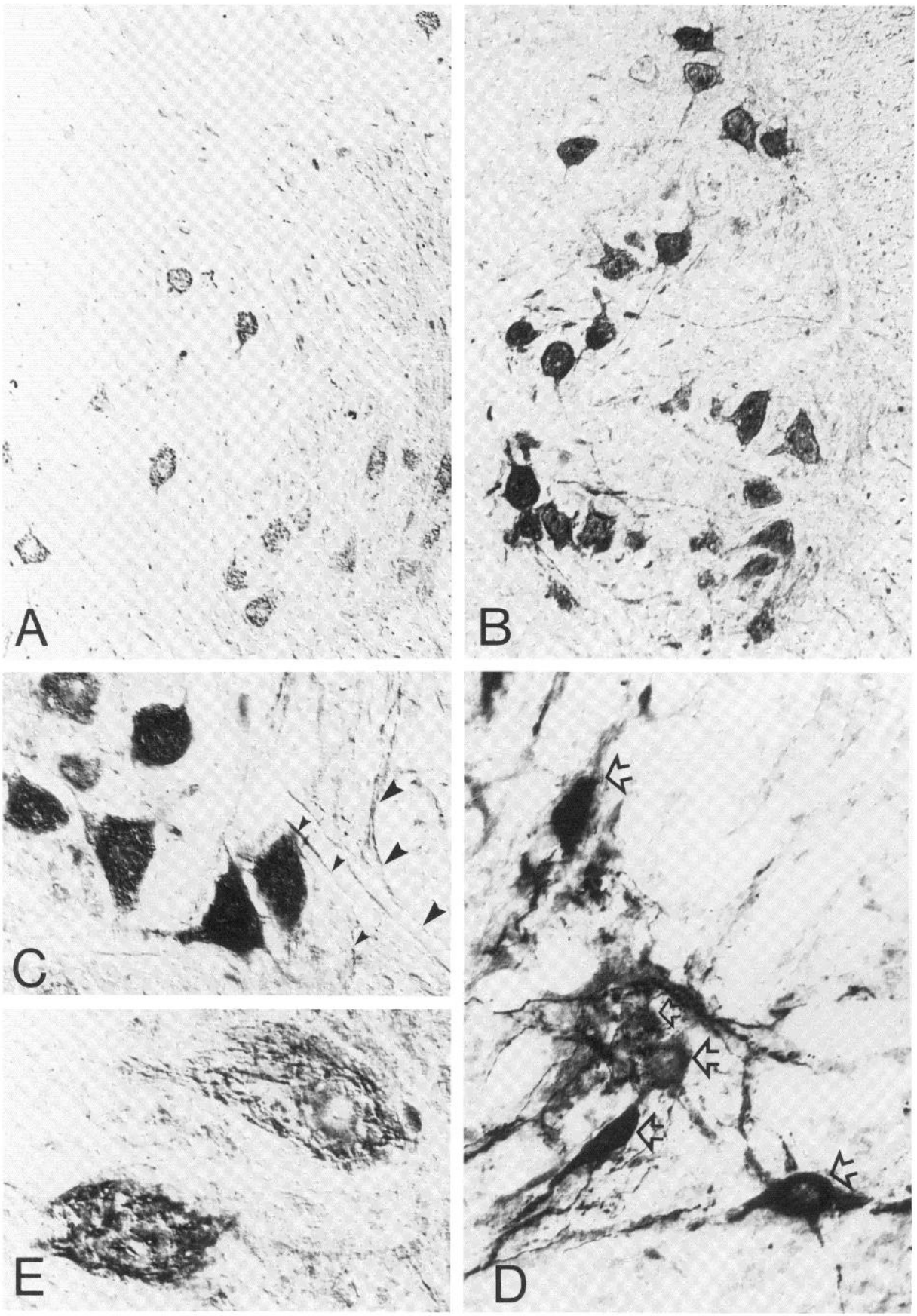

Figure 3. Large CGRP-immunoreactive cells in the ventral spinal cord of the rat (*A* to *D*) and the pig (*E*). *A*, CGRP-immunoreactive cells in the rat lumbar ventral horn. Magnification × 110. *B*, Cells in the same region following colchicine treatment. The immunostain of both cells and fibers is markedly increased. Magnification × 110. *C*, CGRP-immunoreactive cells in the ventral horn of the rat cervical spinal cord. Note fibers entering the white matter (*arrowheads*). Magnification × 330. *D*, CGRP-immunoreactive fibers and cell bodies (*arrows*) in the parasympathetic nucleus of the rat sacral spinal cord. Magnification × 360. *E*, High power photomicrograph of CGRP-immunoreactive cells in the ventral horn of the pig. Note the granular appearance of the immunoreaction. Magnification × 560.

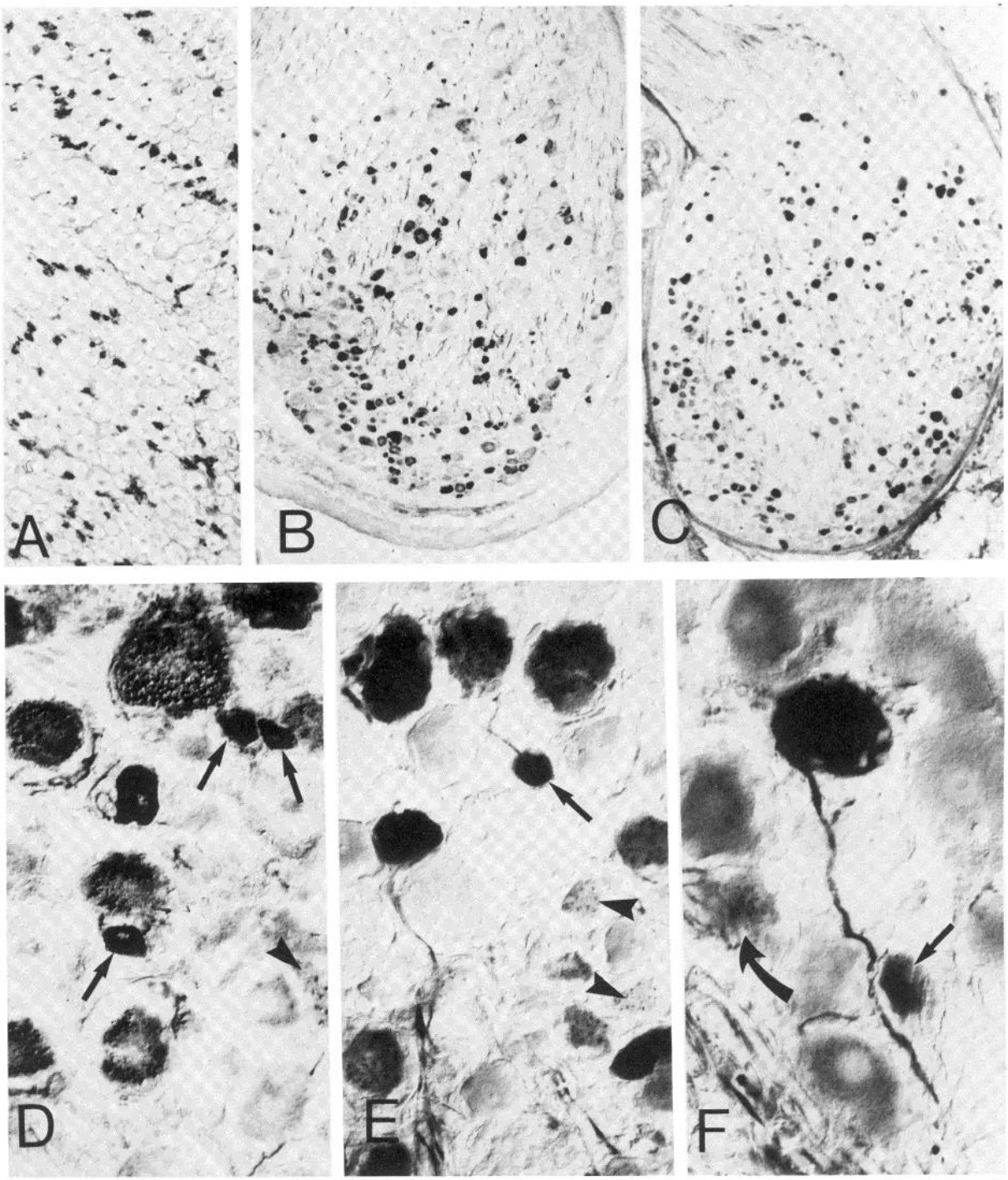

Figure 4. *A*, Numerous CGRP-immunoreactive fibers in the sacral dorsal roots of the cat, seen here as immunoreactive clusters (transverse section). Magnification × 140. *B*, CGRP-immunoreactive cells and fibers in a lumbar dorsal root ganglion from a guinea pig. Magnification × 125. *C*, CGRP-immunoreactive cells and fibers in a lumbar dorsal root ganglion from a cat. Magnification × 87. *D* to *F*, High power photomicrographs of CGRP-immunoreactive fibers and cells in (*D*) guinea pig (magnification × 380), (*E*) rat (magnification × 360), and (*F*) cat (magnification × 580) dorsal root ganglia. Both small- (*small arrows*) and intermediate-sized cells are immunoreactive. No large cells were immunoreactive. The intensity of staining ranged from very dense (*small arrows*) to light (*arrowheads*) in neurons with a granular cytoplasmic immunoreactive. Some cells with pericellular plexuses were seen (*curved arrow*). In *F*, an intensely stained cell with a single emergent process is obvious.

thoracic segments. The results are summarized in Tables III and IV. In the lumbar and sacral dorsal root ganglia of the cat, the levels of CGRP were 4 times greater than those recorded for substance P, in agreement with the larger number of CGRP-immunoreactive cells detected by immunocytochemical analysis (Table V).

Gel permeation chromatography

Gel permeation chromatography of pooled rat dorsal cord extracts revealed three peaks of CGRP-like immunoreactivity. The largest peak, with a K_{av} of 0.38 ± 0.02, co-eluted with synthetic CGRP and accounted for approximately 70% of the total CGRP-like immunoreactivity.

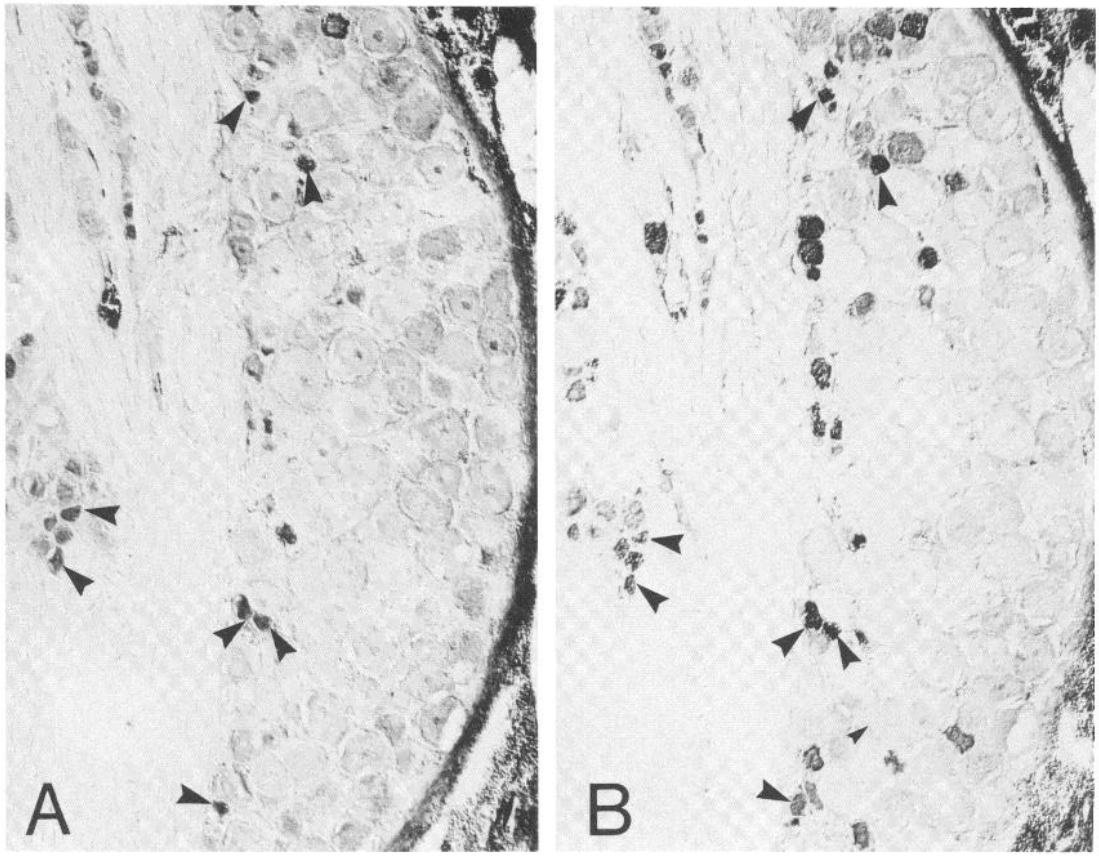

Figure 5. Serial sections through a thoracic dorsal root ganglion of the cat immunostained for substance P (*A*) and CGRP (*B*). All substance P-immunoreactive cells are also immunoreactive for CGRP. Some examples are shown by *arrowheads*. Cells immunoreactive for CGRP only were also present. Magnification × 105.

TABLE II

Ratios of substance P-immunoreactive to CGRP-immunoreactive cells in cat dorsal root ganglia

	Cat 1		Cat 2		Cat 3		Cat 4		Cat 5		Cat 6	
	SP[a]	CGRP	SP	CGRP	SP	CGRP	SP	CGRP	SP	CGRP	SP	CGRP
No. of sections	8		7		6		7		6		6	
Mean	62.9	150.0	64.9	203.3	61.9	167.7	55.0	115.1	110.8	241.0	38.8	124.7
SEM	15.8	17.0	5.1	20.8	5.7	14.7	6.7	12.7	18.1	20.8	4.8	20.0
Geometric mean of CGRP/SP	2.8[b]		3.1[b]		2.7[b]		2.1[b]		2.3[b]		3.2[b]	

[a] SP, substance P.

[b] $p < 0.001$ for testing whether the quotient is greater than 1.

TABLE III

CGRP immunoreactivity in normal rat spinal cord[a]

	Whole	Dorsal	Ventral	Dorsal Root Ganglia
	pmol/gm of wet weight			
Cervical	97.4 ± 8.8	225.7 ± 30.0	15.7 ± 2.7	129.3 ± 18.1
Thoracic	112.8 ± 12.4	320.0 ± 34.2	38.3 ± 11.8	258.4 ± 61.0
Lumbar	158.1 ± 12.8	337.0 ± 34.9	27.6 ± 8.0	140.2 ± 17.0
Sacral	216.9 ± 32.9	340.6 ± 74.6	35.1 ± 10.6	225.4 ± 46.9

[a] $n = 6$. Values are mean ± SEM.

Experimental procedures

Rhizotomy

In order to investigate the origin of CGRP-immunoreactive fibers and terminals in the spinal cord, changes in distribution and concentration of the peptide were studied after dorsal rhizotomy in cats and rats.

In both the cat and the rat there was a striking decrease of CGRP nerve fibers from the ipsilateral dorsal roots, laminae I, II, III, and V (areas which receive terminals of primary afferent fibers), and in Lissauer's tract (Fig. 6). In the cat the immunostain in lamina X above the central canal was also unilaterally decreased, and in the rat the number of fibers ventral to the central canal was less than in unoperated rats. No change in fiber distribution was observed in the ventral horn. Substance P immunoreactivity was also decreased in the ipsilateral dorsal horn, but the reduction was less than for CGRP (Fig. 6*B*). The depletion of CGRP-immunoreactivity from the lumbar and sacral dorsal spinal cord of the cat following rhizotomy was statistically significant ($p < 0.05$, Student's paired t test). The results are summarized in Table IV. There was no significant change in ventral levels. In addition, comparison of CGRP-immunoreactivity in normal and operated animals (contralateral peptide levels) showed no significant differences.

TABLE IV

CGRP immunoreactivity in normal cat spinal cord and cat spinal cord following unilateral lumbosacral rhizotomy[a]

Spinal Cord	Normal		Rhizotomy			
			Ipsilateral		Contralateral	
	Dorsal	Ventral	Dorsal	Ventral	Dorsal	Ventral
	pmol/gm of wet weight		*pmol/gm of wet weight*			
Cervical	64.0 ± 9.2	1.6 ± 1.4	ND[b]		ND	
Thoracic	88.5 ± 10.5	4.1 ± 1.4	ND		ND	
Lumbar	124.7 ± 25.8	3.2 ± 2.3	5.6 ± 1.2[c]	6.6 ± 2.1	105.0 ± 36.0	4.1 ± 0.7
Sacral	286.6 ± 80.8	12.4 ± 9.1	62.2 ± 1.4[c]	5.6 ± 1.4	279.5 ± 64.0	43.0 ± 0.6

[a] For both normal and operated cats, $n = 3$.
[b] ND, not done.
[c] $p < 0.05$, Student's t test.

TABLE V

CGRP and substance P immunoreactivity in cat dorsal root ganglia[a]

	CGRP	SP[b]
	pmol/gm of wet weight	
Lumbar	142.1 ± 35.0	33.0 ± 11.1[c]
Sacral	198.3 ± 39.6	48.1 ± 10.0[d]

[a] $n = 4$.
[b] SP, substance P.
[c] $p < 0.010$.
[d] $p < 0.025$, CGRP versus SP; Student's unpaired t test.

Intraspinal colchicine

Following injection of colchicine into the spinal cord, the staining of the large cells in the ventral horn was more intense, but cell bodies were not observed in the dorsal horn (Fig. 3, *A* and *B*). However, serial sections immunostained for vasoactive intestinal polypeptide revealed numerous cells in the dorsal horn which are not usually apparent in the untreated rat (Gibson et al., 1984b).

Discussion

CGRP is widely distributed throughout the central nervous system (Amara et al., 1982; Rosenfeld et al., 1983). The localization of this peptide within sensory, motor, and integrative systems suggested a peptide with many roles. Here we have aimed to map its distribution in the entire spinal cord and dorsal root ganglia of several mammalian and one non-mammalian species with particular attention to sensory and motor areas.

In the spinal cord, CGRP-immunoreactive fibers and terminals were abundant in all levels of the spinal cord in all of the species studied. These fibers were most numerous in the dorsal horn and were heavily concentrated in Lissauer's tract, laminae I to III, lamina V, and lamina X, the region around the central canal. In general, the distribution of CGRP remained constant throughout the length of the spinal cord, except for the thoracic segments, where CGRP-immunoreactive fibers were densely aggregated around the cells of the sympathetic column and in sacral segments where fibers were more numerous and prominent in the central canal area and around the autonomic parasympathetic nuclei. This apparent caudal increase is a common feature for many peptides (Anand et al., 1983; Gibson et al., 1984b, c) and, in the case of CGRP, is thought to be related to a greater gray:white matter ratio as there is a higher concentration of peptide in the gray matter (Gibson et al., 1984c).

In the dorsal horn of the spinal cord, CGRP immunoreactivity is localized to all laminae which contain interneurons that respond to both noxious (mechanical and thermal) and innocuous stimuli (Willis and Coggeshall, 1978; Light and Perl, 1979; Cervero and Iggo, 1980; Honda and Perl, 1981; Nahin et al., 1983). In addition, CGRP is present in a large number of small cells in the dorsal root ganglia which give rise to C and A δ fibers and are responsible for relay of nociceptive stimuli (Cervero and Iggo, 1980). Thus, the distribution of CGRP suggests a role in sensory transmission, and central administration of CGRP has recently been shown to have an antinociceptive effect (Bates et al., 1984).

We have also attempted to elucidate further the origin of CGRP immunoreactivity in the spinal cord. In early studies others have demonstrated that the major source of substance P, another transmitter candidate, was from afferent fibers originating in the dorsal root ganglia (Takanashi and Otsuka, 1975; Otsuka and Konishi, 1977; Hökfelt et al., 1980; Barbut et al., 1981; Buck et al., 1982). Since CGRP has a distribution similar to that of substance P (Barber et al., 1979; Gibson et al., 1981) in the upper dorsal horn and is present in a far greater number of dorsal root ganglion cells, these cells seemed a likely source of peptide. Following lumbosacral dorsal rhizotomy in the cat and cervical dorsal rhizotomy in the rat there was a depletion of CGRP-immunoreactive nerves from dorsal roots, laminae I to III, V, and X, and Lissauer's tract, all areas that are known to receive terminals from primary sensory neurons. In the ventral cord there was a negligible change in fiber distribution.

It is therefore possible to speculate that the higher proportion of CGRP is derived from dorsal root ganglia and, indeed, the dorsal roots are abundantly packed with CGRP-containing fibers. Examination of the deafferentated limbs of the cats and rats and of the operation site led us to believe that the rhizotomies were successful and did not involve either the cord or ventral roots. Dorsal rhizotomy resulted in a considerable decrease of CGRP from the dorsal horn. The origin of the small amount of CGRP present in the dorsal and ventral spinal cord following dorsal rhizotomy is unknown. In our hands, intraspinal colchicine pretreatment did not reveal a population of CGRP-immunoreactive interneurons in the dorsal horn, although the treatment allowed for visualization of other peptide-containing cells in the same animal (Gibson et al., 1984b). Therefore, the residual CGRP immunoreactivity is most likely to originate from supraspinal regions, from ascending projections of afferent fibers (from segments below the level of rhizotomies), or from aberrant afferent fibers in the ventral root (Coggeshall et al., 1973).

We have demonstrated a degree of coexistence between substance P and CGRP in the dorsal root ganglia. Substance P was chosen for this study since it is the most abundant peptide in the dorsal root ganglion, although somatostatin-, cholecystokinin/gastrin-, and vasoactive intestinal polypeptide-containing cells have also been recorded (Hökfelt et al., 1980; Buck et al., 1982; Dalsgaard et al., 1982; Honda et al., 1983; Otten et al., 1983). Substance P was found only in small-diameter cells and was always present with CGRP, although many additional cells contained CGRP only. Some cells of intermediate size in which CGRP only was identified probably correspond to the B_2 type (Rambourg et al., 1983). However, an elaborate classifi-

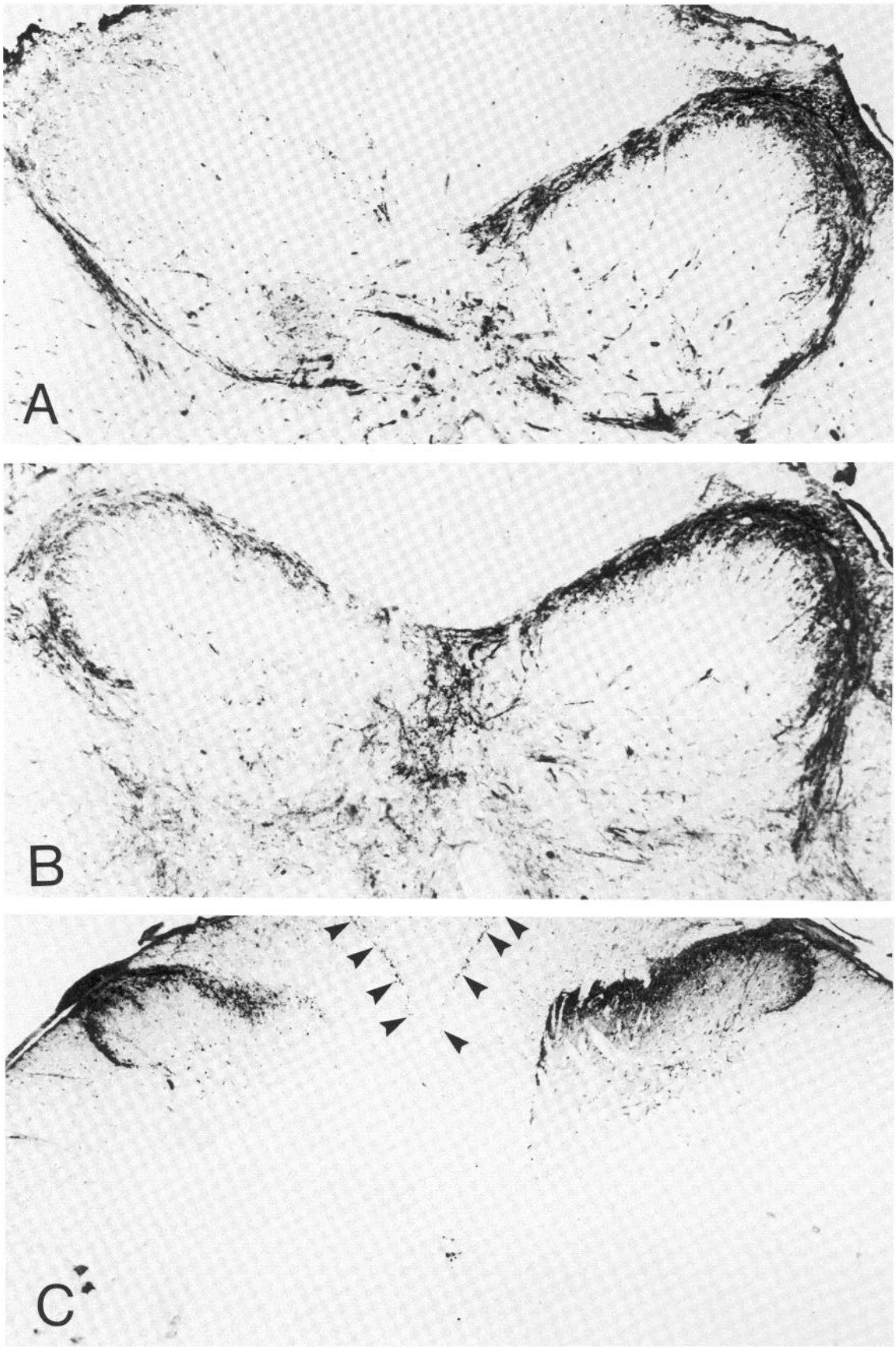

Figure 6. A and *B*, Serial sections the sacral (S_2) segment of the cat spinal cord following unilateral lumbosacral rhizotomy. In *A*, CGRP immunoreactivity is markedly reduced from Lissauer's tract, laminae I to III, and lamina X ipsilateral to the lesion. There is a similar, although less dramatic, effect on substance P immunoreactivity. Magnification × 60. *C*, Following unilateral cervical rhizotomy in the rat there is a loss of CGRP-immunoreactive fibers from the medial ipsilateral dorsal horn. In this section the distribution of CGRP-immunoreactive fibers (similar to that in normal unoperated animals) in the fasciculi proprii (*arrowheads*) is clearly seen. Magnification × 85.

cation of dorsal root ganglion cell type was not possible at the light microscopic level. As yet, we are unable to deduce whether substance P and CGRP are present in the same nerve fibers, although fibers immunoreactive to substance P and CGRP have been demonstrated around the same dorsal root ganglion cell. However, in the rat, regions where the anatomical localization of SP and CGRP is similar include: small dorsal root ganglion cells, dorsal horn, Lissauer's tract, and, in lamina X, a ventral fiber bundle below the central canal.

In all species studied, except guinea pig, cells with large somata, immunoreactive for CGRP, were present in the ventral spinal cord. In the untreated rats practically all large cells in the ventral cord were positively stained, although in some cases the immunostain was weak. In the somatic regions of the spinal cord, large CGRP-immunoreactive neurons were especially noted in areas associated with innervation of large muscles, i.e., fore- and hindlimbs and trunk muscles. The findings suggest that CGRP is present in motoneurons, and our own preliminary experiments using the retrograde tracer, True Blue, according to the method of Sharkey et al. (1983), injected into the rat sciatic nerve indicate that the majority of motoneurons in the lumbar spinal cord are immunoreactive for CGRP, although some interneurons in the ventral horn may also be immunoreactive for CGRP (Brodal, 1969; Ranson, 1959). However, the fact that CGRP immunostaining of the large cells in the ventral spinal cord is enhanced by colchicine treatment invites speculation that this peptide is synthesized in the cell body. These findings of CGRP immunoreactivity in regions which are associated with motor function are in accord with Rosenfeld et al. (1983), who have described CGRP-containing motoneurons in the caudal region of the nucleus ambiguus, an area which controls visceral motor regulation.

In conclusion, the presence of this new peptide which is widely distributed through various systems is likely to prove an important link in the understanding of functional integration in the central nervous system.

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