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Neuroplasticity in the smooth muscle of the myenterically and extrinsically denervated rat jejunum

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Abstract. The objective of this study was to examine the effects of two different denervation procedures on the distribution of nerve fibers and neurotransmitter levels in the rat jejunum. Extrinsic nerves were eliminated by crushing the mesenteric pedicle to a segment of jejunum. The myenteric plexus and extrinsic nerves were eliminated by serosal application of the cationic surfactant benzyldimethyltetradecylammonium chloride (BAC). The effects of these two denervation procedures were evaluated at 15 and 45 days. The level of norepinephrine in whole segments of jejunum was initially reduced by more than 76% after both denervation procedures, but by 45 days the level of norepinephrine was the same as in control tissue. Tyrosine hydroxylase (noradrenergic nerve marker) immunostaining was absent at 15 days, but returned by 45 days. However, the pattern of noradrenergic innervating axons was altered in the segment deprived of myenteric neurons. Immunohistochemical studies showed protein gene product 9.5 (PGP 9.5)-immunoreactive fibers in whole-mount preparations of the circular smooth muscle in the absence of the myenteric plexus and extrinsic nerves. At 45 days, the number of nerve fibers in the circular smooth muscle increased. Vasoactive intestinal polypeptide (VIP)-immunoreactive fibers, a subset of the PGP 9.5 nerve fibers, were present in the circular smooth muscle at both time points examined. Choline acetyltransferase (CAT) activity and VIP and leucine enkephalin levels were measured in separated smooth muscle and submucosa-mucosal layers of the denervated jejunum. VIP and leucine-enkephalin levels were no different from control in tissue that was extrinsically denervated alone. However, the levels of these peptides were elevated two-fold in the smooth muscle 15 and 45 days after myenteric and extrinsic denervation. In the submucosa-mucosa, VIP and leucine enkephalin levels also were elevated two-fold at 15 days, but comparable to control at 45 days. CAT activity was equal to control in the smooth muscle but elevated two-fold in the submucosa-mucosa at both times. These results provide evidence for innervation of the circular smooth muscle by the submucosal plexus. Moreover, these nerve fibers originating from the submucosal plexus proliferate in the absence of the myenteric plexus. Furthermore, the myenteric neurons appear to be essential for normal innervation of the smooth muscle by the sympathetic nerve fibers. It is speculated that the sprouting of the submucosal plexus induced by myenteric plexus ablation is mediated by increased production of trophic factors in the hyperplastic smooth muscle.

Key words: Choline acetyltransferase – Enkephalin – Norepinephrine – Vasoactive intestinal peptide – Protein gene product 9.5 – Enteric nervous system – Sympathetic nervous system – Jejunum – Denervation – Rat (Sprague Dawley)

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Introduction

The enteric nervous system of the gastrointestinal tract consists of two primary ganglionated plexuses, the myenteric plexus which is situated between the longitudinal and circular smooth muscle, and the submucosal plexus which lies within the submucosa (Furness and Costa 1987). A variety of neurotransmitters such as acetylcholine, GABA, serotonin, and a diversity of peptides have been localized to neurons intrinsic to the gut wall (Schultzburg et al. 1980). The intrinsic ganglion cells of the myenteric and submucosal plexuses of the gastrointestinal tract also are innervated by postganglionic sympathetic fibers, which reach the gut along with the mesenteric arteries. Sympathetic nerve fibers richly innervate the arteries that enter the gut wall and project both orad and caudad after entry (Dowman 1952; Norberg 1964; Jacobowitz 1965; Gershon 1981). Parasympathetic nerve fibers from the dorsal motor nucleus of the vagus predominately innervate the myenteric plexus (Connors et al. 1983; Kirchgessner and Gershon 1989; Berthoud et al.

1990). The pathway by which the preganglionic parasympathetic nerve fibers enter the intestine is unclear (Kirchgessner and Gershon 1989; Zhang et al. 1991).

There are pathological conditions in humans (Krishnamurthy and Schuffler 1987) and experimental manipulations in animal models in which nerve perturbations occur. The absence of enteric neurons in Hirschsprung's disease (Ehrenpreis 1971) and in Chagas' disease (Ferreira-Santos and Carril 1964) is associated with an increase in the thickness of the smooth muscle. Interestingly in Hirschsprung's disease, there is an increase in the number of extrinsic adrenergic fibers in these thickened segments. In the aganglionic terminal bowel of the piebald mouse, the muscularis mucosae is increased in thickness (Tennyson et al. 1986). This change in muscle thickness and the absence of intrinsic neurons suggests a relationship between the size of the muscle and its neural innervation.

This laboratory has developed a model in which the myenteric plexus and extrinsic nerve fibers in a segment of rat jejunum are eliminated by serosal application of the cationic surfactant benzyldimethyltetradecylammonium chloride (BAC). This treatment eliminates at least 96% of the myenteric neurons but has no effect on the number and function of submucosal neurons (Herman and Bass 1989; See et al. 1990a). BAC treatment eliminates the myenteric plexus and extrinsic nerves by destruction of the longitudinal smooth muscle and a portion of the circular smooth muscle. The surviving smooth muscle rapidly regenerates and both the longitudinal and circular smooth muscle are increased in thickness (See et al. 1988). An increase in the levels of certain neurotransmitters (Dahl et al. 1987) as well as hypertrophy of VIP submucosal neurons (See et al. 1990b) have been observed. Furthermore, pharmacological studies have suggested that there is innervation of the circular smooth muscle, presumably by the submucosal plexus (Herman and Bass 1990a). Taken together, these results suggest that after elimination of the myenteric plexus, there is enhanced innervation of the circular smooth muscle by the submucosal plexus.

In this study we have used biochemical and morphological techniques to examine the innervation of the smooth muscle by the submucosal plexus from a segment of jejunum in the absence of the myenteric plexus and extrinsic nerves. Since extrinsic sympathetic innervation also is eliminated, the innervation of the smooth muscle by noradrenergic nerves was examined in the presence and absence of the myenteric plexus. The results of this study provide evidence that there is increased innervation of the myenterically denervated circular smooth muscle by the submucosal plexus. The results also suggest that the myenteric plexus is important for the normal distribution of noradrenergic innervation in the jejunum.

Materials and methods

Denervation procedures

All procedures in this study that involve the use of animals were approved by the University of Wisconsin-Madison Clinical Health Sciences Animal Care Committee. Male Sprague-Dawley rats (Harlan Sprague-Dawley Inc., Madison, Wis.) weighing 220–225 g were housed singly and maintained on 12 h light/dark cycles with food and water freely available throughout this study. Animals were anesthetized by i.p. injection of pentobarbital and chloral hydrate anesthetic and a 5 cm portion of jejunum, 6 cm caudad to the ligament of Trietz, was exteriorized through a midline incision. The segment to be denervated by either of the techniques described below was marked by serosal sutures placed at the mesenteric border.

Extrinsic denervation of the segment of jejunum was accomplished by crushing the paravascular nerve bundles and the mesenteric arteries in three consecutive arcades with a fine forceps for 10 s at a minimum of three points between the superior mesenteric artery and the wall of the jejunum (Furness and Costa 1978; See et al. 1990b). A similar length of jejunum was exteriorized in a second group of animals, but the mesenteric nerves were left intact (sham operated control).

Myenteric plexus and extrinsic innervation of the segment were eliminated by the serosal application of a cationic surfactant (Fox et al. 1983; Herman and Bass 1989). One to two milliliters of 2 mM benzyldimethyltetradecylammonium chloride (Aldrich Chemical Co., Milwaukee, Wis.) in 0.9% saline was applied to the serosal surface of the delineated segment with a glass pipet every 5 min for 30 min (BAC treated). In a second group of animals, a segment of jejunum was treated in a similar manner with 0.9% saline alone (vehicle control). The exposed segment in all treatment groups was rinsed with saline, returned to the peritoneal cavity and the incision closed.

Measurement of choline acetyltransferase, leucine-enkephalin, and vasoactive intestinal peptide

Preparation of tissue. Six to eight animals in each treatment group were sacrificed by a blow to the head followed by cervical dislocation 15 and 45 days after each respective treatment. A central 3 cm portion of the 5 cm segment of treated jejunum was dissected, rinsed with cold 0.1 M phosphate-buffered saline pH 7.4 (PBS) and slit open along the mesenteric border. The smooth muscle (longitudinal and circular) was separated from the submucosa and mucosa on a Sylgard lined Petri dish with the aid of a dissecting microscope. The separated layers of the jejunum were weighed and frozen immediately in liquid nitrogen and stored at −80° C until time of assay. Tissue also was obtained from untreated animals of the same age and weight.

Assay for choline acetyltransferase. The smooth muscle and the submucosa-mucosal layers from treated and control segments were separately homogenized in cold 25 mM phosphate buffer pH 7.4 (10 ml/g wet wt), containing 0.1 mM mercaptoethanol, 1 mM ethylenediaminetetraacetic acid (EDTA) and 2 mM phenylmethylsulfonylfluoride. The homogenates were centrifuged at 27000 x*g* for 30 min. Choline acetyltransferase (CAT) activity was measured by a method similar to Fonnum (1975). A 20 µl aliquot of supernatant was incubated at 37° C for 30 min with a 100 µl reaction mixture containing 50 mM phosphate pH 7.4, 150 mM NaCl, 20 mM EDTA, 0.1 mM neostigmine, 8 mM choline, 0.2 mM acetyl-coenzyme A and 80,000 cpm [^{3}H]acetyl-coenzyme A (Amersham, Arlington Heights, Ill.).

The reaction was terminated by the addition of 5 ml of 10 mM phosphate buffer, pH 7.4. Two milliliters of tetraphenol boron in acetonitrile (5 mg/ml) and 10 ml of scintillation cocktail (0.5%

PPO, 90% toluene, 10% butanol) were added. The contents were mixed and counted in a liquid scintillation counter. All enzyme assays were carried out under conditions in which the reaction was linear with time and amount. A small amount of labelled product was produced in the presence of cholinesterase and does not represent CAT activity. Therefore, CAT activity was calculated from the difference in labeled product obtained with a reaction mixture containing neostigmine and another in which cholinesterase was substituted for neostigmine. Aliquots of each sample were assayed in duplicate. One unit of enzyme activity was defined as that which catalyzes the formation of one picomole of [^{3}H]acetylcholine per minute.

Radioimmunoassays for leucine-enkephalin and VIP. The smooth muscle and the submucosa-mucosal layers from treated and control segments were extracted separately in cold 0.1 M HCl (30 ml per g wet wt), homogenized and centrifuged at 12000 x*g* for 30 min. Aliquots of the resultant supernatants were dried under vacuum and reconstituted in 0.1 M TRIS buffer. pH 7.4, containing 0.1% gelatin, 0.01% thimerosal, 0.002% neomycin and 0.1% Triton X-100 (all from Sigma Chemical, St. Louis, Mo.). This extraction procedure results in the recovery of 85% of the leu-ENK and 88% of the VIP from the homogenate.

For the determination of leu-ENK, 100 µl of various concentrations of leu-ENK (Sigma Chemical, St. Louis, Mo.) or reconstituted aliquots were added to polystyrene tubes. One hundred microliters of antiserum raised in our laboratory (Lindberg et al. 1979) diluted to give an bound to free ratio of 0.5–0.6 in the absence of unlabeled leu-ENK were added, followed by 100 µl containing 12,000 cpm ^{125}I-leu-ENK (Amersham, Arlington Heights, Ill.). The tubes were incubated for 22–24 h at 4° C, then 100 µl normal rabbit serum and 1 ml ice cold 25% polyethylene glycol 6000–8000 were added. Tubes were vortexed, allowed to stand 60 min at 4° C and centrifuged at 7500 x *g* for 20 min. The supernatants were removed and the pellets counted in a scintillation counter.

For the determination of VIP, 100 µl of various concentrations of VIP (Bachem, Torrance, Calif.) or reconstituted aliquots were added to polystyrene tubes, then 100 µl of antiserum (Epstein and Poulsen 1991) diluted to give a bound to free ratio of 0.5–0.6 in the absence of unlabeled VIP were added and the mixture incubated 22–24 h at 4° C. One hundred microliters containing 14000 cpm ^{125}I-VIP (Amersham, Arlington Heights, Ill.) were added and the mixture incubated an additional 22–24 h at 4° C. Then, 100 µl each of normal rabbit gamma globulin and goat anti-rabbit serum were added. After a 2 h incubation at room temperature, 1 ml of cold 3% polyethylene glycol 8000 was added, and the tubes were centrifuged at 2000 x *g* for 45 min. The supernatant was removed and the pellet counted in a scintillation counter.

Norepinephrine determination

A central 1 cm portion of the 5 cm segment of denervated and control jejunum was weighed, immediately frozen in liquid nitrogen and stored at −80° C. Tissues were homogenized (15 ml per g wet weight) in ice cold 3 N formic acid/acetone 15:85 (v/v) and centrifuged for 10 min at 900 x *g*. The pellets were resuspended in 3 N formic acid/acetone (15 ml per g wet weight), recentrifuged and the supernatants pooled. The combined supernatants were extracted with 1.0 ml of heptane/chloroform 8:1 and centrifuged for 2 min at 900 x *g*. The aqueous portions containing the norepinephrine were dried in a vacuum centrifuge, reconstituted in 0.3 ml HPLC buffer, filtered and stored at −80° C until analysis. This extraction procedure results in a 93% percent recovery of the norepinephrine.

Norepinephrine was fractionated by a Bioanalytical Systems HPLC with a 250 mm × 4.6 mm C-18 reverse phase column. The flow rate was maintained at 0.7 ml per min. The HPLC buffer was 0.1 M citrate-disodium phosphate, pH 3.5, containing 0.012% sodium octyl sulphate, 0.015% EDTA and 6% methanol by volume. Five microliters of the reconstituted extracts were injected on the HPLC and norepinephrine was detected electrochemically with a glassy carbon detector set at a potential of 0.7 volts versus the reference electrode (Loullis et al. 1979). Norepinephrine standard solutions (0.0 to 10.0 ng) were processed the same as the tissue extracts. Norepinephrine was identified and quantified by the retention time and peak height of the standard solutions and tissue extracts spiked with a known amount of norepinephrine.

Immunohistochemistry

A central 1 cm portion of the 5 cm segment from denervated jejunum and a 1 cm segment from untreated jejunum were dissected and rinsed with cold 0.1 M phosphate-buffered saline, pH 7.4 (PBS). Tissues were slit open along the mesenteric border and pinned under light tension on a bed of Sylgard with the mucosal surface uppermost. The orad and caudad ends of the segments were identified. Tissues were fixed with 4% formaldehyde in PBS for 16–24 h at 4° C, then rinsed in PBS. The mucosa was removed and the longitudinal and circular smooth muscle layer was separated from the submucosa with the aid of a dissecting microscope. The smooth muscle was processed for the immunocytochemical demonstration of neurons and nerve fibers with antibody against protein gene product 9.5 (PGP 9.5), a neuron-specific cytoplasmic protein (Lundberg et al. 1988). Nerve subpopulation were identified with antisera to vasoactive intestinal polypeptide (VIP) and the catecholamine synthesizing enzyme tyrosine hydroxylase (TH). The individual whole-mounts were incubated for 36–48 h at 4° C in primary antiserum diluted with PBS containing 0.3% Triton X-100, 3% goat serum and 0.1% sodium azide. Whole-mounts were rinsed with PBS, uncubated in biotinylated goat anti-rabbit or goat anti-mouse sera for 2 h at room temperature. Whole-mounts were rinsed in PBS, incubated in avidin-peroxidase and reacted with diaminobenzidine (Vectro Laboratories, Burlingame, Calif.).

The specificity of the immunohistochemical reaction was checked in three types of control experiments: preparations were exposed (1) to the second antibody alone, (2) to the primary antibody which had been preincubated with pure antigen, or (3) to the second antibody which had been preincubated with the host animal's serum. No nerve cell bodies or processes were visualized in any of these control tests. Cross-reactivity with other peptides or proteins containing amino acid sequences recognized by the different antisera connot be excluded. It is therefore appropriate to refer to the immunoreactive material as VIP-like, TH-like and PGP 9.5-like. For simplicity however, the shorter terms are used.

Data analysis

Results are expressed as the mean ± standard error of the mean (SEM). A one-way analysis of variance was used to compare the data. Significant differences between means were judged by the Newman Keuls procedure with a probability error rate of 0.05. Neurotransmitter content in the vehicle treated, sham operated and untreated jejunum was statistically the same, therefore the data from these treatment groups were combined and labeled as control.

Nomenclature

The term "extrinsic denervation" is used to denote the procedure in which the extrinsic nerve fibers to a segment of jejunum are eliminated by surgically clamping paravascular nerve bundles and the mesenteric artery. "Myenteric and extrinsic denervation" is used to denote the procedure in which the myenteric plexus is permanently eliminated and the extrinsic nerve fibers to the segment of jejunum are eliminated by BAC treatment.

Results

Norepinephrine levels and tyrosine hydroxylase immunostaining

The level of norepinephrine (Fig. 1A) in whole segments of jejunum 15 days after crushing the paravascular nerves (extrinsic denervation alone) is reduced 88% from 40.2 ± 2.4 to 4.9 ± 1.0 ng/cm. In segments of jejunum in which the myenteric plexus and extrinsic nerves have been eliminated by BAC treatment (myenteric and extrinsic denervation), the level of norepinephrine is reduced 76% to 9.7 ± 1.5 ng/cm. However, by 45 days (Fig. 1B), the levels of norepinephrine are comparable to control (50.8 ± 3.7 ng/cm) after either extrinsic denervation alone (43.7 ± 3.7 ng/cm) or myenteric and extrinsic denervation (50.7 ± 6.3 ng/cm).

Norepinephrine levels were also measured 2 cm orad and 2 cm caudad to the BAC treated segment. The norepinephrine levels in the orad tissue (45.0 ± 10.6 ng/cm) and caudad tissue (35.8 ± 5.0 ng/cm) 15 days after BAC treatment are not significantly different from control 40.2 ± 2.4 ng/cm). However, by 45 days, the norepinephrine level in the orad tissue (84.1 ± 4.1 ng/cm) is significantly increased while in the caudad tissue (59.2 ± 9.8 ng/cm) the level is comparable to control (50.8 ± 3.7 ng/cm).

In whole-mount preparations of the smooth muscle from untreated jejunum (Fig. 2A), there is a network of tyrosine hydroxylase immunoreactive fibers that are present primarily in association with the ganglia of the myenteric plexus and in the internodal strands. An occasional fiber can be seen in the smooth muscle. Fifteen days after extrinsic denervation alone (Fig. 2B), no tyrosine hydroxylase immunoreactive fibers are detectable in the smooth muscle. However, by 45 days tyrosine hydroxylase immunoreactive fibers are seen again surrounding the myenteric ganglia, in the internodal strands (Fig. 2C) and in association with blood vessels in the smooth muscle (Fig. 2D).

As shown in Fig. 3A, no tyrosine hydroxylase immunoreactive fibers are detectable in the smooth muscle 15 days after the myenteric plexus and extrinsic nerves are eliminated by BAC treatment. However, by 45 days (Fig. 3B) tyrosine hydroxylase containing varicose nerve fibers are visible as single fibers in the smooth muscle. These nerve fibers are not seen in association with blood vessels as is the pattern seen in tissue that is only extrinsically denervated.

Tissue weight, CAT activity, leucine-enkephalin and VIP levels in the smooth muscle and submucosa-mucosa layers

Changes in tissue weight. As shown in Fig. 4, there is no change in the wet weight per centimeter of the separate layers of jejunum after extrinsic denervation alone. However, the wet weight per centimeter of both the smooth muscle and submucosa-mucosa is significantly increased 15 and 45 days after BAC treatment. Since there is a change in tissue wet weight, we have compared

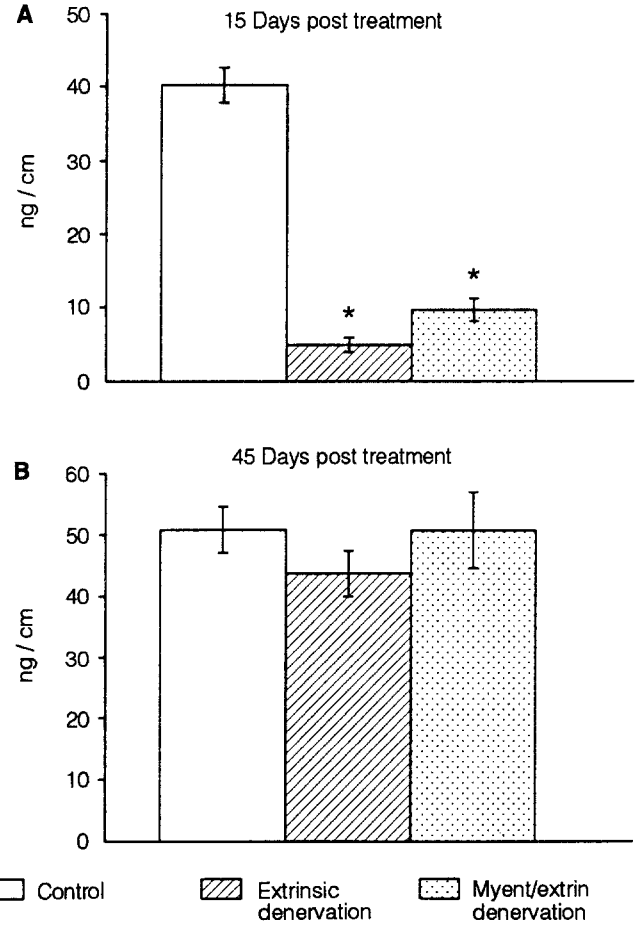

Fig. 1. Norepinephrine levels in segments of rat jejunum (**A**) 15 days and (**B**) 45 days after extrinsic denervation alone ($n=8$), after myenteric and extrinsic denervation (myent/extrin, $n=8$) and in control ($n=15$). Data are expressed as the means $\pm$ SEM. * Indicates a significant difference from control ($P<0.05$)

the activity of CAT and levels of norepinephrine, leucine-enkephalin and VIP per centimeter length of jejunum. If the neurotransmitter content was compared by wet weight or protein content, the change would reflect the change in wet weight and protein as much as it would reflect a change in the content of the neurotransmitters.

Choline acetyltransferase activity

Extrinsic denervation alone. Fifteen days after extrinsic denervation alone (Fig. 5A), CAT activity in the smooth muscle does not differ from control (60.9 ± 5.9 vs. 50.1 ± 5.6 units/cm). However, CAT activity in the submucosa-mucosa is significantly decreased compared to control (114.7 ± 10.7 vs. 161.0 ± 13.6 units/cm). By 45 days (Fig. 5B), CAT activity is significantly increased in the smooth muscle compared to control (71.3 ± 8.0 vs. 45.5 ± 3.2 units/cm). In the submucosa-mucosa, CAT activity is still decreased although not statistically from control (108.8 ± 21.5 vs. 148.6 ± 9.9 units/cm).

Myenteric and extrinsic denervation. CAT activity (Fig. 5) in the smooth muscle is comparable to control

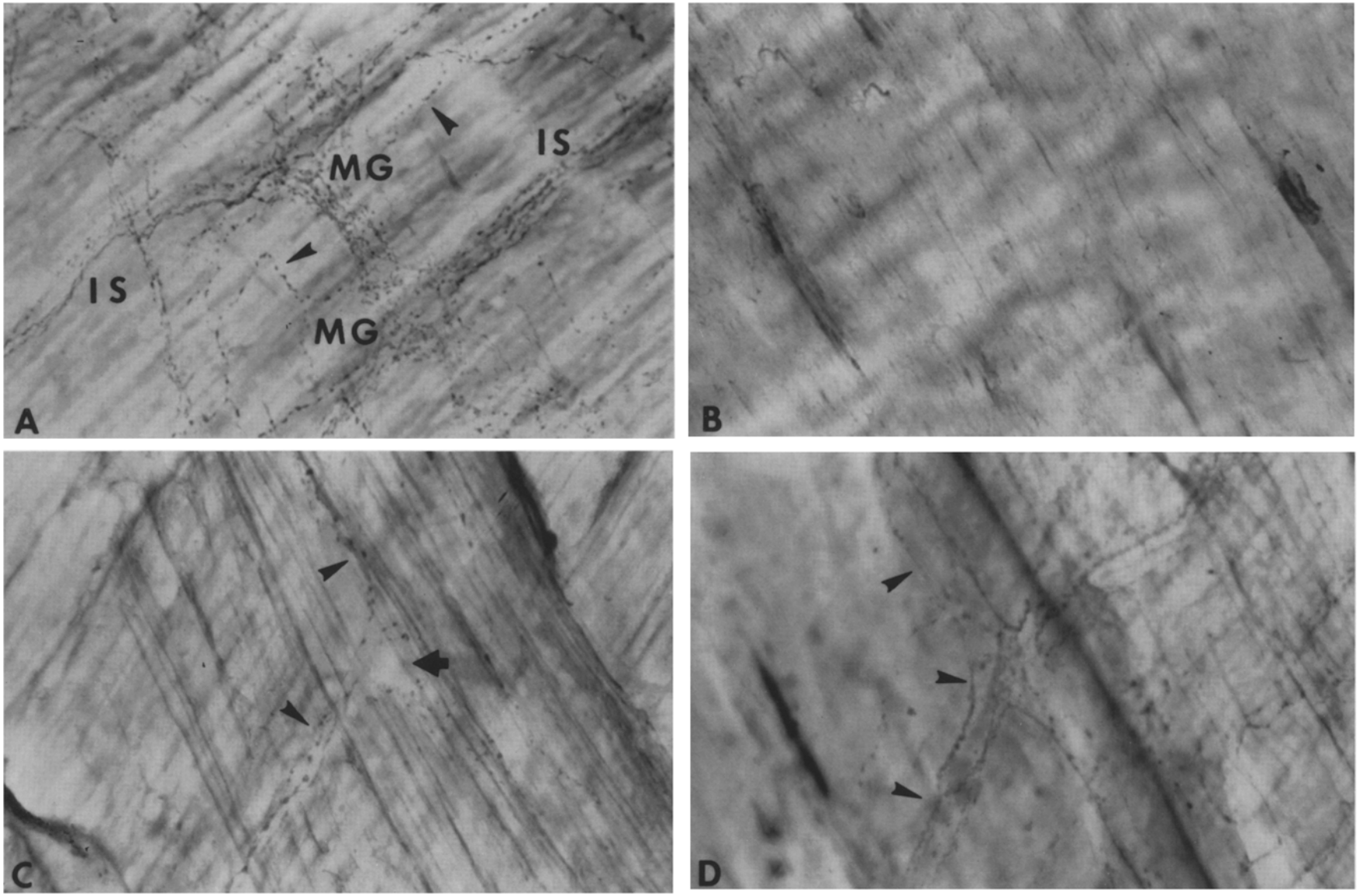

Fig. 2A–D. Tyrosine hydroxylase (TH) immunoreactivity in whole-mounts of the smooth muscle from rat jejunum. **(A)** In control smooth muscle, TH immunoreactivity is in the myenteric ganglia (*MG*) and internodal strands (*IS*). Occasional TH-immunoreactive fibers are seen within the smooth muscle (*arrowheads*). **(B)** No TH immunoreactivity is detectable 15 days after elimination of the extrinsic innervation to the segment. **(C)** Forty-five days after extrinsic denervation, TH immunoreactivity (*arrowheads*) is seen surrounding an apparent myenteric ganglion (*arrow*) and **(D)** blood vessel in the smooth muscle

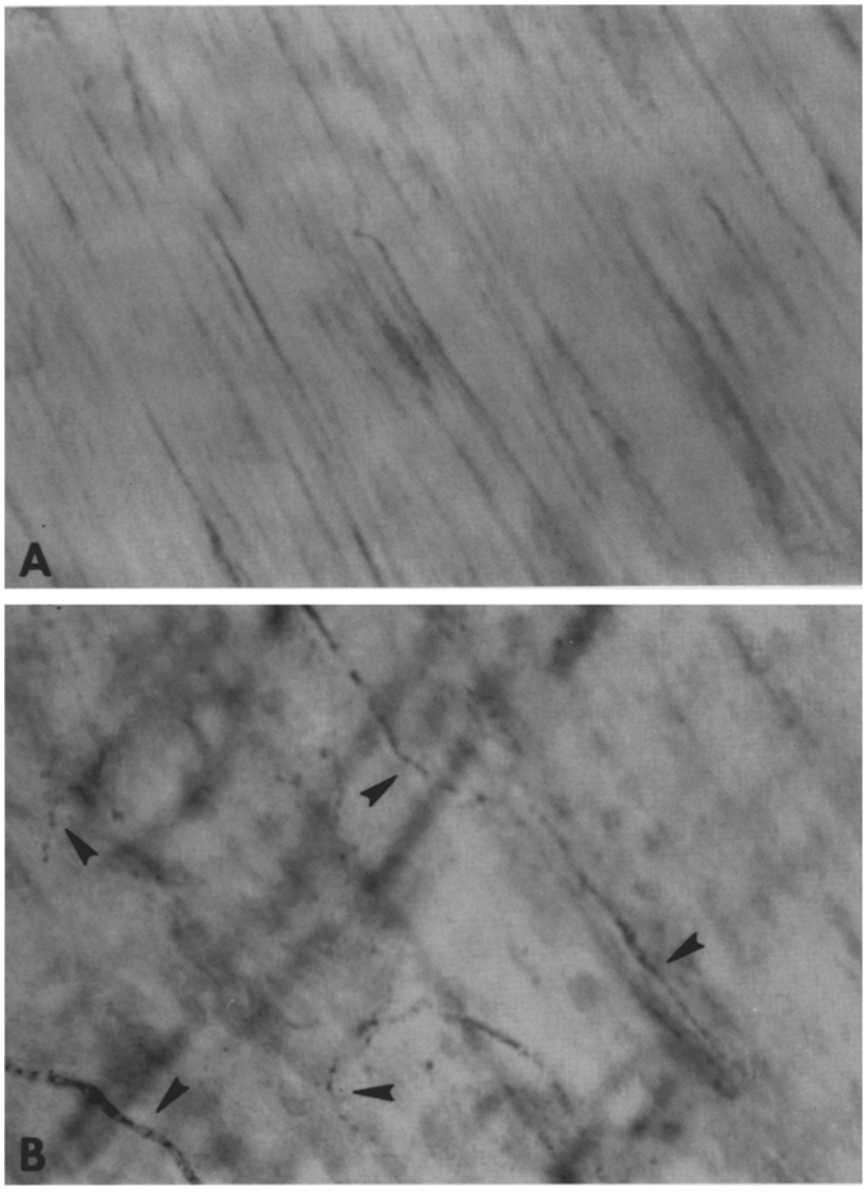

Fig. 3A, B. Tyrosine hydroxylase (TH) immunoreactivity in whole-mounts of the smooth muscle from rat jejunum. (**A**) No TH immunoreactivity is detectable 15 days after myenteric and extrinsic denervation. (**B**) By 45 days, TH immunoreactivity (*arrowheads*) is seen as single fibers in the smooth muscle that are not in association with blood vessels. (control; see Figure 2A)

both 15 days (60.0 ± 6.0 vs. 50.1 ± 5.6 units/cm) and 45 days (37.2 ± 4.7 vs. 45.5 ± 3.2 units/cm) after BAC treatment. However, CAT activity is increased two-fold compared to control in the submucosa-mucosa both 15 days (344.5 ± 19.6 vs. 161.0 ± 13.6 units/cm) and 45 days (241.0 ± 23.6 vs. 148.6 ± 9.9 units/cm) after BAC treatment.

Levels of leucine enkephalin and VIP

Extrinsic denervation alone. Extrinsic denervation alone does not alter the content of leu-ENK and VIP in either the smooth muscle or submucosa-mucosa after 15 days and 45 days compared to control (Figs. 6, 7).

Myenteric and extrinsic denervation. As shown in Fig. 6A and Fig. 7A there is a two-fold increase in the levels of leu-ENK (779 ± 106 vs. 323 ± 29 pg/cm) and VIP (75.2 ± 11.1 vs. 30.9 ± 4.1 ng/cm) compared to control in the smooth muscle 15 days after BAC treatment. In the submucosa-mucosa, there also is a significant increase in the level of leu-ENK (1659 ± 98 vs. 971 ± 90 pg/cm) and VIP (127 ± 18.9 vs. 66.9 ± 3.8 ng/cm) compared to control. Forty-five days after BAC treatment (Fig. 6B and Fig. 7B), the levels of leu-ENK (866 ± 83 vs. 352 ± 37 pg/cm) and VIP (60.8 ± 7.7 vs. 23.6 ± 1.7 ng/cm) remain significantly elevated in the smooth muscle. However, in the submucosa-mucosa the levels of leu-ENK (1498 ± 132 vs. 1296 ± 61 pg/cm) and VIP (90.0 ± 8.5 vs. 77.5 ± 6.8 ng/cm) are comparable to control.

PGP 9.5 and VIP immunostaining

In whole-mount preparations of the smooth muscle from control jejunum, PGP 9.5 immunoreactivity is present in association with the ganglia of the myenteric plexus

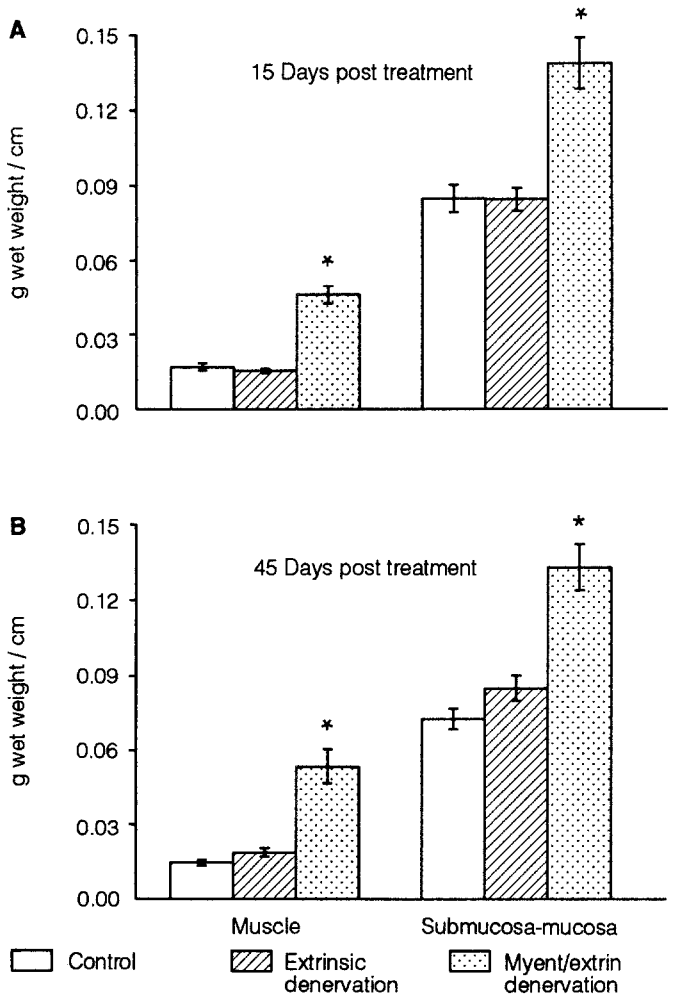

Fig. 4. Wet weight of the smooth muscle and submucosa-mucosa from segments of rat jejunum (**A**) 15 days and (**B**) 45 days after extrinsic denervation alone ($n=13$), after myenteric and extrinsic denervation (myent/extrin, $n=12$) and in control ($n=18$). Data are expressed as the means ± SEM. * Indicates a significant difference from control ($P<0.05$)

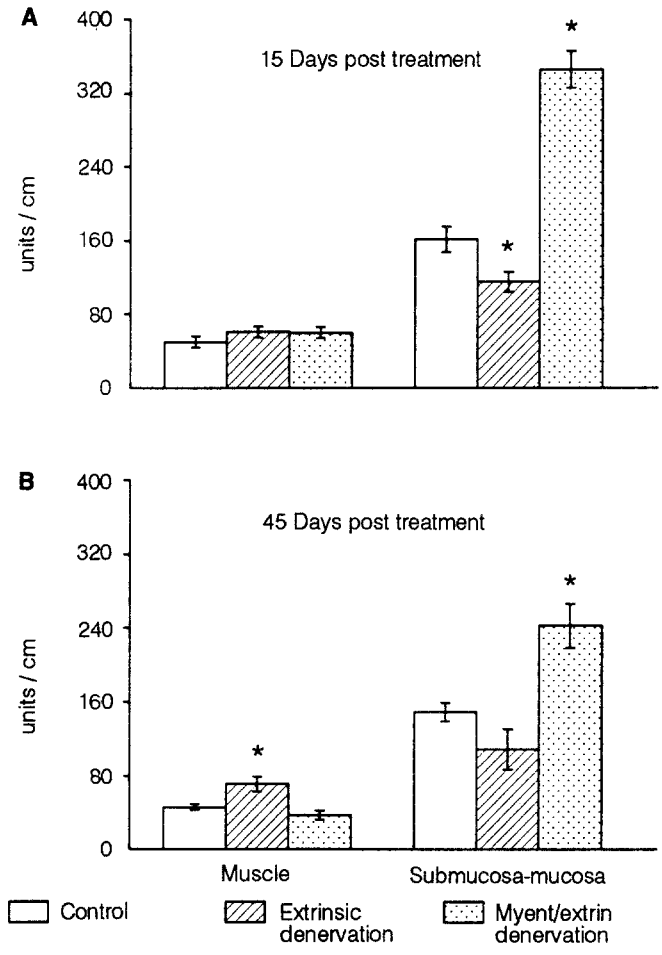

Fig. 5. Choline acetyltransferase activity in the smooth muscle and submucosa-mucosa from segments of rat jejunum (**A**) 15 days and (**B**) 45 days after extrinsic denervation alone ($n=6$), after myenteric and extrinsic denervation (myent/extrin, $n=6$) and in control ($n=12$). Data are expressed as the means ± SEM. * Indicates a significant difference from control ($P<0.05$)

and in the internodal strands (Fig. 8A). Single fibers are also found running parallel to the circular muscle layer. Fifteen days after myenteric and extrinsic denervation by BAC, no myenteric ganglia or internodal strands are detectable (Fig. 8B). However, a few fibers are found projecting parallel to the circular muscle and running within the smooth muscle bundles. These fibers are seen throughout the entire 1 cm length of muscle examined and do not appear to be more prevalent at the orad or caudad ends. Forty-five days after myenteric and extrinsic denervation the number of PGP 9.5-immunoreactive fibers within the circular muscle layer is greater than at 15 days (Fig. 8C). No myenteric ganglia or internodal strands are observed in the smooth muscle.

In whole-mount preparations of the smooth muscle from control jejunum, VIP immunoreactivity is present in the ganglia of the myenteric plexus and in internodal strands (Fig. 8D). As shown in Fig. 8E and 8F, no VIP-positive myenteric ganglia or internodal strands are detectable at 15 and 45 days after myenteric and extrinsic denervation. However, a small number of VIP-staining fibers are present within the circular muscle layer. These fibers generally project parallel to the smooth muscle bundles and do not appear to be greater in number at the orad or caudad ends of the segment of treated jejunum examined.

Discussion

The present study demonstrates that there is innervation of the circular smooth muscle of the rat jejunum by nerve fibers originating from the submucosal plexus. This is clearly evident by the presence of PGP 9.5-immunoreactive nerve fibers in the circular smooth muscle after removal of the myenteric plexus and extrinsic nerves. Furthermore, these nerve fibers increase in number with time. VIP-immunoreactive nerve fibers are also present in the circular smooth muscle, however the number of VIP nerve fibers does not change with time.

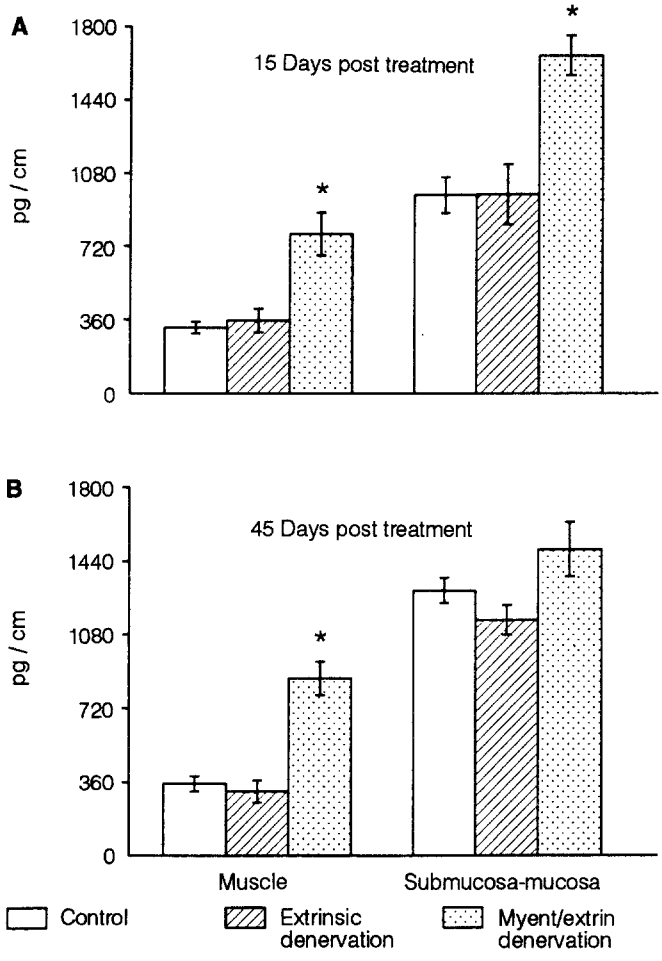

Fig. 6. Leucine-enkephalin levels in the smooth muscle and submucosa-mucosa from segments of rat jejunum (**A**) 15 days and (**B**) 45 days after extrinsic denervation alone ($n=7$), after myenteric and extrinsic denervation (myent/extrin, $n=8$) and in control ($n=14$). Data are expressed as the means ± SEM. * Indicates a significant difference from control ($P<0.05$)

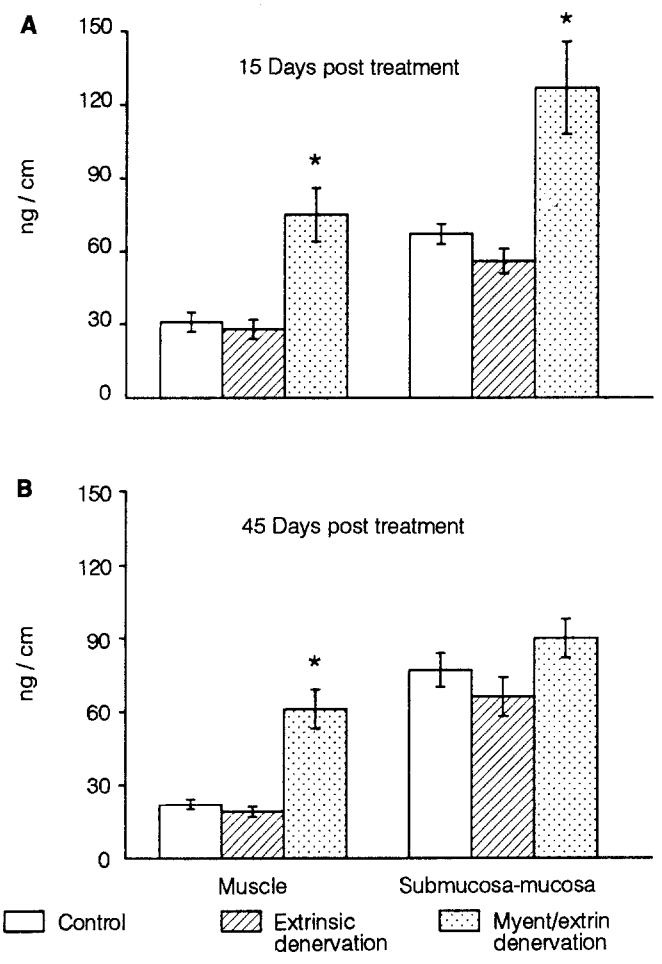

Fig. 7. Vasoactive intestinal peptide levels in the smooth muscle and submucosa-mucosa from segments of rat jejunum (**A**) 15 days and (**B**) 45 days after extrinsic denervation alone ($n=7$), after myenteric and extrinsic denervation (myent/extrin, $n=8$) and in control ($n=14$). Data are expressed as the means ± SEM. * Indicates a significant difference from control ($P<0.05$)

This study does not allow the distinction between new nerve fiber growth into the circular muscle or proliferation of preexisting nerve fibers from the submucosal plexus.

Our laboratory had previously shown an increase in neurotransmitters in myenterically and extrinsically denervated segments of whole jejunum (Dahl et al. 1987). In the present study, CAT activity and increased levels of leucine-enkephalin and VIP are found in the smooth muscle as well as the submucosa-mucosa. Thus, the present study indicates that the observed increase in neurotransmitters in whole segments of jejunum can be attributed to an increase in neurotransmitters in both the smooth muscle and the submucosa-mucosa. The finding of neurotransmitters in the smooth muscle in the absence of the myenteric plexus is evidence in support of submucosal plexus innervation of the circular smooth muscle. Moreover, the increase in neurotransmitters is associated with an increase in number of nerve fibers in the circular smooth muscle originating from the submucosal plexus.

Pharmacological studies of myenterically denervated jejunum from our laboratory provide further evidence that the circular smooth muscle is innervated by the submucosal plexus with time. Herman and Bass (1990a) have shown the contractility of myenterically denervated circular smooth muscle became subsensitive to carbachol with time. This subsensitive response was antagonized by hexamethonium, tetrodotoxin and Botulinum toxin A (Herman and Bass 1990b). These studies imply that the change in response to carbachol and the other drugs is mediated by enhanced innervation of the circular smooth muscle from the submucosal plexus.

The submucosal plexus in the absence of the myenteric plexus retains a physiological function. In an in vivo study, See et al. (1990a) demonstrated the relationship between contraction of the circular smooth muscle and mucosal ion secretion does not require the myenteric plexus. The nerves involved in this reflex originate from the submucosal plexus and are stimulated by contraction of the smooth muscle. Thus, the submucosal plexus functionally innervates the smooth muscle in the rat jejunum.

Evidence for submucosal plexus innervation of the circular smooth muscle is limited. Other investigators, after the surgical removal of the myenteric plexus in

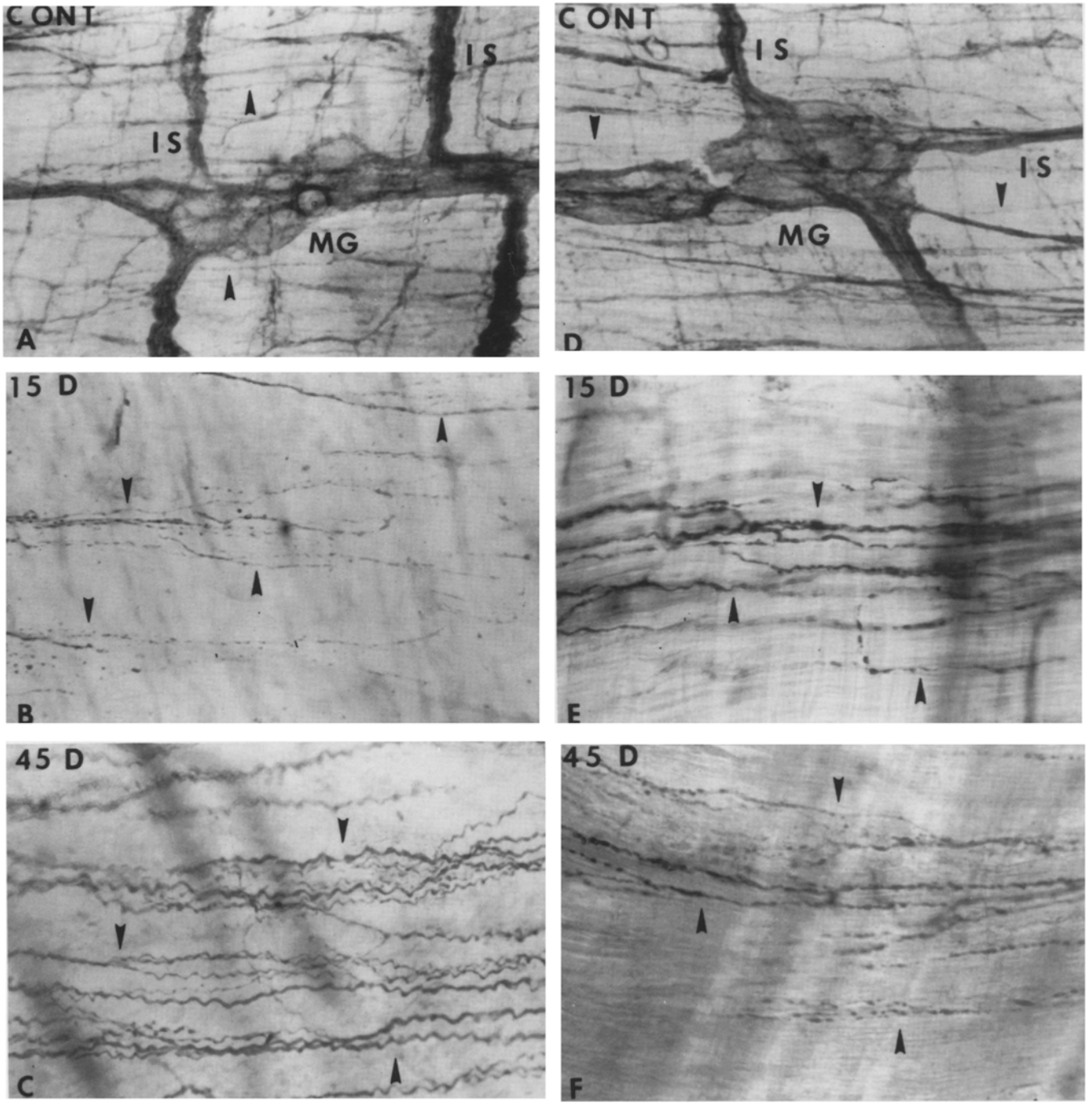

Fig. 8. PGP 9.5 (**A–C**) and VIP (**D–F**) immunoreactivity in whole-mounts of the smooth muscle from rat jejunum. All micrographs were taken with the circular muscle oriented left to right. In control smooth muscle, PGP 9.5 (**A**) and VIP (**D**) immunoreactivity is seen in the myenteric ganglion (*MG*) and internodal strands (*IS*) of the myenteric plexus and in fibers (*arrowheads*) in the smooth muscle. Fifteen days (15D) after the myenteric plexus and extrinsic innervation is eliminated by BAC treatment, PGP 9.5 (**B**) and VIP (**E**) fibers (*arrowheads*) are seen in the circular muscle. Forty-five days (45D) after BAC treatment, the number of PGP 9.5 (**C**) fibers is increased while the number of VIP (**F**) fibers is similar as that at 15 days. Note the absence of myenteric ganglia and internodal strands

the rat (Ekblad et al. 1987) and dog (Furness et al. 1990) have demonstrated a small number of VIP fibers that survive beneath the myectomy. The authors suggest that the submucosal plexus may be the source of these fibers. It is possible that fibers originating from the myenteric plexus enter the submucosa and run along the gut before returning to the smooth muscle. In our experiments this seems unlikely, since the denervated segment of jejunum is 5 cm in length and normal orad and caudad projections of nerve fibers rarely exceed 8 mm (Ekblad et al. 1987; Furness et al. 1990). Domoto et al. (1990) found VIP-containing nerves in the circular smooth muscle of

the human colon originating from both the myenteric and submucosal ganglia. Sanders and Smith (1986) demonstrated that neurons emanating from the submucosal plexus innervate the circular muscle in the proximal colon of the dog. These findings along with our present study suggest that in most species there are two intrinsic sources of innervation of the circular smooth muscle: the myenteric plexus which supplies the majority of nerve fibers innervating the longitudinal and circular smooth muscle, and the submucosal plexus which innervates the circular smooth muscle.

Our study also examined the effects of extrinsic denervation alone on neurotransmitters in the jejunum. The levels of leucine-enkephalin and VIP did not change in either the smooth muscle or submucosa-mucosa. These results are consistent with effects of chemical sympathectomy in the rat by guanethidine in which no change from control was observed after five weeks (Nelson et al. 1988).

The activity of CAT in the submucosa-mucosa however, is decreased by extrinsic denervation. Although the route by which parasympathetic nerve fibers enter the jejunum is a matter of controversy, it may be that a portion of the parasympathetic fibers enter the gut along with the mesenteric blood vessels (Berthoud et al. 1990; Zhang et al. 1991). Kirchgessner and Gershon (1989) injected the dorsal motor nucleus of the vagus with an anterograde tracer and identified only vagal efferent fibers in the myenteric plexus that reach the intestine by an intraenteric route that is not lesioned by crushing mesenteric nerves. Berthoud et al. (1990) injected the dorsal motor nucleus and identified vagal innervation in the myenteric and submucosal plexuses. The authors suggest that the label found in the submucosal plexus is of vagal afferent origin and may reach the intestine via the mesentery. The decrease in CAT activity observed in our study after extrinsic denervation is consistent with vagal afferent innervation of the submucosal plexus via the mesentery.

Another focus of our study was to examine sympathetic innervation of the smooth muscle after denervation. The levels of norepinephrine after extrinsic denervation alone and after myenteric and extrinsic denervation are reduced 88% and 76% respectively 15 days after treatment but return to control levels by 45 days. Chemical sympathetic denervation in the rat by guanethidine decreased the level of norepinephrine 90% for up to 6 months (Johnson and O'Brien 1976) and dopamine-β-hydroxylase for at least 5 weeks (Nelson et al. 1988). The difference between surgical and chemical depletion of norepinephrine levels can be explained by the methods employed. High doses of guanethidine cause the degeneration of adrenergic neuron cell bodies (Burnstock et al. 1971), whereas the surgical interruption of the nerve fibers does not. In a study by Hill et al. (1985), sympathetic reinnervation of both the arteries and myenteric plexus was observed within 8 weeks after extrinsic denervation by freezing the mesenteric arteries supplying the gut wall. In the present study adrenergic reinnervation of myenteric ganglia and blood vessels is observed 45 days after extrinsic denervation, however when the myenteric neurons are permanently eliminated by BAC treatment, the pattern of adrenergic nerve fiber innervating axons is altered. Nerve fibers were seen as single fibers in the smooth muscle and not associated with blood vessels. Hill et al. (1985) suggest the target (myenteric plexus) plays a role in the reinnervation of the gut by the sympathetic nervous system. Similarly, our results suggest that the normal pattern of sympathetic innervation may require the myenteric plexus.

Ganglion cells adapt to changes in the size of their targets by modulating neuron cell body size and the complexity of axonal and dendritic processes (Purves 1988). We observed an increase in the wet weight of both the smooth muscle and submucosa-mucosa in myenterically denervated jejunum due to hyperplasia of the smooth muscle cells (See et al. 1988) and proliferation of mucosal cryptstem cells (See et al. 1990b). Accompanying the increase in mass is the hypertrophy of VIP containing neurons in the submucosal plexus (See et al. 1988), and as shown in our present study a proliferation of nerve fibers and an increase in the level of neurotransmitters. Gabella (1984) reported an increase in size of neurons in the myenteric plexus in response to hypertrophy of the guinea pig small intestine. A study of the submandibular gland by Voyvodic (1989) showed that ganglion cells innervating an enlarged target develop more complex dendritic arborizations and an increase in cell body size. Steers et al. (1990) found that hypertrophy and hyperplasia of the obstructed urinary bladder is accompanied by hypertrophy of postganglionic neurons innervating the enlarged bladder. Thus, all these observations support the conclusion that neuronal size and complexity are influenced by the relative size of the target they innervate.

A possible molecular mechanism to explain neuronal changes in the myenterically denervated smooth muscle is the uptake and transport of one or more target derived trophic factors. Neurotrophic factors are retrograde messengers and are thought to modulate neuronal survival, process outgrowth and transmitter synthesis (Heumann et al. 1984; Thoenen and Edgar 1985; Lander 1987). One possible trophic molecule is nerve growth factor (NGF). NGF promotes the survival and growth of sensory and catecholamine containing sympathetic neurons (Thoenen and Edgar 1985). Snider (1988) has shown that exogenous NGF given to neonatal rats can enhance dendritic growth as well. Finally, the length and complexity of dendritic arborizations of autonomic ganglion cells is correlated with the difference in body weight of closely related species (Purves and Lichtman 1985; Purves et al. 1986). As the amount of trophic factor produced by a target organ may bear some relationsip to its size, the ability of NGF to influence neuronal cell size and dendritic outgrowth provides a plausible link between neuroplasticity of innervating neurons and the size of the target.

In conclusion, the presence of nerve fibers in the circular smooth muscle along with the finding of neurotransmitters, support the conclusion that the circular

smooth muscle is innervated by nerve fibers originating from the submucosal plexus. Moreover, there is enhanced innervation of the circular smooth muscle by these nerve fibers in the absence of the myenteric plexus. Furthermore, the myenteric plexus appears important for establishing the normal pattern of extrinsic sympathetic innervation to the smooth muscle. It is speculated that the sprouting of the submucosal plexus induced by myenteric plexus ablation is mediated by increased production of trophic factors in the hyperplastic smooth muscle.

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