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AXONAL BLOCKADE INDUCES THE EXPRESSION OF VASOACTIVE INTESTINAL POLYPEPTIDE AND GALANIN IN RAT DORSAL ROOT GANGLION NEURONS

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SUMMARY

Nerve growth factor (NGF) undergoes retrograde transport from peripheral target organs, and has been recently reported to regulate the production of some neuropeptides in dorsal root ganglion (DRG) neurons. Therefore, to ascertain whether or not the expression of calcitonin gene-related peptide (CGRP), vasoactive intestinal polypeptide (VIP), and galanin was regulated by the retrograde transport of factors such as NGF, we carried out an immunocytochemical analysis using vinblastine as an axonal transport blocker and a monoclonal antibody to the NGF receptor (NGFR) as a marker of NGF-responsive neurons.

The percentage of CGRP-containing DRG neurons (L5) was decreased by sciatic nerve transection or by the application of higher doses of vinblastine (0.3-0.6 mM) to the sciatic nerve. VIP and galanin were expressed in some DRG neurons after the application of a low dose of vinblastine (0.15 mM), which can block axonal flow without causing neuronal damage. The expression of these peptides was not affected by dorsal rhizotomy. About 70% of the CGRP-containing neurons also expressed NGFR, while most of the VIP-containing or galanin-containing neurons lacked NGFR. These findings indicate that the depletion of peripheral target-derived neurotrophic factor(s) other than NGF by axonal blockade may induce the gene expression of VIP and galanin.

INTRODUCTION

Nerve growth factor (NGF) is one of the target-derived neurotrophic factors (NTFs), and has been shown to play an important role in the differentiation, survival, and regeneration of sympathetic and sensory neurons[1,12]. It is considered that NGF is produced by the target tissues of these neurons, internalized into nerve terminals after binding with the nerve growth factor receptor (NGFR), and then transported in a retrograde fashion to the neuronal cell body[1]. Therefore, NGF-responsive neurons may contain NGFR. Recently, NGF has been shown to regulate production of neuropeptides in mature sensory neurons[13].

Various kinds of neuropeptides have been discovered in rat dorsal root ganglion (DRG) cells[10], they can be divided into two groups according to their responses to peripheral nerve transection or crush injury. One group of neuropeptides, which includes substance P (SP) and calcitonin gene-related peptide (CGRP), have been found to decrease after peripheral axotomy[16,17]. It is considered that the production of these peptides is down-regulated by depletion of NTF(s) caused by the blockade of retrograde axonal transport. NGF may be one of these NTFs because its application to rat sensory neurons leads to an increase of these peptides[7,13], and because the continuous application of NGF to the cut ends of sciatic nerves prevents a decrease of SP levels in the rat DRG[6]. The other group consists of vasoactive intestinal peptide (VIP), the VIP-related peptide

with amino-terminal histidine and carboxy-terminal isoleucine (PHI, a peptide which is coded on VIP precursor mRNA), and galanin. These peptides and their mRNAs have been shown to increase after peripheral nerve injury[9,14,16,17,20]. It is still unclear what kind of signals induce the gene expression of these peptides[15]. One possible mechanism is that their synthesis is normally suppressed by NTF(s) transported from the periphery. In this case, absence of the depressor following the blockade of axonal flow may allow upregulation of these peptides. These peptides show very low levels in the normal rat DRG (expression by less than 5% of all DRG neurons). Another possibility is that axonal degeneration causes the synthesis of unknown NTF(s) which are transported to the cell body to activate the production of these peptides.

Therefore, in order to ascertain whether or not the expression of CGRP, VIP, and galanin depends on retrograde axonal transport, we carried out an immunocytochemical analysis in the rat using vinblastine, which interferes with axonal flow without causing any axonal degeneration[5]. In addition, whether sensory neurons containing these peptides were responsive to NGF or not was also examined using a monoclonal antibody for the NGFR as a marker of NGF-responsive neurons.

MATERIALS AND METHODS

Male Wistar rats weighing about 200 g were used in this study. All the animals were operated on under sodium pentobarbital anesthesia (50 mg/kg). The animals were divided into two groups. The first group underwent axotomy of one sciatic nerve at the level of the hip joint (n=2) or rhizotomy of a unilateral dorsal root at the L5 spinal level (n=2). The sciatic nerve or dorsal root were ligated and cut distal to the DRG. The second group received the local application of vinblastine (an axonal flow blocker) to one sciatic nerve. The sciatic nerve was exposed over a 10 mm length at the hip level and surrounded with cotton wool soaked in 0.15 mM (n=3), 0.3 mM (n=3) or 0.6 mM (n=3) vinblastine (Sigma) in 0.9% saline for 15 min. Cotton wool soaked in saline alone was used on the contralateral side (the control; n=3). Then, in order to wash out vinblastine, rinsing with saline was performed three times. All the animals in the two groups were kept alive for 7 days after these procedures.

Rats were perfused under deep sodium pentobarbital anesthesia (75 mg/kg) with 0.9% saline (100 ml) followed by 400 ml of cold Zamboni's fixative[21]. The bilateral DRGs at the L5 spinal level were excised, postfixed in the same fixative overnight, and kept in 30% (w/v) sucrose in 0.1 M phosphate buffer for 2 days at 4 °C. The DRGs (L5) were cut into 15-μm sections in a cryostat at -20 °C and mounted on gelatin-coated glass slides.

For immunocytochemical staining by the indirect immunofluorescent method of Coons[3], slide-mounted sections

were incubated for 2 days at 4 °C with mouse anti-NGFR (1:1,000), rabbit anti-CGRP (Amersham; 1:2,000), rabbit anti-VIP (Ortho; 1:25) or rabbit anti-galanin (Serotec; 1:2,000) antiserum diluted with 0.02 M PBS containing 0.3% Triton X. The samples were then washed in 0.02 M PBS and further incubated overnight at 4 °C with sheep anti-mouse Ig (Amersham; 1:250) conjugated with Texas red (TR) or sheep anti-rabbit IgG (Cappel; 1:1,000) conjugated with fluorescein isothiocyanate (FITC). After a final wash, the samples were air-dried and coverslipped with a mixture of glycerine and PBS (1:1). For double staining of NGFR and CGRP, VIP, or galanin, the first incubation was performed with a mixture of mouse anti-NGFR monoclonal antibody (1:1,000) and rabbit anti-CGRP polyclonal antibody (1:2,000), rabbit anti-VIP polyclonal antibody (1:25) or rabbit anti-galanin polyclonal antibody (1:2,000) diluted with 0.02 M PBS containing 0.3% (v/v) Triton X. The second antibodies were a mixture of sheep anti-mouse Ig serum conjugated with TR (1:250) and sheep anti-rabbit IgG serum conjugated with FITC (1:1,000) diluted with 0.02 M PBS containing Triton X.

Preparations were viewed and photographed under an Olympus fluorescence microscope equipped with B and G excitation filters to assure exclusive visualization of FITC and TR fluorescence. Preliminary control experiments showed no cross-reactivity in the double-staining immunocytochemical procedure described above.

A anti-NGFR monoclonal antibody (clone 192-IgG)[2] was derived by immunizing BALB/c mice with soluble protein from PC12 membranes and fusion of splenocytes with SP2/0-Ag14 mouse myeloma cells. Two classes of NGFR (high and low affinity) have been described, and this NGFR antibody is thought to recognize both classes of receptor[2,8]. Polyclonal antibodies to CGRP, VIP and galanin were raised against synthetic rat CGRP, natural porcine VIP, and natural porcine galanin, respectively. The specificities of these antibodies were checked by absorption tests. No immunoreactive structures were observed in sections stained with antiserum pre-absorbed with the corresponding synthetic peptide at the final concentration of 10^{-6} M.

Sciatic nerves of some rats in the second group were examined by means of electron microscopy. The animals were perfused under deep sodium pentobarbital anesthesia with 0.9% saline followed by fixative containing 1% glutaraldehyde and 3% paraformaldehyde in 0.1 M PB at 4 °C. Sciatic nerves of application site were excised, postfixed in the same fixative for 4 hs. and washed with 0.1 M PB. Samples were then osmicated (1% osmium tetroxide in 0.1 M PB for 60 min), stained (1% uranyle acetate in distilled water for 60 min), and embeded in Epon Araldite. Thin sections were cut with a diamond knife and viewed under the electron microscope.

RESULTS

NGFR-like immunoreactive (IR), CGRP-IR, VIP-IR, and galanin-IR neurons in the L5 DRG after peripheral axotomy,

dorsal rhizotomy, or the application of vinblastine are shown in Figs. 1-4, and the quantitative data are shown in Table I and Fig. 5. The results of double staining for NGFR and CGRP, VIP, or galanin are shown in Table II. Electron microscopic photographs of sciatic nerves treated with vinblastine of various concentrations are shown in Fig. 6.

NGFR-like immunoreactivity

The antibody to NGFR immunostained the DRG neuronal perikarya granularly and diffusely. Half of the DRG neurons on the control side displayed NGFR-like immunoreactivity (Fig. 1B), and cells of various sizes were positive. Approximately 75% of the NGFR-IR neurons also showed CGRP-like immunoreactivity (Figs. 1A, 1B). NGFR-IR neurons decreased to 60-70% of the control levels after peripheral axotomy (Fig. 1D). About 75% of the NGFR-IR neurons still showed CGRP-like immunoreactivity, which coincided with the proportion in the control DRGs (Figs. 1C, 1D). The proportion of NGFR-IR neurons was not changed by the application of 0.15 mM vinblastine (Fig. 2B). However, NGFR-IR neurons were decreased to about 75% and 60% of the control level by the application of 0.3 mM and 0.6 mM vinblastine, respectively (Figs. 2D, 2F). Dorsal rhizotomy did not change percentage of NGFR-IR neurons (Fig. 1F).

CGRP-like immunoreactivity

CGRP-like immunoreactivity was detected in half of the DRG neurons on the control side and various sized cells were again involved (Fig. 1A). Approximately 70% of the CGRP-IR neurons displayed NGFR-like immunoreactivity (Figs. 1A, 1B). The percentage of CGRP-IR neurons was decreased to about 70% of the control level by peripheral axotomy (Fig. 1C), and about 40% of these CGRP-IR neurons showed NGFR-like immunoreactivity (Figs. 1C, 1D). CGRP-IR neurons were not affected by the application of 0.15 mM vinblastine, as was the case with NGFR-IR neurons (Fig. 2A). However, the proportion of CGRP-IR neurons decreased to about 80% and 70% of the control level following treatment with 0.3 mM and 0.6 mM vinblastine, respectively (Figs. 2C, 2E). Dorsal rhizotomy had no effect on the CGRP-IR neurons (Fig. 1E).

VIP-like immunoreactivity

VIP-like immunoreactivity was shown in the perinuclear perikarya of DRG neurons (Fig. 3). Only about 1% of the DRG neurons on the control side displayed VIP-like immunoreactivity (Fig. 3A). However, after peripheral axotomy it was found that 20-25% of DRG neurons displayed VIP-like immunoreactivity (Fig. 3B). The percentage of VIP-IR neurons was also increased by the application of vinblastine. About 20-25% of DRG neurons were positive for VIP-like immunoreactivity at any dose of vinblastine applied, either 0.15 mM (Fig. 3C), 0.3 mM, or 0.6 mM (Fig. 3E). These neurons were mainly small cells of less than 30 μm in diameter. After 0.15 mM vinblastine application which did not affect NGFR-IR neurons, about 10% of the VIP-IR neurons

showed NGFR-like immunoreactivity (Figs. 3C, 3D). Conversely, about 5% of the NGFR-IR neurons were positive for VIP-like immunoreactivity. The proportion of VIP-IR neurons was unaffected by dorsal rhizotomy (Fig. 3F).

Galanin-like immunoreactivity

The galanin-like immunostaining pattern was similar to that of VIP (Fig. 4). Three to four percent of the DRG neurons on the control side displayed galanin-like immunoreactivity (Fig. 4A). The proportion of galanin-IR neurons was increased by peripheral axotomy (Fig. 4B), with about 70% of DRG neurons becoming positive for galanin. These neurons were of various sizes. The incidence of galanin-IR neurons was also increased by vinblastine application. About 40-50% of DRG neurons showed galanin-like immunoreactivity following the use of 0.15 mM (Fig. 4C) and 0.3 mM vinblastine. These neurons were mainly small cells. Following the application of 0.6 mM vinblastine, 50-70% of the DRG neurons displayed galanin-like immunoreactivity (Fig. 4E). Both large neurons more than 30 μm in diameter and small neurons displayed immunoreactivity. After 0.15 mM vinblastine was applied, about 10% of the galanin-IR or NGFR-IR neurons showed immunoreactivity for the other peptide (Figs. 4C, 4D). The proportion of Galanin-IR neurons was unaffected by dorsal rhizotomy (Fig. 4F).

Electron microscopical analysis for sciatic nerve after vinblastine application

Low dose of vinblastine (0.15 mM) caused no apparent morphological changes in myelinated and unmyelinated fibers (Fig. 6B). Densities of both myelinated and unmyelinated fibers were slightly decreased. Both myelinated and unmyelinated fibers were about 80% of those control neurons (Fig. 6A), respectively. Since no sign of degeneration was observed in these nerve fibers, the decrease of densities may be due to increased endoneurial fluid, as previously pointed out by Fitzgeraled et al[5]. In contrast, most of unmyelinated fibers and some myelinated fibers were degenerated in nerves treated with 0.3 mM vinblastine (Fig. 6C). High dose of vinblastine (0.6 mM) caused complete disappearance of unmyelinated fibers and more pronounced destruction of myelins (Fig. 6D).

DISCUSSION

An antibody to NGFR was used as a marker of NGF-responsive neurons in this study. Expression of NGFR has been found in 40-70% of rat DRG neurons examined both in situ and in culture[12,18]. It has been demonstrated that the expression of NGFR is closely related to that of various peptides in DRG neurons. For examples, all the SP-containing DRG neurons show NGFR expression, while majority of somatostatin-containing neurons lack the receptor[19]. The present study showed that half of the DRG neurons displayed NGFR-like immunoreactivity and that three quarters of such

neurons were positive for CGRP. Conversely, about 70% of the CGRP-containing neurons showed NGFR expression. These proportions were not changed after the application of 0.15 mM vinblastine (Figs. 2A, 2B, and data not shown). Such a high incidence of the combined expression of CGRP-like and NGFR-like immunoreactivity may support the notion that NGF regulates CGRP synthesis[13].

Peripheral axotomy induced a decrease of both CGRP- and NGFR-positive neurons to about two-thirds of the respective control values. The incidence of NGFR-like immunoreactivity in CGRP-containing neurons also decreased from 70% to 40%. These findings may indicate that CGRP-containing neurons with NGFR expression are more likely to be affected by peripheral axotomy. In contrast to CGRP and NGFR, peripheral axotomy induced a 10- to 20-fold increase of VIP-positive and galanin-positive neurons. The number of galanin-positive neurons appearing after peripheral axotomy was about three times that of VIP-positive cells. Galanin was found in both small and large neurons, while VIP was mainly found in small neurons. Galanin-like and VIP-like immunoreactivity were both expressed by some small neurons (in preparation; H.K.).

The percentages of NGFR-positive and peptide-positive neurons were not affected by dorsal rhizotomy. DRG neurons have both central and peripheral branches and are considered to be controlled by different NTFs derived from both the peripheral and central target structures[4]. NGF may trophically support DRG neurons from the periphery[11], while brain-derived neurotrophic factor (BDNF) is one of the NTFs thought to be derived from the spinal cord[1]. Thus, the neurotrophic mechanism of the central branches of DRG neurons differs from that of their peripheral branches and may have no effect on CGRP, VIP, galanin, and NGFR expression.

Application of vinblastine to nerve trunks at an appropriate concentration causes axonal transport blockade without neuronal damage[5]. It has been reported that axonal degeneration may be produced by higher concentration of vinblastine, and that unmyelinated fibers are more sensitive to this than myelinated ones[5]. The present electron microscopical analysis has clearly shown that low dose of vinblastine (0.15 mM) induced no or very few damage, if any, of unmyelinated fibers, while not only unmyelinated fibers but also myelinated fibers were almost totally destroyed by 0.6 mM vinblastine application. These findings are in line with previous study of Fitzgerald et al[5]. The histological feature caused by 0.6 mM vinblastine application may well explain the fact the decreased proportions of CGRP- or NGFR-positive neurons after 0.6 mM vinblastine application was comparable to those induced by peripheral axotomy.

On the other hand, the percentage of these neurons was not affected by 0.15 mM vinblastine, which is also reasonably explained by the present histological examination. The fact that VIP-like and galanin-like immunoreactivity were enhanced after low dose of vinblastine treatment (0.15 mM), may indicate that VIP and galanin gene expression can be influenced by axonal blockade itself. This suggests the

presence of some as yet unknown (or known) suppression factors for these peptides that undergo retrograde transport. The depression of CGRP-like and NGFR-like immunoreactivity after peripheral nerve axotomy or vinblastine application may partly be explained by the absence of NGF. However, gene expression of galanin, as well as VIP[15], may not be controlled by NGF, because most of the DRG neurons positive for these peptides after axonal blockade lacked NGFR-like immunoreactivity.

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FIGURE LEGENDS

Fig. 1
Immunofluorescent photomicrographs showing CGRP-IR neurons (A, C, E) and NGFR-IR neurons (B, D, F) in DRG after peripheral axotomy (C and D in the same section) or dorsal rhizotomy (E, F), and in the control group (A and B in the same section). Double-labeled neurons are indicated with arrows. Calibration bar=100 μm.

Fig. 2
Immunofluorescent photomicrographs showing CGRP-IR neurons (A, C, E) and NGFR-IR neurons (B, D, F) in the DRG after the application of vinblastine at 0.15 mM (A and B in the same section), 0.3 mM (C, D), and 0.6 mM (E, F). Double-labeled neurons are indicated with arrows. Calibration bar=100 μm.

Fig. 3
Immunofluorescent photomicrographs showing VIP-IR neurons (A, B, C, E and F) and NGFR-IR neurons (D) in the DRG after peripheral axotomy (B), the application of vinblastine at 0.15 mM (C and D in the same section) and 0.6 mM (E), or dorsal rhizotomy (F). The control is shown in (A). Double-labeled neurons are indicated with arrows. Calibration bar=100 μm.

Fig. 4
Immunofluorescent photomicrographs showing galanin-IR neurons (A, B, C, E and F) and NGFR-IR neurons (D) in the DRG after peripheral axotomy (B), the application of vinblastine at 0.15 mM (C and D in the same section) and 0.6 mM (E), or dorsal rhizotomy (F). The control is shown in (A). Double-labeled neurons are indicated with arrows. Calibration bar=100 μm.

Fig. 5
Bar charts showing the percentages of NGFR-IR, CGRP-IR, VIP-IR, and galanin-IR neurons in the DRG after peripheral axotomy, dorsal rhizotomy, and the application of vinblastine at 0.15 mM, 0.3 mM, and 0.6 mM. The control group is also shown.

Fig. 6
Electron microscopic photographs showing sciatic nerves treated with vinblastine at concentrations of 0 mM (A; control), 0.15 mM (B), 0.3 mM (C), and 0.6 mM (D). Calibration bar=5 μm.

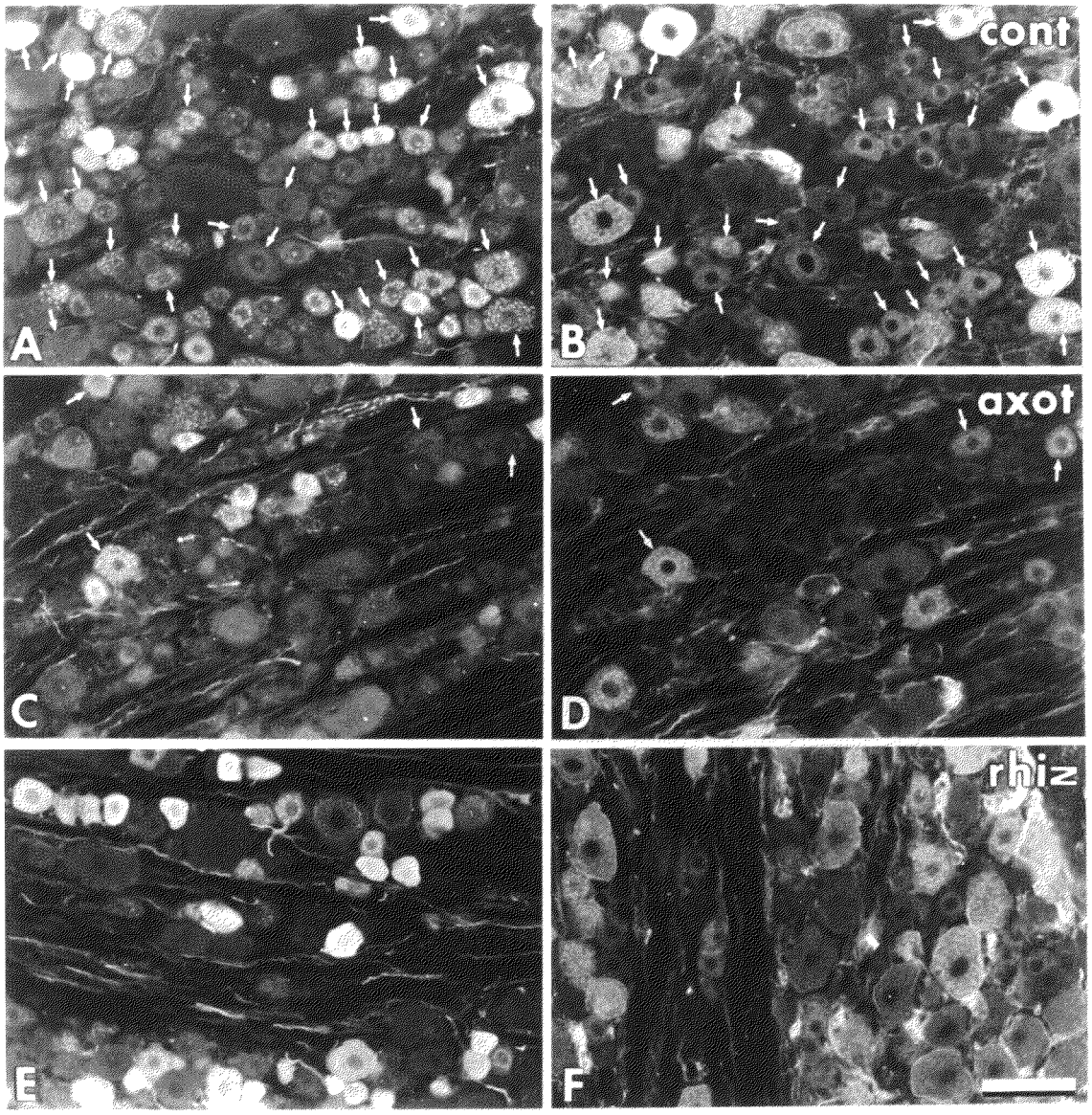

Fig. 1

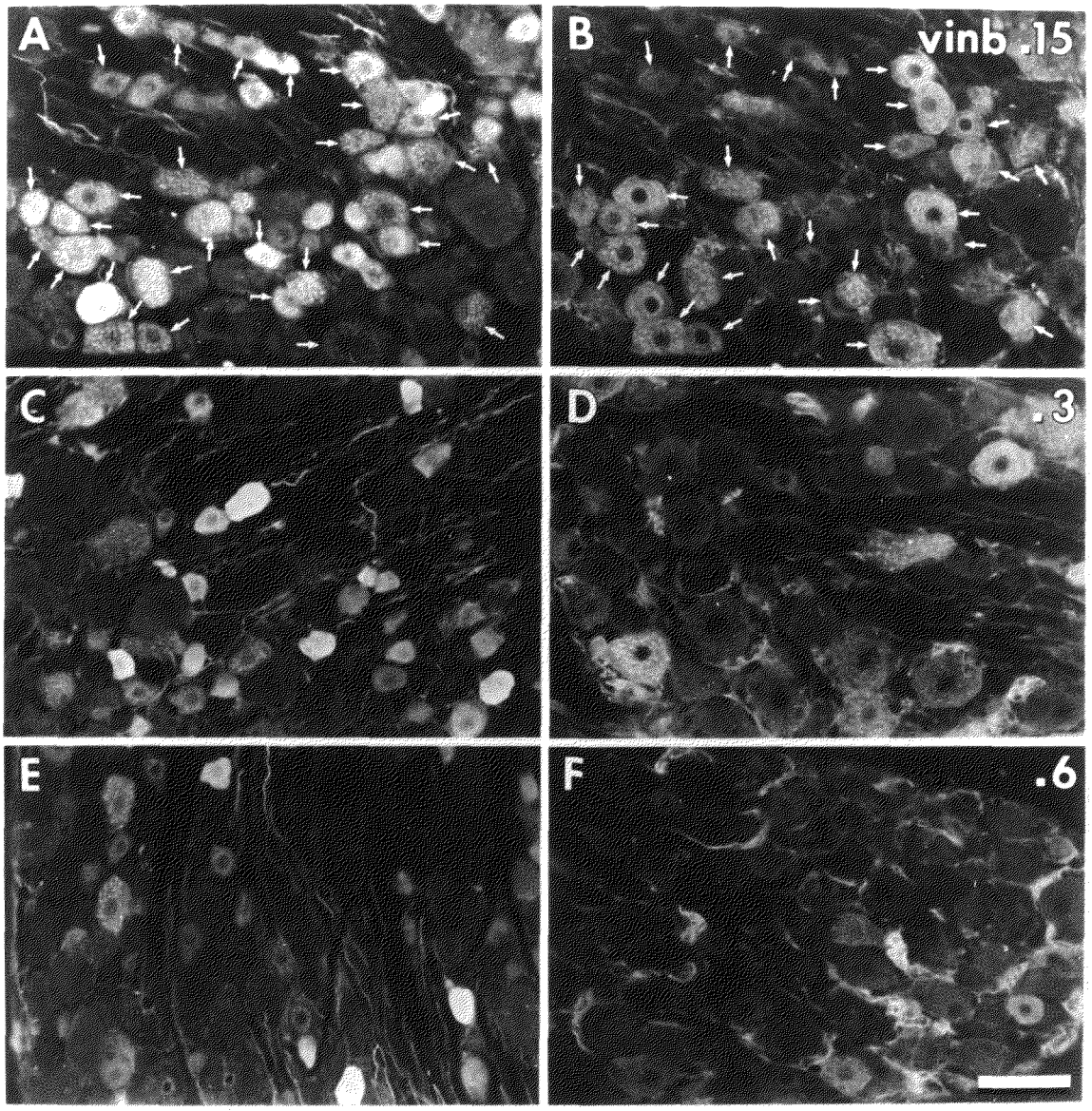

Fig. 2

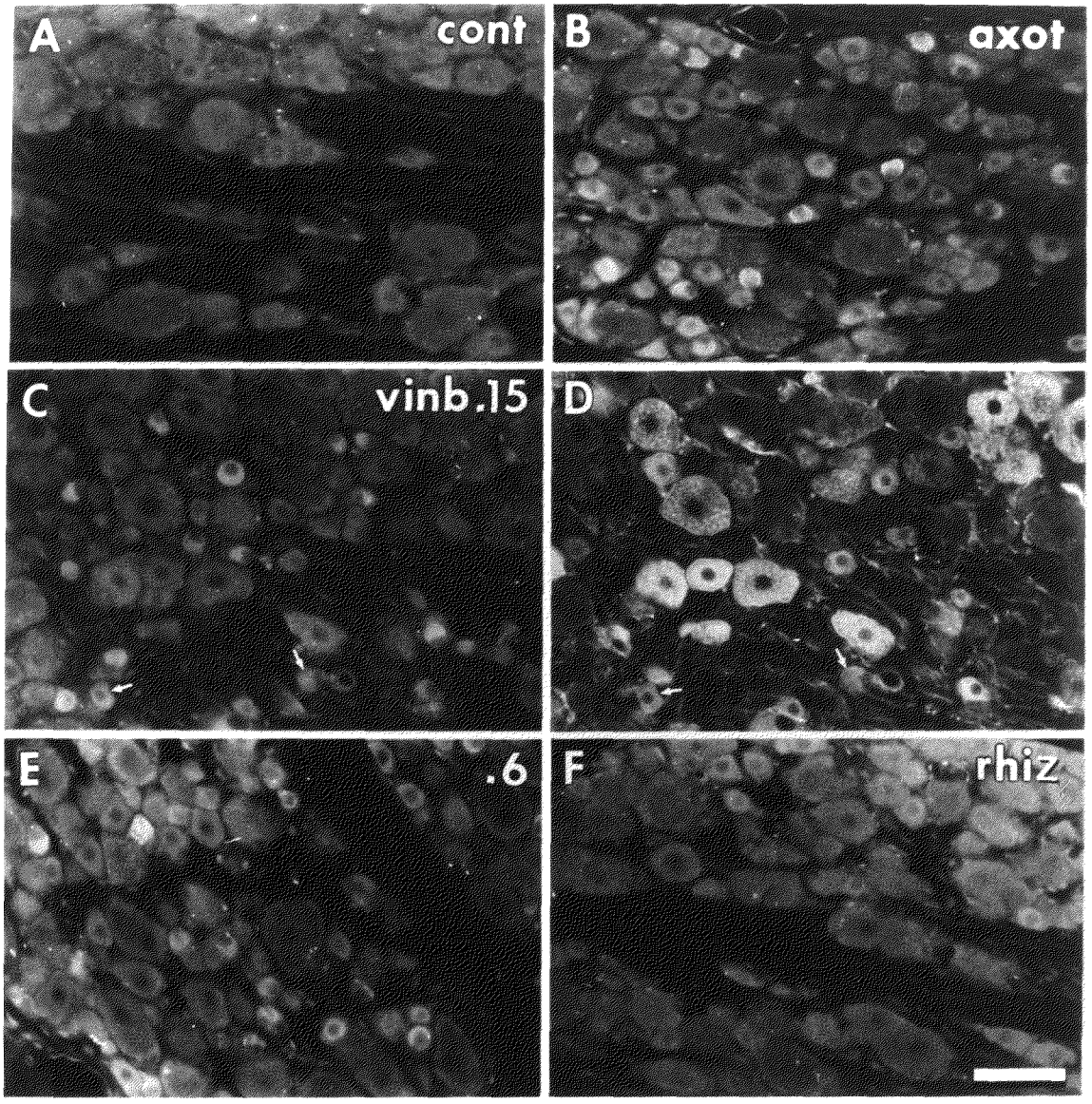

Fig. 3

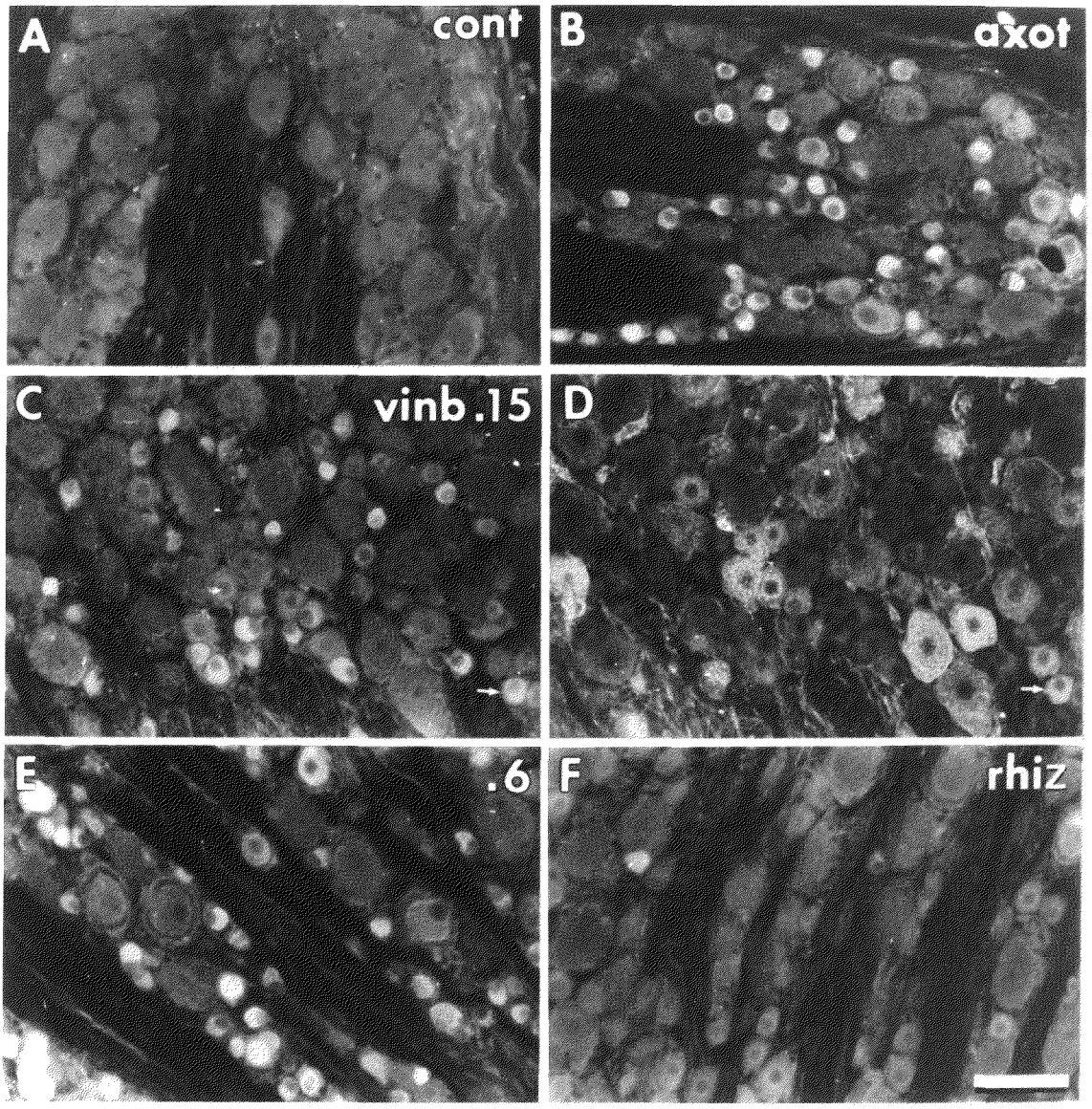

Fig. 4

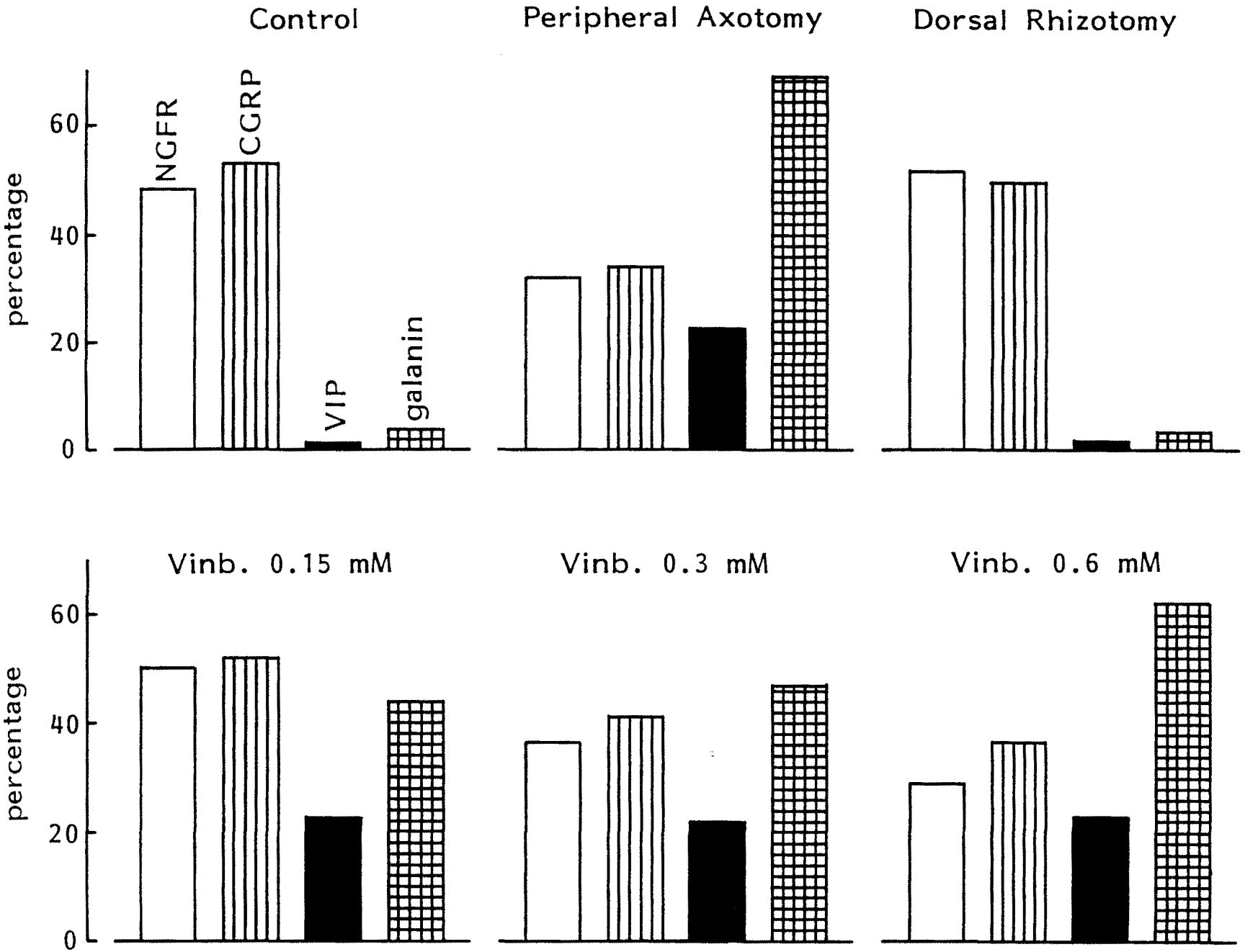
Control
Peripheral Axotomy
Dorsal Rhizotomy
percentage
60
40
20
0
NGFR
CGRP
VIP
galanin
Vinb. 0.15 mM
Vinb. 0.3 mM
Vinb. 0.6 mM

Fig. 5

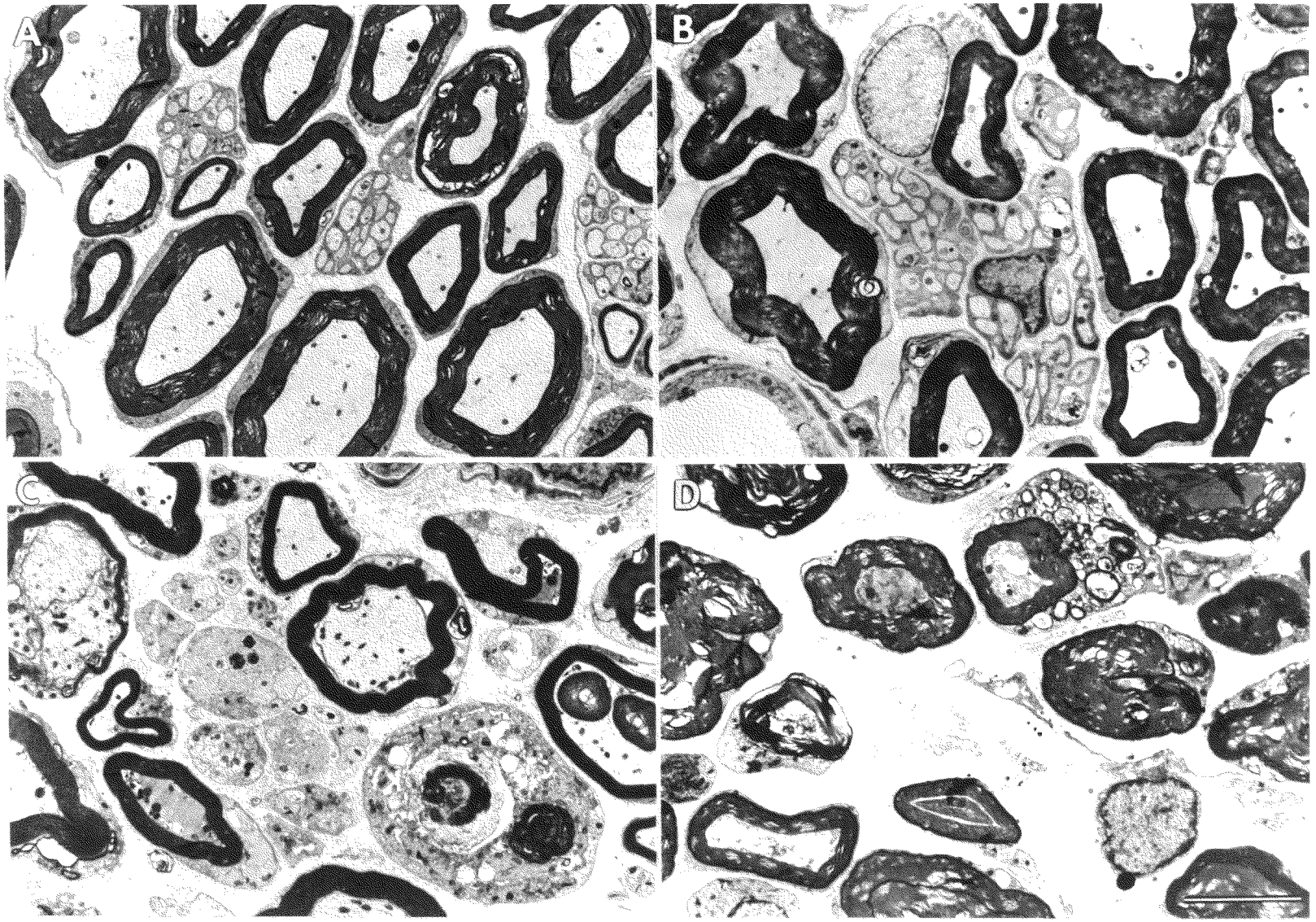

Fig. 6

Table I The numbers and percentages of NGFR-IR, CGRP-IR, VIP-IR and Galanin-IR neurons

	CONTROL	PERIPHERAL AXOTOMY	DORSAL RHIZOTOMY	VINBLASTINE 0.15 mM	VINBLASTINE 0.3 mM	VINBLASTINE 0.6 mM
NGFR	183/390(46.9%)	132/386(34.2%)	174/324(53.7%)	197/381(51.7%)	104/288(36.1%)	82/289(28.4%)
	232/466(49.8%)	187/623(30.0%)	140/285(49.1%)	97/202(48.0%)	55/148(37.2%)	174/581(29.9%)
mean	48.4%	32.1%	51.4%	49.9%	36.6%	29.2%
CGRP	306/582(52.6%)	160/445(36.0%)	139/286(48.6%)	200/393(50.9%)	149/378(39.4%)	95/247(38.5%)
	346/641(54.0%)	203/629(32.3%)	136/267(50.9%)	227/432(52.5%)	76/176(43.2%)	173/493(35.1%)
mean	53.3%	34.1%	49.8%	51.7%	41.3%	36.8%
VIP	4/247(1.6%)	72/305(23.6%)	3/210(1.4%)	48/213(22.5%)	64/315(20.3%)	37/169(21.9%)
	2/241(0.8%)	54/253(21.3%)	4/257(1.6%)	67/301(22.3%)	73/309(23.6%)	27/110(24.5%)
mean	1.2%	22.5%	1.5%	22.4%	22.0%	23.2%
Galanin	12/294(4.1%)	154/215(71.6%)	9/254(3.5%)	95/211(45.0%)	135/262(51.7%)	112/158(70.9%)
	9/308(2.9%)	123/185(66.5%)	7/218(3.2%)	84/198(42.4%)	107/254(42.1%)	54/101(53.5%)
mean	3.5%	69.1%	3.4%	43.7%	46.9%	62.2%

Table II The results of double staining for NGFR and CGRP, VIP, or galanin

	NGFR	CGRP	DOUBLE*	*/NGFR	*/CGRP
CONTROL	160	171	118	73.8%	69.0%
	112	130	85	75.9%	65.4%
mean				74.9%	67.9%
PERIPHERAL	47	72	33	70.2%	45.8%
AXOTOMY	37	83	29	78.4%	34.9%
mean				74.3%	40.4%

	NGFR	VIP	DOUBLE*	*/NGFR	*/VIP
VINBLASTINE	135	67	4	3.0%	6.0%
(0.15 mM)	115	48	6	5.2%	12.5%
mean				4.1%	9.3%

	NGFR	Galanin	DOUBLE*	*/NGFR	*/Galanin
VINBLASTINE	108	65	9	8.3%	13.8%
(0.15 mM)	92	105	7	7.6%	6.7%
mean				8.0%	10.3%