

## Extrinsic neural control of nitric oxide synthase expression in the myenteric plexus of rat jejunum

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1. The role of extrinsic innervation in the expression of nitric oxide synthase (NOS) in the myenteric plexus remains unknown. In this study, we investigated the effect of extrinsic denervation on NOS expression in rat jejunum.
2.  $N^G$ -nitro-L-arginine methyl ester (L-NAME)-sensitive non-adrenergic, non-cholinergic (NANC) relaxations induced by transmural nerve stimulation were significantly increased in muscle strips obtained from rats treated with splanchnic ganglionectomy or 6-hydroxy-dopamine (6-OH-dopamine). Truncal vagotomy or treatment of the coeliac ganglia with capsaicin did not significantly affect NANC relaxations.
3. The number of NOS-immunopositive cells in the myenteric plexus was significantly increased in tissues obtained from rats treated with splanchnic ganglionectomy or 6-OH-dopamine.
4. Western blot analysis and Northern blot analysis showed a significant increase in the density of the immunoreactive NOS band and the NOS mRNA band, respectively, of the tissues obtained from rats treated with splanchnic ganglionectomy or 6-OH-dopamine.
5. Truncal vagotomy or treatment of the coeliac ganglia with capsaicin did not significantly affect the density of NOS band or NOS mRNA band.
6. It is concluded that neuronal NOS expression in the myenteric plexus is independent of vagus nerve and is negatively regulated by the splanchnic nerves in the rat small intestine.

Small bowel transplantation is a life-saving option for patients who suffer from chronic intestinal failure (Todo, Reyes, Furukawa, Abu-Elmagd, Lee & Tzakis, 1995). However, abnormal motility of the transplanted small bowel can lead to various complications such as malabsorption and diarrhoea (Todo *et al.* 1995). Extrinsic and intrinsic nerves regulate the motility of the small intestine (Andrews, Grundy & Lawes, 1980) and transplantation causes complete disruption of the extrinsic innervation to the graft. The denervation may damage the neuronal function in the myenteric plexus of the grafted small intestine. The abnormal release of excitatory and inhibitory neurotransmitters in the denervated bowel may alter the motility pattern. It has been demonstrated that the increase in intestinal contractile motility after transplantation is associated with a marked increase of excitatory non-adrenergic, non-cholinergic (NANC) neurotransmitter (probably substance P) (Ishii, Kusunoki, Fujita, Yamamura & Utsunomiya, 1994; Kusunoki, Ishii, Nakao, Fujiwara, Yamamura & Utsunomiya, 1995).

Nitric oxide (NO) is an important inhibitory NANC neurotransmitter in the gastrointestinal tract, including the small

intestine. NO released in response to stimulation of the myenteric plexus causes relaxation of the smooth muscle in the gastrointestinal tract (Bredt, Hwang & Snyder, 1990; Bult, Boeckxstaens, Pelckmans, Jordaens, Van Maercke & Herman, 1990; Boeckxstaens, Pelckmans, Bogers, Bult, De Man & Oosterbosch, 1991; D'Amato, Curro & Montuschi, 1992; Takahashi & Owyang, 1995). The rapid relaxation induced by NANC stimulation is significantly antagonized by the NO biosynthesis inhibitors,  $N^G$ -nitro-L-arginine (L-NNA) and  $N^G$ -nitro-L-arginine methyl ester (L-NAME), in the small intestine (Boeckxstaens, Pelckmans, Bult, De Man, Herman & Van Maercke, 1990; Bult *et al.* 1990). In addition, NO synthase (NOS) has been detected in the myenteric plexus (Bredt *et al.* 1990; Aimi, Kimura, Kinoshita, Minami, Fujimura & Vincent, 1993) and in the central nervous system (Bredt *et al.* 1990). These observations suggest that NO neurons in the myenteric plexus play an important role in mediating intestinal relaxation.

We have recently shown that the L-NAME-sensitive NANC relaxation was significantly enhanced in the grafted small intestine, suggesting an enhanced functioning of the NO

pathway in the myenteric plexus (Nakao, Ishii, Kusunoki, Yamamura & Utsunomiya, 1997). It is conceivable that the increased activity of the NO pathway is, in part, caused by extrinsic denervation following transplantation. The physiological role of extrinsic innervation on NO neurons in the myenteric plexus of the small intestine remains unknown.

We have shown that the expression of NOS, a key enzyme responsible for the release of NO, in the myenteric plexus is controlled by the vagus nerve in the rat stomach (Nakamura, Takahashi, Campbell, Taniuchi & Owyang, 1996). However, it is not known if the extrinsic vagus nerve and splanchnic nerve regulate NOS expression in the myenteric plexus of the small intestine. In this study, we investigated the effect of extrinsic denervation on NOS expression in rat jejunum. We demonstrate that splanchnic denervation causes enhanced NOS mRNA expression and NOS synthesis in the jejunal myenteric plexus, resulting in enhanced NANC relaxation of the jejunum. This goes some way towards explaining the abnormal motility that occurs after transplantation of the small bowel.

## METHODS

### Animal preparation

Male Sprague–Dawley rats (body weight, 220–250 g) were fasted overnight and anaesthetized with an intramuscular injection of xylazine and ketamine (13 and 87 mg (kg body wt)<sup>-1</sup>, respectively).

To investigate the role of the vagus and splanchnic nerves in the expression of NOS in the jejunal myenteric plexus, bilateral abdominal truncal vagotomy and splanchnic ganglionectomy were performed. The splanchnic ganglionectomy was carried out by deflecting the stomach and spleen to the right of the rat to facilitate identification of the nerves and coeliac ganglion. Sham-operated rats served as controls. Five days after the operation, rats were anaesthetized with an intramuscular injection of xylazine and ketamine (13 and 87 mg (kg body wt)<sup>-1</sup>, respectively) and killed by decapitation. Intestinal tissues were obtained for *in vitro* studies.

To investigate the role of catecholamines upon the mediation of NANC relaxation, 6-hydroxy-dopamine (6-OH-dopamine, 100 mg kg<sup>-1</sup> day<sup>-1</sup>, i.p.) was administered for 3 days before the experiment. Pretreatment with 6-OH-dopamine has been shown to lower the concentration of catecholamines in the rat stomach (Raybould, Roberts & Dockray, 1987). Rats treated with a vehicle, i.e. 0.9% saline (250 µl for 3 days, i.p.), served as controls.

To investigate if the splanchnic afferent neurons are involved in the mediation of NOS synthesis, the coeliac–superior mesenteric ganglia were treated with capsaicin. Capsaicin (10 mg) was sonicated with 0.1 ml Tween 80 for 10 min and mixed with olive oil to 1 ml. During anaesthesia, coeliac–superior mesenteric ganglia were exposed, as described by Holzer & Raybould (1992). A small piece of gauze soaked in capsaicin was placed around the ganglia for 30 min. The surrounding tissue was covered with gauze. The gauze was frequently replaced to minimize the spread of capsaicin. The area was thoroughly rinsed with olive oil, followed by saline, and dried with sterile swabs. The abdominal incision was closed. Treatment of the rats with vehicle was carried out in the same way with 10% Tween 80 in olive oil. Experiments were performed 7 days after the perineural capsaicin treatment.

All animal experiments were carried out in accordance with the National Institutes of Health *Guide for the Care and Use of Laboratory Animals*. The procedure used for the capsaicin treatment was approved by The University of Michigan Committee on Use and Care of Animals (UCUCA, approval no. 5027A). All efforts were made to minimize animal suffering and reduce the number of animals used in the experiments.

### NANC relaxation in response to transmural stimulation

Longitudinal muscle strips (15 mm in length) from the jejunum were suspended between two platinum electrodes in a 30 ml organ bath filled with Krebs–Henseleit buffer. Mechanical activity was recorded on a polygraph through isometric transducers. Muscle strips were stretched in 1 mm increments and repeatedly exposed to carbachol (10<sup>-6</sup> M) to determine  $L_0$ , the length at which the maximal active tension response developed. The resting tension was kept constant by readjusting it during the equilibration period. Concentration–response curves were constructed for each muscle strip, using sodium nitroprusside (10<sup>-8</sup> M to 10<sup>-5</sup> M), an activator of soluble guanylate cyclase, and carbachol (10<sup>-8</sup> M to 10<sup>-5</sup> M). To study NANC relaxation, the muscle strips were exposed to transmural electrical stimulation (70 V, 2 ms, 0.5–20 Hz, for 60 s) in the presence of atropine (10<sup>-6</sup> M) and guanethidine (10<sup>-6</sup> M). Experiments were performed after a 60 min equilibration period. Antagonists were given 10 min before the transmural stimulation. To study NO-dependent relaxation, the muscle strips were stimulated in the presence of atropine (10<sup>-6</sup> M), guanethidine (10<sup>-6</sup> M) and L-NAME (10<sup>-4</sup> M).

Transmural stimulation induced a triphasic response in rat jejunum: a rapid relaxation (first phase), followed by a phasic contraction (second phase), and a delayed relaxation (third phase). In response to transmural stimulation, L-NAME significantly inhibited the first phase of relaxation without affecting the third phase. L-NAME-sensitive NANC relaxation was studied at several frequencies in the range 0.5–20 Hz. Results were expressed as the percentage of the maximum L-NAME-sensitive NANC relaxation in each muscle strip. The maximum L-NAME-sensitive NANC relaxation in control rats was expressed as 100% and results were compared between the muscle strips obtained from control rats (sham operation or vehicle treatment) and treated rats (splanchnic ganglionectomy, 6-OH-dopamine, vagotomy or capsaicin).

### NADPH diaphorase histochemistry

NOS in the myenteric plexus and the central nervous system has been shown to be an NADPH diaphorase (Bredt, Glatt, Hwang, Fotuhi, Dawson & Snyder, 1991a; Dawson, Bredt, Fotuhi, Hwang & Snyder, 1991; Hope, Michael, Knigge & Vincent, 1991; Young, Furness, Shuttleworth, Bredt & Snyder, 1992). The NADPH diaphorase staining technique specifically labels neurons containing NOS (Bredt *et al.* 1991a; Dawson *et al.* 1991; Hope *et al.* 1991; Young *et al.* 1992). As previously reported, NADPH diaphorase-positive neuronal cell bodies and fibres were found throughout the entire rat gastrointestinal tract, including the small intestine (Belai, Schmidt, Hoyle, Hassall, Saffrey & Moss, 1992; Aimi *et al.* 1993). Most of the myenteric neurons that were NOS immunopositive in all regions of the gut also stained for NADPH diaphorase (Belai *et al.* 1992). The jejunal whole muscle layers were obtained from rats treated with sham operation, vagotomy or splanchnic ganglionectomy. The tissues were fixed overnight in 4% paraformaldehyde at 4 °C.

Histochemical staining for NADPH diaphorase was performed using the method previously described by Aimi (1993). NADPH diaphorase activity was rendered visible by incubating the tissues in 0.1 M phosphate buffer (pH 8.0) containing 0.01 mM β-NADPH,

0.02 mM Nitroblue Tetrazolium, and 0.3% Triton X-100, at 37 °C for 2 h. Washing the whole-mount preparations in 0.1 M phosphate buffer terminated the reaction. After several washings with 0.1 M phosphate buffer, the whole muscle layers were placed on glass slides, air dried, and cover-slipped with Gelmount. As previously described (Takahashi, Nakamura, Itoh, Sima & Owyang, 1997), the number of NADPH diaphorase-positive cells were counted in fifty ganglia in each preparation, and the average number of NADPH diaphorase-positive cells per ganglion was determined in each preparation.

#### NOS immunohistochemistry

Tracey (Tracey, Nakane, Pollock & Forstermann, 1993) demonstrated that NADPH diaphorase activity does not always colocalize with NOS activity. This suggests that NADPH diaphorase may not be NOS. Therefore, we performed immunohistochemistry using NOS antibody to detect neurons containing NOS in the myenteric plexus. Animals that had undergone a sham operation, vagotomy, splanchnic ganglionectomy, 6-OH-dopamine treatment, or capsaicin treatment were anaesthetized with pentobarbitone and perfused with 300 ml of 0.1 M phosphate-buffered saline (PBS, pH 7.4), followed by 300 ml of ice-cold fixative (4% paraformaldehyde in 0.1 M PBS). A segment of the jejunum was removed and embedded in paraffin.

Sections, 3 µm thick, of paraffin-embedded tissue were washed three times with PBS, 5 min each time. Sections were blocked for 1 h in 0.1 M PBS containing 4% normal goat serum and 0.1% Triton X-100. Sections were then incubated with antisera raised in rabbits against purified soluble NOS extract from rat cerebellum (nNOS antibody; Santa Cruz, CA, USA), at a dilution of 1:1000 in PBS for 18 h at 4 °C. The antibody to neuronal NOS (nNOS) has been shown to have no cross-reactivity with the antibody to the endothelial type of NOS (eNOS) or to the inducible type of NOS (iNOS) (Saffrey, Hassall, Hoyle, Belai, Moss & Schmidt, 1992). After washing in PBS, sections were incubated with biotinylated anti-rabbit IgG for 1 h at room temperature. Horseradish peroxidase staining was done using an avidin-biotin-labelled kit (Vectastain ABC kit). Diaminobenzidine and nickel chloride were used as chromogens. Sections were dehydrated and mounted. The total number of NOS-immunopositive cells in each ganglion of the myenteric plexus was counted microscopically, as previously described (Takahashi *et al.* 1997).

#### PGP 9.5 immunohistochemistry

It has been demonstrated that antisera raised against neuronal cytosolic protein, protein gene product (PGP) 9.5, stains the generic neurons in the CNS and myenteric plexus (Eaker & Sallustio, 1994). To investigate if vagotomy, splanchnic ganglionectomy, 6-OH-dopamine treatment, or capsaicin treatment affects the number of generic neurons of the myenteric plexus, PGP 9.5 immunohistochemistry was performed using paraffin-embedded sections that were only 3 µm apart from the sections used for NOS immunohistochemistry. The total number of generic neurons in the myenteric plexus (i.e. PGP 9.5-immunopositive cells) was counted in each ganglion. The percentage of NOS-immunopositive cells in each ganglion (the number of NOS-immunopositive cells/the number of PGP 9.5-immunopositive cells per ganglion) was evaluated in each slide.

#### Western blot analysis of NOS synthesis

The jejunal longitudinal muscle layers adherent with myenteric plexus were obtained from rats treated with sham operation, vagotomy, splanchnic ganglionectomy, vehicle, 6-OH-dopamine or capsaicin. Soluble homogenates of these samples were prepared in a lysis buffer containing 50 mM Tris-HCl (pH 7.4), EGTA (0.1 mM),

dithiothreitol (1 mM), leupeptin (10 µg ml<sup>-1</sup>), aprotinin (10 µg ml<sup>-1</sup>), phenylmethylsulphonyl fluoride (1 mM), and Triton X-100 (0.1%). Western blot analysis of the jejunal tissue was performed as described by Matsumoto, Mitchell, Schmidt, Kohlhaas, Warner & Forstermann (1992). Equal amounts of protein (each sample, 10 µg) were separated by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE, 7.5% w/v gel) and transferred to a nitrocellulose membrane. All procedures were done in Tris buffer (40 mM, pH 7.5) containing 0.3 M NaCl and 0.3% Tween 20. The membrane was blocked with bovine serum albumin (6% w/v), and subsequently incubated with specific polyclonal nNOS antibody (1:1000 dilution) and a horseradish peroxidase conjugate of affinity-purified goat antibody to rabbit IgG. The immune complexes were detected on photographic film by H<sub>2</sub>O<sub>2</sub>-luminol chemiluminescence. An imaging analyser measured the band density.

#### Western blot analysis of PGP 9.5

Quantitative changes in the generic neurons in the myenteric plexus after vagotomy, splanchnic ganglionectomy, 6-OH-dopamine treatment, or capsaicin treatment were studied by Western blot analysis of PGP 9.5. The jejunal longitudinal muscle layers adherent with myenteric plexus were obtained from rats treated with sham operation, vagotomy, splanchnic ganglionectomy, vehicle, 6-OH-dopamine, or capsaicin. Equal amounts of protein (each sample, 10 µg) were separated by SDS-PAGE (15% w/v gel) and transferred to a nitrocellulose membrane. After blocking with bovine serum albumin (6% w/v), the membranes were incubated with specific polyclonal PGP 9.5 antibody (1:10 000 dilution) (Eaker & Sallustio, 1994).

#### Northern blot analysis of NOS mRNA expression

For Northern blot analysis, the total RNA was isolated from the homogenized jejunal longitudinal muscles layers adherent with the myenteric plexus obtained from rats treated with sham operation, vagotomy, splanchnic ganglionectomy, vehicle, 6-OH-dopamine or capsaicin. Extraction and preparation of RNA was performed using TRIzol Reagent. The amount of RNA was estimated by measuring absorbance at 260 nm. Following isolation, the total RNA samples (20 µg) were electrophoresed on an agarose gel and transferred onto a nylon membrane. As demonstrated by Huang, Dawson, Brecht, Snyder & Fishman (1993), we used a 2947 bp rat neuronal NOS cDNA probe that extends 2.1 kb in the 3' direction beyond the first exon (244–3191) of the cloned cDNA (Brecht, Hwang, Glatt, Lowenstein, Reed & Snyder, 1991b). This probe was made from original cDNA (provided by Dr Snyder of The Johns Hopkins University School of Medicine) cut by the restriction endonuclease, *Aat* II, as previously reported (Nakamura *et al.* 1996). The cDNA probe (2.9 kb) was labelled with [<sup>32</sup>P]dCTP (111 TBq mmol<sup>-1</sup>) by the random-primed labelling method. The samples were prehybridized at 65 °C for 3 h and hybridized with the labelled probes at 65 °C for 16 h. The samples were washed twice (5 min each time) in 2 × standard saline citrate (SSC), 0.1% sodium dodecyl sulphate (SDS) at 65 °C, followed by two 5 min washes in 0.2 × SSC, 0.1% SDS at 65 °C. To confirm equivalent loading of RNA in the various lanes, the membrane was washed and rehybridized with a probe against glyceraldehyde 3-phosphate dehydrogenase (GAPDH). The samples were autoradiographed with intensifying screens at -80 °C. An imaging analyser measured the radioactivity of the tissue.

#### Data analysis

The results are expressed as means ± s.e.m. Statistical analysis was performed by analysis of variance (ANOVA) or Student's *t* test. *P* values < 0.05 were considered significant.

## Materials

Atropine, aprotinin, capsaicin, diaminobenzidine, guanethidine, leupeptin, NADPH (reduced form), nickel chloride, Nitroblue Tetrazolium, 6-OH-dopamine, Triton X-100, Tween 80 and phenylmethylsulphonyl fluoride were obtained from Sigma. L-NAME was obtained from Research Biochemicals. Antibody to constitutive nNOS was obtained from Santa Cruz (Santa Cruz, CA, USA). Antibody to PGP 9.5 was obtained from Ultraclone (Isle of Wight, UK). Anti-rabbit IgG and an avidin-biotin-labelled kit were obtained from Vector (Burlingame, CA, USA). [<sup>32</sup>P]dCTP and random-primer labelling (Rediprime) were obtained from Amersham. TRIzol was obtained from Life Technologies (Gaithersburg, MD, USA). Aat II and dithiothreitol were obtained from Boehringer Mannheim.

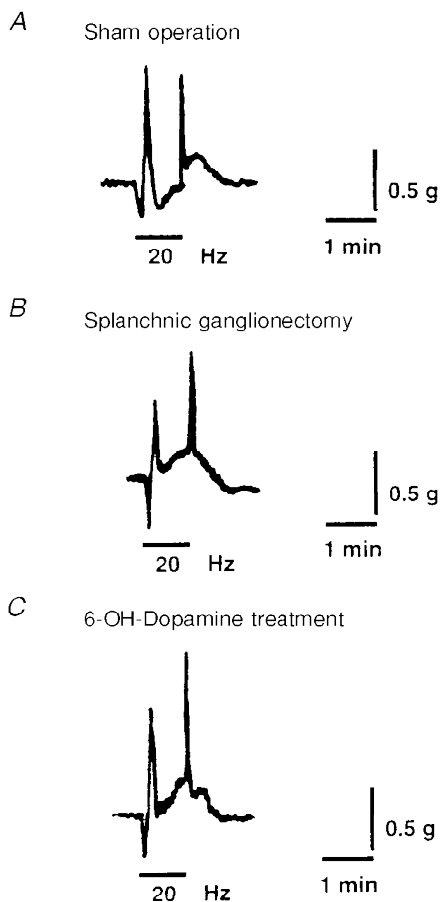
## RESULTS

### NANC relaxation

Transmural stimulation of the jejunal longitudinal muscle strips for 1 min induced a triphasic response comprising a rapid relaxation (first phase), a phasic contraction (second phase), and a delayed relaxation (third phase). A significant off-contraction was observed immediately after transmural stimulation was stopped (Fig. 1A). Tetrodotoxin completely abolished these responses (data not shown), suggesting that these were neurally mediated events. As previously described (Takahashi *et al.* 1997), the first phase of NANC relaxation was significantly antagonized by L-NAME,

suggesting mediation by neural release of NO from the jejunal myenteric plexus. Atropine ( $10^{-6}$  M) and guanethidine ( $10^{-6}$  M) significantly reduced the second phase of contraction and the third phase of relaxation, respectively, suggesting mediation by the release of acetylcholine and noradrenaline. The muscle strips obtained from rats treated with splanchnic ganglionectomy or 6-OH-dopamine did not exhibit the third phase of delayed relaxation (Fig. 1B), confirming that these treatments abolished the noradrenaline pathway in the jejunum.

To investigate whether NO-dependent relaxation is affected by splanchnic ganglionectomy or truncal vagotomy, transmural stimulation studies using the jejunal muscle strips pretreated with atropine ( $10^{-6}$  M) or guanethidine ( $10^{-6}$  M) were performed. Significant frequency-dependent (0.5–20 Hz) NANC relaxations (the first phase of rapid relaxation) in the presence of atropine and guanethidine were observed in preparations from control and treated rats (Fig. 2). Note that L-NAME-sensitive NANC relaxations in response to transmural stimulation were significantly enhanced in the muscle strips obtained from rats treated with splanchnic ganglionectomy ( $P < 0.0001$ , ANOVA) and 6-OH-dopamine ( $P < 0.05$ , ANOVA), compared with sham-operated rats and vehicle-treated rats (Figs 2 and 3). NANC relaxations in vagotomized rats and capsaicin-treated rats did not show any significant changes, compared



**Figure 1.** Motor responses of jejunal muscle strips to transmural nerve stimulation in control rats (A), rats treated with splanchnic ganglionectomy (B) and rats treated with 6-OH-dopamine (C)

In control rats, transmural stimulation (70 V, 2 ms, 20 Hz, for 60 s) of longitudinal muscle strips induced a triphasic response: a rapid relaxation (first phase), a phasic contraction (second phase), and a delayed relaxation (third phase). An off-contraction was observed immediately after transmural stimulation was stopped. The delayed relaxation (third phase) was not observed in the muscle strips obtained from rats treated with splanchnic ganglionectomy or 6-OH-dopamine.

with sham-operated rats and vehicle-treated rats. As a control, muscle relaxations evoked by exogenously applied sodium nitroprusside ( $10^{-6}$  M) remained unchanged in the preparations from rats treated with splanchnic ganglionectomy and 6-OH-dopamine, compared with sham-operated rats and vehicle-treated rats (Fig. 2).

**NADPH diaphorase histochemistry**

The NADPH diaphorase-positive cells and fibres were clearly observed in the jejunal myenteric plexus of sham-operated rats. The number of NADPH diaphorase-positive cells in the myenteric plexus was significantly increased in the muscle strips obtained from rats treated with splanchnic ganglionectomy ( $11.2 \pm 2.1$  cells per ganglion), compared with sham-operated rats ( $5.6 \pm 1.6$  cells per ganglion). (Fig. 4, mean  $\pm$  s.e.m.,  $n = 4$ ,  $P < 0.05$ , Student's *t* test). In contrast, the number of NADPH diaphorase-positive cells in the myenteric plexus was not significantly different in the muscle strips obtained from vagotomized rats

( $5.3 \pm 1.5$  cells per ganglion), compared with sham-operated rats.

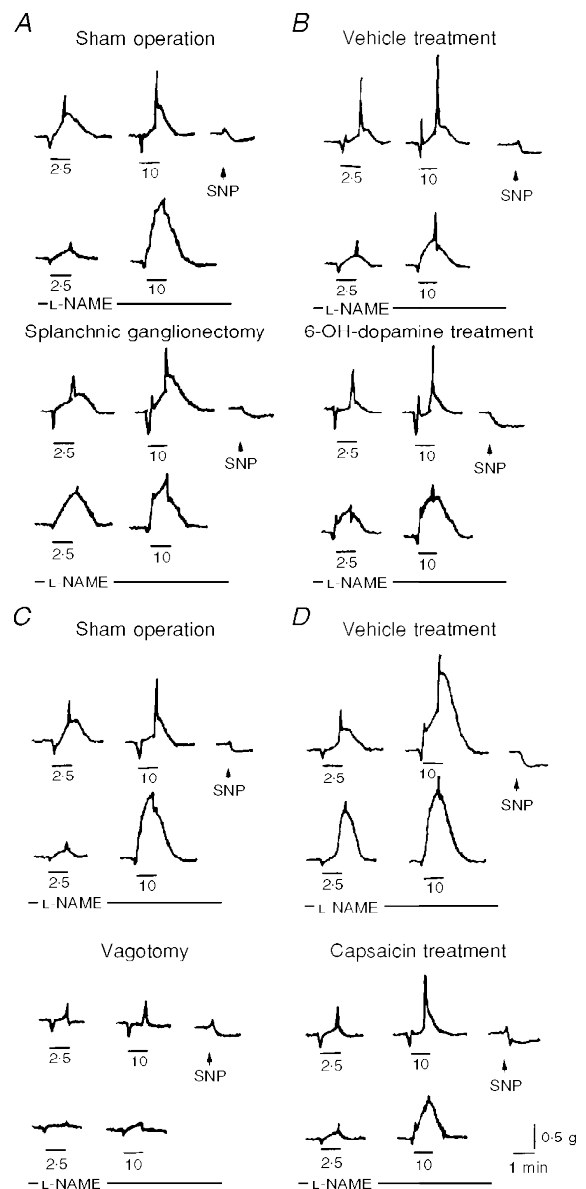
**NO immunohistochemistry**

NOS-immunoreactive neuronal cell bodies were found throughout the myenteric plexus of the jejunum of sham-operated rats. Non-neuronal tissues such as muscle cells, endothelium, mucosal cells and macrophages were unstained (Fig. 5). The number of NOS-immunopositive cells was significantly increased in rats treated with splanchnic ganglionectomy or 6-OH dopamine but not in vagotomized rats, compared with sham-operated rats or vehicle-treated rats (Fig. 5). On the other hand, the number of PGP 9.5-immunopositive cells in the jejunal myenteric plexus was not significantly different among these rats (data not shown).

The percentage of NOS-immunopositive cells per ganglion in sham-operated rats and vehicle-treated rats was

**Figure 2. Tracings of NANC relaxations in response to transmural nerve stimulation in the presence of atropine ( $10^{-6}$  M) and guanethidine ( $10^{-6}$  M) in rats treated with splanchnic ganglionectomy (A), 6-OH-dopamine (B), vagotomy (C) or capsaicin (D)**

L-NAME significantly inhibited NANC relaxation in response to transmural stimulation (70 V, 2 ms, 2.5 and 10 Hz, for 60 s) in muscle strips obtained from control rats, 6-OH-dopamine-treated rats, and rats treated with splanchnic ganglionectomy. NANC relaxation was significantly enhanced in muscle strips obtained from rats treated with splanchnic ganglionectomy or 6-OH-dopamine. Relaxation induced by sodium nitroprusside ( $10^{-6}$  M) was not significantly different between control rats and rats treated with splanchnic ganglionectomy or 6-OH-dopamine.



$11.6 \pm 1.8$  and  $10.6 \pm 1.1\%$ , respectively. The percentage of NOS-immunopositive cells per ganglion was significantly increased to  $22.6 \pm 2.9\%$  and  $18.9 \pm 1.8\%$  in rats treated with splanchnic ganglionectomy or 6-OH-dopamine, respectively (mean  $\pm$  s.e.m.,  $n = 4$ ,  $P < 0.05$ , Student's *t* test). In contrast, vagotomy or capsaicin treatment did not affect the percentage of NOS-immunopositive cells per ganglion ( $12.4 \pm 1.5$  and  $11.5 \pm 1.5\%$ , respectively).

#### Western blot analysis of NOS synthesis

The longitudinal muscle layers with adherent myenteric plexus may contain endothelial NOS (eNOS), as well as neuronal NOS (nNOS). The molecular weights of nNOS and eNOS are 155 kDa and 130 kDa, respectively (Forstermann, Pollock, Tracey & Nakane, 1994). nNOS antibody has been shown to have no cross-reactivity with eNOS antibody (Saffrey *et al.* 1992). Therefore, we performed Western blot analysis to detect the quantitative changes of nNOS synthesis in the longitudinal muscle tissue of rat jejunum.

The NOS-immunoreactive band at 155 kDa obtained from jejunal longitudinal muscle corresponded well with that obtained from rat cerebellum (data not shown). The NOS-

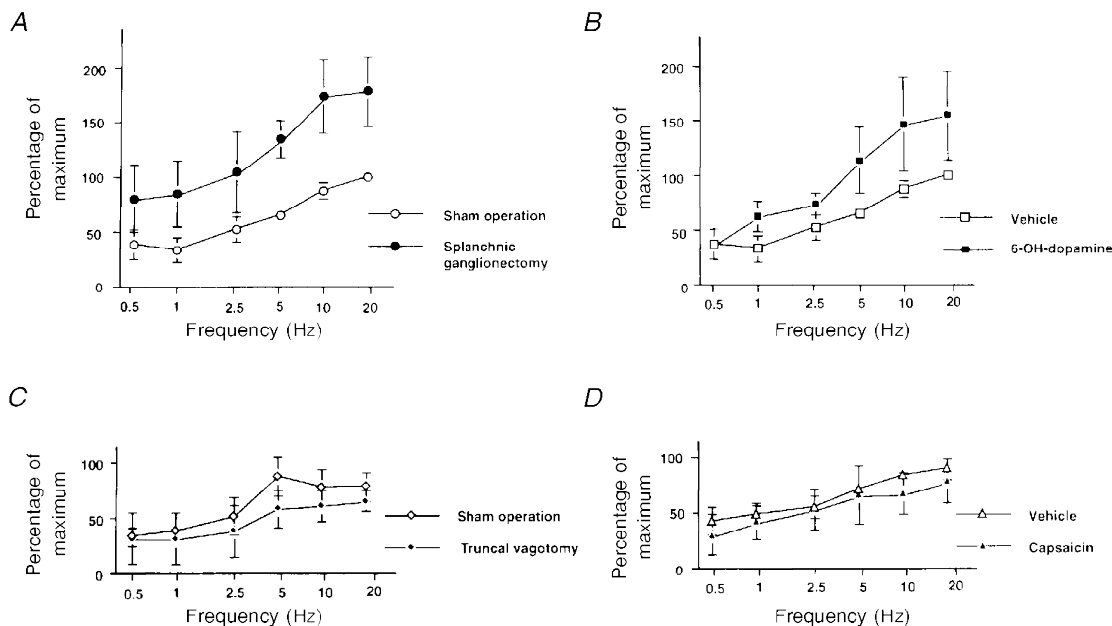
immunoreactive band at 155 kDa was clearly observed in tissue from controls (Fig. 6).

A significant increase in the density of the NOS-immunoreactive band was observed in tissue from rats treated with splanchnic ganglionectomy and 6-OH-dopamine, compared with sham-operated and vehicle-treated rats. Vagotomy and capsaicin-treatment had no effects on the density of the NOS-immunoreactive band (Fig. 6).

The density of the NOS-immunoreactive band was significantly increased to  $161 \pm 28\%$  of sham-operated rats and to  $122 \pm 6\%$  of vehicle-treated rats following splanchnic ganglionectomy ( $n = 6$ ) and 6-OH-dopamine treatment ( $n = 6$ ), respectively (Fig. 7, mean  $\pm$  s.e.m.,  $P < 0.05$ , Student's *t* test).

#### Western blot analysis of PGP 9.5

The density of the PGP 9.5-immunoreactive band at 27 kDa obtained from jejunal tissue was not significantly changed in the tissues obtained from rats treated with splanchnic ganglionectomy ( $n = 3$ ,  $104 \pm 8\%$ ), 6-OH-dopamine ( $n = 5$ ,  $97 \pm 7\%$ ), vagotomy ( $n = 3$ ,  $96 \pm 8\%$ ), and capsaicin



**Figure 3.** NANC relaxation in response to transmural nerve stimulation in rats treated with splanchnic ganglionectomy (*A*), 6-OH-dopamine (*B*), vagotomy (*C*) or capsaicin (*D*)

Transmural nerve stimulation (70 V, 2 ms, 0.5–20 Hz, for 60 s) evoked NANC muscle relaxation in a frequency-dependent manner (0.5–20 Hz). NANC relaxation was significantly enhanced in the muscle strips from rats treated with splanchnic ganglionectomy (d.f. = 1, 48;  $F = 26.042$ ;  $P < 0.0001$ ; ANOVA) or 6-OH-dopamine (d.f. = 1, 36;  $F = 4.697$ ;  $P < 0.05$ ; ANOVA), compared with sham-operated rats or vehicle-treated rats. NANC relaxation was not significantly different in the muscle strips obtained from capsaicin-treated rats or vagotomized rats, compared with sham-operated rats or vehicle-treated rats. Results were obtained from four to five muscle strips from four to five control rats (sham operated and vehicle treated); five muscle strips from five rats treated with splanchnic ganglionectomy; four muscle strips from four 6-OH-dopamine-treated rats, four muscle strips from four capsaicin-treated rats, and four muscle strips from four vagotomized rats.

( $n = 3$ ,  $104 \pm 11\%$ ), compared with sham-operated rats and vehicle-treated rats (Fig. 6, mean  $\pm$  s.e.m.).

#### Northern blot analysis of NOS mRNA

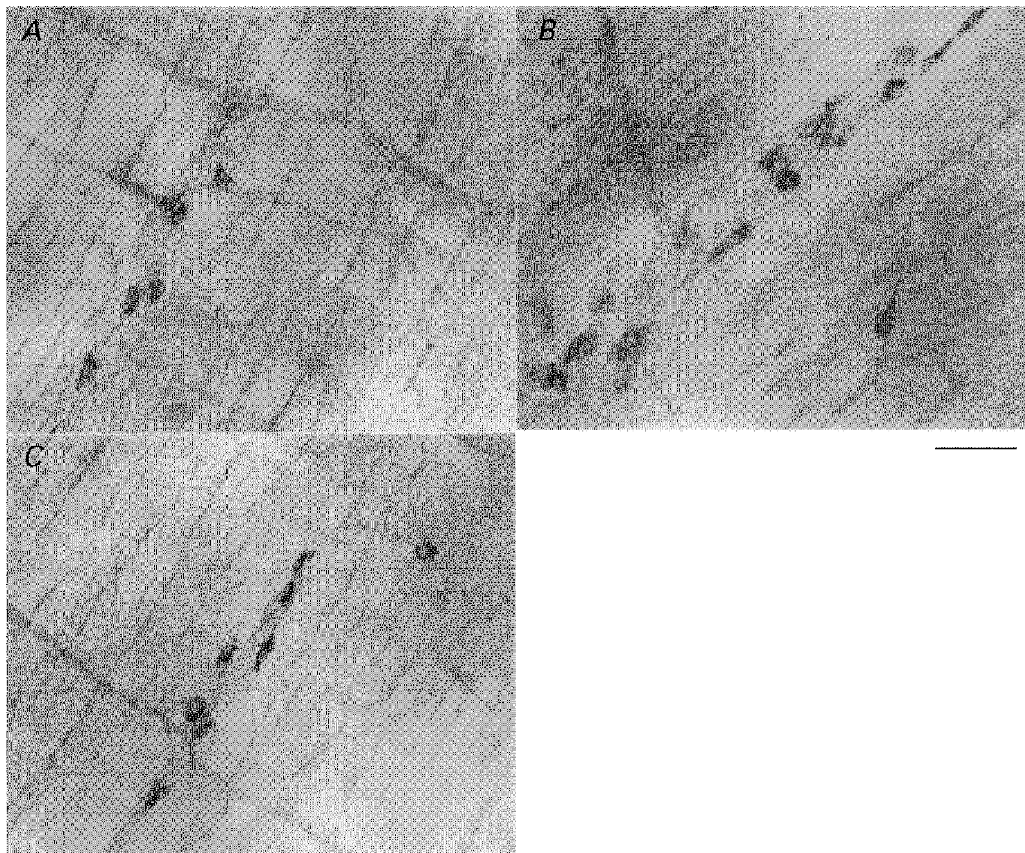
We investigated whether upregulation of NOS mRNA expression enhanced NOS synthesis in the jejunal myenteric plexus in rats treated with splanchnic ganglionectomy. NOS mRNA expression at 9.5 kb was clearly observed in tissue obtained from sham-operated rats. It has been demonstrated that NOS mRNA expression is abundant in rat cerebellum (Bredt *et al.* 1990, 1991*a, b*). The NOS mRNA band obtained from rat jejunal longitudinal muscle corresponded well with the NOS mRNA band from rat cerebellum (data not shown).

The density of NOS mRNA in jejunal tissue obtained from rats treated with splanchnic ganglionectomy was significantly increased to  $243 \pm 58\%$  of that obtained from sham-operated rats. Similarly, the density of NOS mRNA in jejunal tissue obtained from rats treated with 6-OH-dopamine was significantly increased to  $202 \pm 27\%$  of that obtained from vehicle-treated rats (Fig. 8, mean  $\pm$  s.e.m.,  $n = 4$ ,  $P < 0.05$ , Student's *t* test). In contrast, NOS mRNA

expression did not change significantly in the tissues obtained from vagotomized rats ( $101 \pm 12\%$  of control) or capsaicin-treated rats ( $98 \pm 8\%$  of control).

## DISCUSSION

Following transplantation of the small bowel, various morphological and functional changes may occur in the myenteric plexus of the grafted intestine. The absence of extrinsic adrenergic inhibitory innervation has been demonstrated in the graft (Taguchi, Zorychta, Sonnino & Guttman, 1989). Previous studies suggested that the excitatory and inhibitory neural pathways of the graft are affected by transplantation and that the excitatory NANC component becomes dominant after transplantation. It has been proposed that the intramural substance P neuron plays an important role in the excitatory neurotransmission of the graft (Ishii *et al.* 1994; Kusunoki, Ishii, Nakao, Fujiwara, Yamamura & Utsunomiya, 1995). We have recently shown that the inhibitory NANC component is also increased in the graft (Nakao *et al.* 1997). We proposed that these changes in the myenteric plexus of the graft may, in part, be



**Figure 4.** NADPH diaphorase histochemistry of the jejunal myenteric plexus in sham-operated rats (*A*), rats treated with splanchnic ganglionectomy (*B*) and vagotomized rats (*C*)

NADPH diaphorase-positive cells and fibres were clearly seen in the jejunal myenteric plexus of control (sham-operated) rats. The number of NADPH diaphorase-positive cells was significantly increased in rats treated with splanchnic ganglionectomy, but not in vagotomized rats. Scale bar, 50  $\mu$ m.

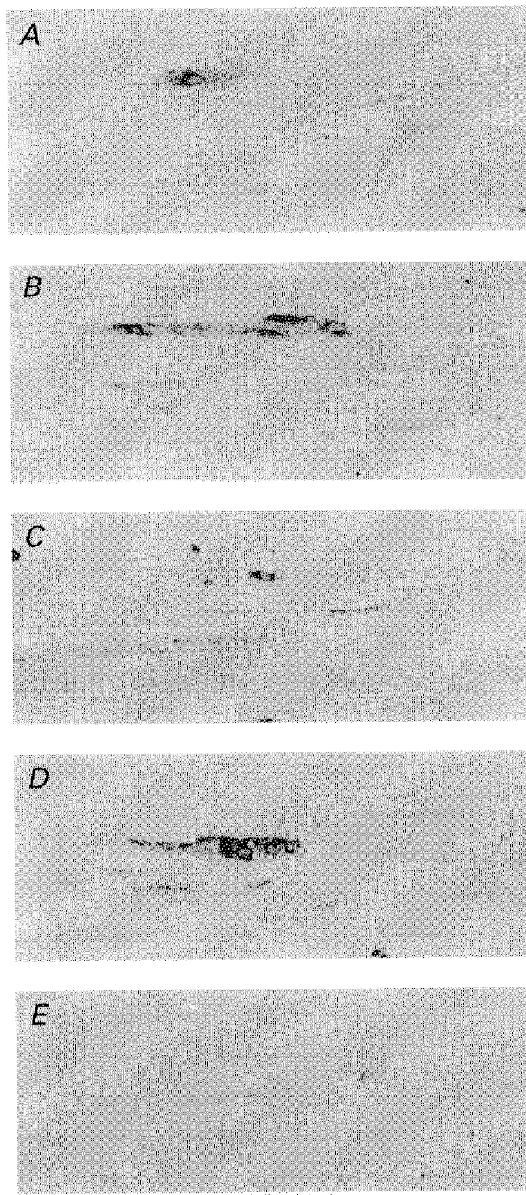
the result of extrinsic denervation of the small intestine. In this study, we investigated the effect of extrinsic denervation on the inhibitory NANC component of the small intestine in rats.

As previously described (Takahashi & Owyang, 1995), NANC relaxation in response to transmural stimulation was significantly antagonized by L-NAME and abolished by tetrodotoxin in muscle strips from rat gastric body, suggesting the mediation by neural release of NO in the gastric myenteric plexus. In our present study, similar results were obtained with longitudinal muscle strips obtained from rat jejunum, indicating the importance of NO as an inhibitory NANC neurotransmitter in the gastrointestinal tract.

We have demonstrated that NANC relaxation was significantly increased in the jejunal longitudinal muscle

strips obtained from rats treated with splanchnic ganglionectomy, compared with sham-operated rats. This increase may be due to increased sensitivity of muscle cells to NO or increased release and synthesis of NO from the myenteric plexus. NO relaxes smooth muscles through a mechanism similar to that of sodium nitroprusside, i.e. by causing an increase in the 3',5'-cyclic guanosine monophosphate (cGMP) content of smooth muscle cells (Marletta, 1993). Jejunal relaxation evoked by exogenously applied sodium nitroprusside was not significantly different in the preparations obtained from rats treated with splanchnic ganglionectomy, compared with sham-operated rats. This suggests that the sensitivity of muscle cells to NO is not affected by splanchnic ganglionectomy.

Splanchnic ganglionectomy interrupts splanchnic afferent fibres, as well as splanchnic efferent fibres. However, NANC relaxation was not significantly affected in the muscle strips



**Figure 5.** NOS immunohistochemistry of the jejunal myenteric plexus in sham-operated rats (*A*), rats treated with splanchnic ganglionectomy (*B*), vagotomized rats (*C*), 6-OH-dopamine-treated rats (*D*) and capsaicin-treated rats (*E*)

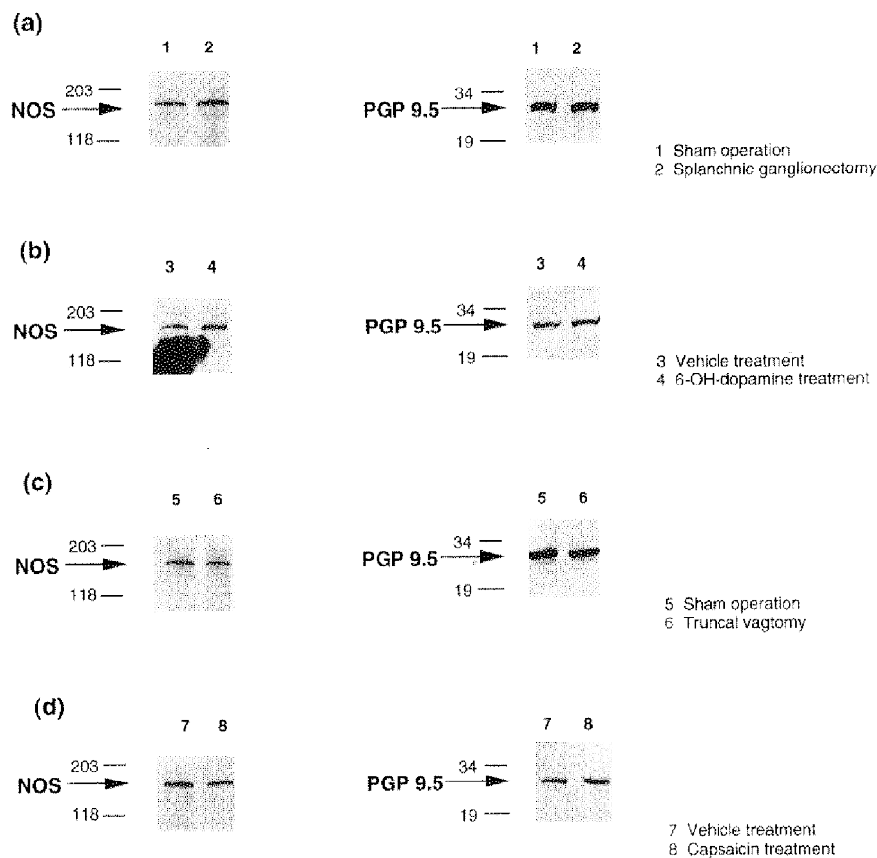
NOS-immunoreactive cells were clearly seen in the myenteric plexus of sham-operated rats. The number of NOS-immunoreactive cells was significantly increased in the tissue of rats treated with splanchnic ganglionectomy or 6-OH-dopamine, but not in rats treated with vagotomy or capsaicin. Scale bar, 100  $\mu$ m.



from rats receiving capsaicin treatment on the coeliac ganglia, compared with vehicle-treated rats. This suggests that splanchnic efferents, rather than splanchnic afferents, play an important role in enhancing NANC relaxation. We have further demonstrated that depletion of catecholamines following 6-OH-dopamine treatment also caused a significant increase in NANC relaxation. In contrast, vagotomy had no effect on NANC relaxation. These observations suggest that the release of catecholamines from the splanchnic efferents is an important factor in modulating the activity of the NO pathway in the jejunal myenteric plexus.

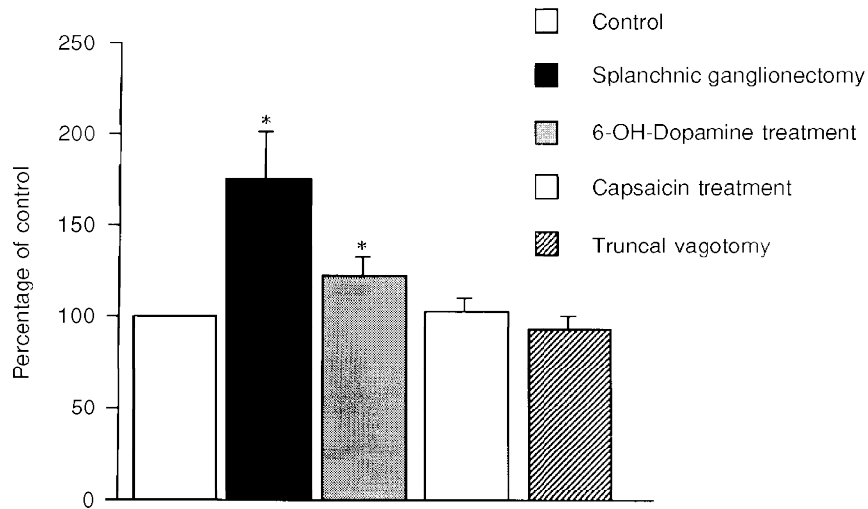
The number of NOS-immunopositive cells and NADPH diaphorase-positive cells in the myenteric plexus was significantly increased in the tissue obtained from rats treated with splanchnic ganglionectomy, but not from vagotomized rats. This strongly indicates that NOS synthesis in the myenteric plexus increased after splanchnic ganglionectomy. This was confirmed by Western blot

analysis, which demonstrated an increase in NOS synthesis after splanchnic ganglionectomy and 6-OH-dopamine treatment, but not after vagotomy or capsaicin-treatment. The increase in NOS synthesis appears to be more pronounced in rats treated with splanchnic ganglionectomy than in those treated with 6-OH-dopamine (Fig. 7). It is possible that 6-OH-dopamine caused incomplete depletion of catecholamines in the group of rats studied, whereas, splanchnic ganglionectomy resulted in complete denervation. In contrast to the increase in NOS synthesis, the density of the PGP 9.5-immunoreactive band was not affected in jejunal tissues obtained from rats treated with vagotomy, splanchnic ganglionectomy, 6-OH-dopamine or capsaicin. Furthermore, the number of PGP 9.5-immunopositive cells in the jejunal myenteric plexus was not affected by splanchnic ganglionectomy and 6-OH-dopamine treatment. This suggests that extrinsic denervation has no effect on the total number of generic neurons in the myenteric plexus of the small intestine.



**Figure 6.** Western blot analysis of NOS and PGP 9.5 in jejunal tissue: sham-operated rats (*a*, lane 1) and rats treated with splanchnic ganglionectomy (*a*, lane 2); vehicle-treated rats (*b*, lane 3) and 6-OH-dopamine-treated rats (*b*, lane 4); sham-operated rats (*c*, lane 5) and vagotomized rats (*c*, lane 6); vehicle-treated rats (*d*, lane 7) and capsaicin-treated rats (*d*, lane 8)

The density of the NOS-immunoreactive band at 155 kDa was significantly increased in jejunal tissue of rats treated with splanchnic ganglionectomy or 6-OH-dopamine, but not in the tissue of vagotomized rats or capsaicin-treated rats. In contrast, the density of the PGP 9.5-immunoreactive band at 27 kDa obtained from the jejunal tissue of rats treated with splanchnic ganglionectomy, 6-OH-dopamine, vagotomy or capsaicin was not significantly different compared with sham-operated rats and vehicle-treated rats.

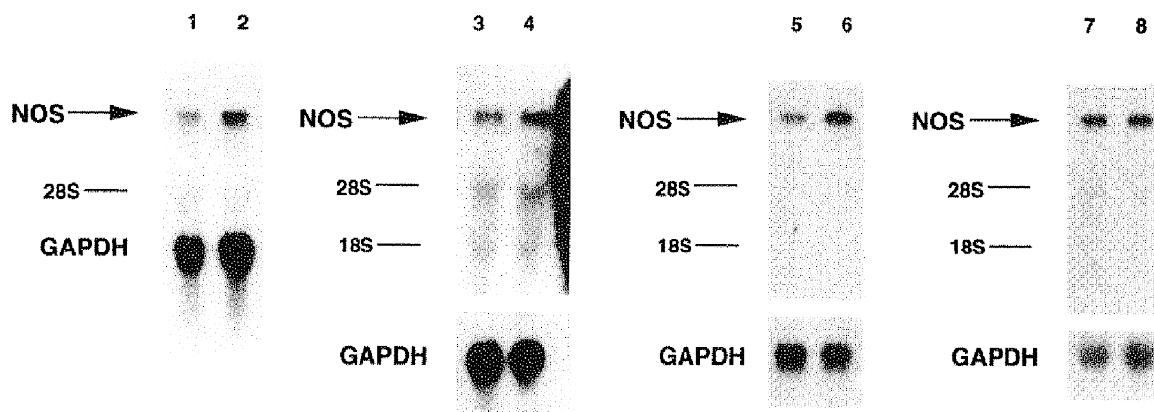


**Figure 7.** The relative density of the NOS-immunoreactive bands obtained from the jejunal tissue of control rats, and from rats treated with splanchnic ganglionectomy, 6-OH-dopamine, capsaicin or vagotomy

The density of the NOS-immunoreactive band was significantly increased to  $161 \pm 28\%$  of sham-operated rats and to  $122 \pm 6\%$  of vehicle-treated rats after splanchnic ganglionectomy ( $n = 6$ ) or 6-OH-dopamine treatment ( $n = 6$ ), respectively, (mean  $\pm$  s.e.m., \* $P < 0.05$ , Student's *t* test).

The increased NOS synthesis in the myenteric plexus following splanchnic ganglionectomy may result from transcription, translation or post-translation. To investigate whether splanchnic ganglionectomy increases gene transcription of NOS in the jejunal myenteric plexus, we

performed Northern blot analysis using cDNA of nNOS. The size of nNOS mRNA and eNOS mRNA has been demonstrated to be 9.5 kb and 4.5 kb, respectively (Forstermann *et al.* 1994). In the present study, we have shown that NOS mRNA expression at 9.5 kb was



**Figure 8.** Northern blot analysis of NOS mRNA in jejunal tissue obtained from sham-operated rats (lane 1), rats treated with splanchnic ganglionectomy (lane 2), sham-operated rats (lane 3), vagotomized rats (lane 4), vehicle-treated rats (lane 5), 6-OH-dopamine-treated rats (lane 6), vehicle-treated rats (lane 7) and capsaicin-treated rats (lane 8)

The density of NOS mRNA in the jejunal tissues obtained from rats treated with splanchnic ganglionectomy was significantly increased to  $243 \pm 58\%$  of that obtained from sham-operated rats ( $n = 4$ ,  $P < 0.05$ , Student's *t* test). However, the density of GAPDH bands was not significantly different between jejunal tissue of sham-operated rats and rats treated with splanchnic ganglionectomy. Similarly, the density of NOS mRNA in the jejunal tissues obtained from 6-OH-dopamine-treated rats was significantly increased to  $202 \pm 27\%$  of that obtained from vehicle-treated rats ( $n = 4$ ,  $P < 0.05$ , Student's *t* test). In contrast, NOS mRNA expression was not significantly changed in the tissues obtained from vagotomized rats ( $n = 4$ ,  $101 \pm 12\%$ ) or capsaicin-treated rats ( $n = 3$ ,  $98 \pm 8\%$ ).

significantly increased in the neuromuscular preparations obtained from rats treated with splanchnic ganglionectomy or 6-OH-dopamine, but not in the preparations obtained from vagotomized rats or rats treated with capsaicin. This indicates that increased NOS synthesis observed in jejunal tissues obtained from rats treated with splanchnic ganglionectomy or 6-OH-dopamine is secondary to increased gene transcription of NOS in the myenteric plexus. This implies that the splanchnic pathway plays an important role in inhibiting NOS mRNA expression in the myenteric plexus.

Yunker & Galligan (1994) demonstrated that the number of NADPH diaphorase-positive cells in the myenteric plexus increased 1 week after surgical extrinsic denervation of guinea-pig ileum. In contrast to our results with rat jejunum, an increased number of NADPH diaphorase-positive cells in the myenteric plexus were also observed after systemic capsaicin treatment, but not after 6-OH-dopamine treatment. This suggests that the loss of primary afferents increased NADPH diaphorase staining in the guinea-pig ileum (Yunker & Galligan, 1994). Therefore control of NOS synthesis may be species specific.

The inhibitory mechanism of splanchnic neuron on NOS mRNA expression remains to be determined. Intracellular  $Ca^{2+}$  is an important factor in gene regulation, including NOS mRNA expression in the myenteric plexus (Nakamura *et al.* 1996). It has been shown that noradrenaline blocks  $Ca^{2+}$  currents by  $\alpha_2$ -adrenoceptor in rat sympathetic neurons (Schofield, 1990). Therefore, it is conceivable that activation of  $\alpha_2$ -adrenoceptor by adrenaline in the myenteric plexus decreases  $Ca^{2+}$  currents, resulting in reduced NOS mRNA expression.

We have previously shown that the vagus nerve controls NOS expression in the rat gastric myenteric plexus. Truncal vagotomy significantly reduced NANC relaxation, NOS synthesis and NOS mRNA expression in gastric tissue (Nakamura *et al.* 1996). In contrast, there were no significant changes observed in NOS expression after truncal vagotomy in rat jejunum in the present study. It has been demonstrated that the distribution of vagus efferent fibres between the stomach and the intestine is significantly different in rats. Ganglia in the myenteric plexus innervated by vagus efferent fibres are more numerous in the stomach than in the small intestine. Innervation of vagus nerve to the ganglia becomes progressively more sparse distally in the small intestine. About 80% of the myenteric neurons in the duodenum are not innervated by vagus efferents (Kirchgessner & Gershon, 1989). This difference in distribution of vagus efferents may explain the differential effects of vagotomy on NOS gene expression in the stomach and small bowel.

In conclusion, we have demonstrated that the splanchnic nerves negatively control NOS expression in the jejunal myenteric plexus by releasing catecholamines in the rat jejunum. In contrast to NOS expression in the stomach, NOS expression in the jejunal myenteric plexus is

independent of the vagus nerve. Extrinsic neural control of NOS expression in the myenteric plexus may play an important role in the regulation of motor function in the small intestine.

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