Virchows Arch (1997) 430:53–61

ORIGINAL ARTICLE

Rishu Guo · Osami Nada · Sachiyo Suita
Tomoaki Taguchi · Kouji Masumoto

The distribution and co-localization of nitric oxide synthase and vasoactive intestinal polypeptide in nerves of the colons with Hirschsprung's disease

Received: 2 April 1996 / Accepted: 18 July 1996

Abstract The distribution and co-localization of nitric oxide synthase (NOS) and vasoactive intestinal polypeptide (VIP) were examined by means of immunohistochemistry and NADPH diaphorase (NADPH-d) histochemistry in the gut of patients with Hirschsprung's disease. In the normoganglionic segment, many nitrergic nerve cells were localized in Auerbach's plexus and nerve fibres were observed preferentially in the circular muscle. The submucosal nitrergic nerve cells were mainly situated in Schabadasch's plexus with occasional cells demonstrable in Meissner's plexus. NOS and VIP were co-localized in most ganglion cells of Auerbach's plexus. In the oligoganglionic segment, a marked reduction of NOS- and VIP- positive nerve cells and fibres was noticed in both the myenteric and submucosal plexuses, and nitrergic fibres had disappeared in the inner layer of the circular muscle. In the aganglionic segment, NOS and VIP were revealed only in extrinsic nerve fasciculi and rami and co-localized in a few fibres. From these observations, the inner layer of the circular muscle of the oligoganglionic segment and the whole of the muscularis propria of the aganglionic segment were considered to be totally lacking in nitrergic innervation. Nitrergic nerves of the human colon comprise both intrinsic and extrinsic elements and the majority of intrinsic nitrergic nerve cells contain VIP. Very low numbers of extrinsic nitrergic fibres contain VIP.

Key words Nitric oxide synthase · NADPH-diaphorase · Vasoactive intestinal polypeptide · Enteric nervous system · Hirschsprung's disease

R. Guo (✉) · S. Suita · T. Taguchi · K. Masumoto
Department of Pediatric Surgery, Faculty of Medicine 60,
Kyushu University, 3-1-1 Maidashi, Higashi-Ku,
Fukuoka 812-82, Japan
Tel.: (81) 92-641-1154, ext. 2483, Fax: (81) 92-632-3003

O. Nada
Department of Medical Technology,
Kyushu University School of Health Sciences,
Fukuoka, Japan

Introduction

Hirschsprung's disease (HD) has been defined pathologically as a congenital absence of the intramural ganglia in the non-peristaltic segment of the bowel. The extrinsic innervation of the gut is disorganized and hyperplastic [23, 24, 45]. Physiological experiments have shown that the lack of non-adrenergic and non-cholinergic (NANC) inhibitory innervation leads to contraction of the affected segment in HD [14, 27]. Immunohistochemical and enzyme-histochemical studies have also revealed that NANC nerves are deficient in the aganglionic bowel with HD. Adenosine 5'-triphosphate (ATP) and vasoactive intestinal polypeptide (VIP) have been proposed as candidates for the neurotransmitter involved in NANC relaxation of smooth muscles in the bowel [6, 15, 19]. It has been suggested that the lack of intrinsic VIP-nergic innervation may be a significant factor in the non-peristaltic state of the dysfunctional bowel [28, 37, 41, 42]. Recently, there is an increasing body of evidence which suggests that nitric oxide (NO) may act as another important NANC neurotransmitter for the mediation of relaxation in the smooth muscle of the gastrointestinal tract [5, 34].

Immunohistochemical assays have elucidated that NO synthase (NOS), an enzyme capable of synthesizing of NO, occurrs in the enteric nervous system of several mammals including humans [8, 13, 17]. Since nitrergic nerve cells have NADPH diaphorase (NADPH-d) activity [10], these nerve cells can also be demonstrated by NADPH-d histochemistry as well as by NOS immunohistochemistry [3, 44, 47]. A considerable body of data is available with regard to the localization of nitrergic nerves in colons affected by HD [2, 9, 32, 43]. Nevertheless, there is little information concerning the nitrergic nerve supply in the oligoganglionic segments of the affected colons. In the present study, the origin and distribution of nitrergic nerves were explored in cases of both short and long segment aganglionosis.

Materials and methods

The bowel specimens were obtained from 12 Japanese children aged 3 months to 10 years, in whom HD was diagnosed using acetylcholinesterase staining. Of these patients 9 cases were short segment aganglionosis and 3 cases were long segment aganglionosis (1 case was total colonic aganglionosis). Two control specimens of colon were obtained from children with anal atresia undergoing a closure of colostomy. The resected gut was immediately flushed with 0.01 M phosphate-buffered saline (PBS, pH 7.4) and opened along the mesenteric border, slightly stretched and pinned onto balsa wood. All tissue samples were fixed in PLP fixative [31] for 2 h at 4° C and washed in 0.05 M phosphate buffer (PB, pH 7.3) containing 7% sucrose, cut into small pieces (about 2×1 cm) and then left overnight at 4° C. After being immersed in PB containing 15% sucrose for at least 1 h, they were further cut into small blocks (about 1×1 cm) and embedded in an embedding matrix (Lipshaw, Pittsburgh, Pa., USA). Cryostat sections of 14 µm thickness were cut, placed onto gelatine-coated glass slides and then air-dried for 10 min with an electric fan. Whole-mount preparations were made according to the method of Costa et al. [7].

The sections were stained by the immunofluorescence technique using biotin-streptavidin-fluorescein isothiocyanate (FITC; Vector Laboratories, Calif., USA) and -Texas red complex (Molecular Probes, Eugene, Ore., USA). The cryostat sections were incubated for 30 min with 10% normal goat serum in a humid box. Excess normal serum was blotted and the sections were incubated overnight at 4° C with primary antiserum to human brain NOS (bNOS), VIP or protein gene product (PGP) 9.5 (Table 1). The sections were washed twice for 10 min each in PBS and incubated for 1 h at room temperature with biotinylated goat anti-rabbit IgG antiserum (Vector), washed in PBS and incubated for 30 min at room temperature with streptavidin-FITC (0.5 µg/ml). After washing, the sections were mounted with a mounting medium for fluorescence microscopy (Vector). Double immunostaining for NOS and VIP was performed by use of rabbit anti-bNOS and guinea-pig anti-VIP antisera. The preparations were preincubated for 30 min with 10% normal goat serum and succesively incubated overnight at 4° C with rabbit anti-bNOS antiserum. They were washed in PBS, then incubated for 30 min with biotinylated goat anti-rabbit IgG antiserum, washed in PBS, incubated for 30 min with Texas red-streptavidin (0.5 µg/ml), then washed in PBS. Subsequently, the same preparations were treated for 30 min with 10% normal goat serum, incubated overnight at 4° C with guinea-pig anti-VIP antiserum, washed in PBS, and then incubated for 30 min with fluorescein-conjugated goat anti-rabbit IgG antiserum (5 µg/ml, Vector). After being washed in PBS and air-dried, they were mounted with a mounting medium. The preparations were examined and photographed under a Zeiss fluorescence microscope (Axioskop 50, Germany) equipped with an epi-illumination system.

NADPH-d activity was demonstrated according to the method of Scherer-Singler et al. [35]. Briefly, cryostat sections and whole-mount preparations were incubated in a solution containing 0.25 mg/ml nitroblue tetrazolium, 1 mg/ml β-NADPH and 0.5% Triton X-100 in 0.1 M TRIS-hydrochloric acid buffer (pH 7.6) for 10–30 min at 37° C.

For combined NOS and NADPH-d, or VIP and NADPH-d staining, cryostat sections were immunostained for NOS or VIP, and then photographed. Subsequently, covers slips were carefully removed and sections were incubated for NADPH-d staining. The same area photographed for fluorescence microscopy was examined and rephotographed under either a Nomarski optics or fluorescence microscope.

Results

The observations on short and long segment aganglionosis differed in a few respects, although essentially they were similar. The following descriptions were made from the diseased colons in short segment aganglionosis, except where otherwise stated. The results obtained are summarized in Table 2.

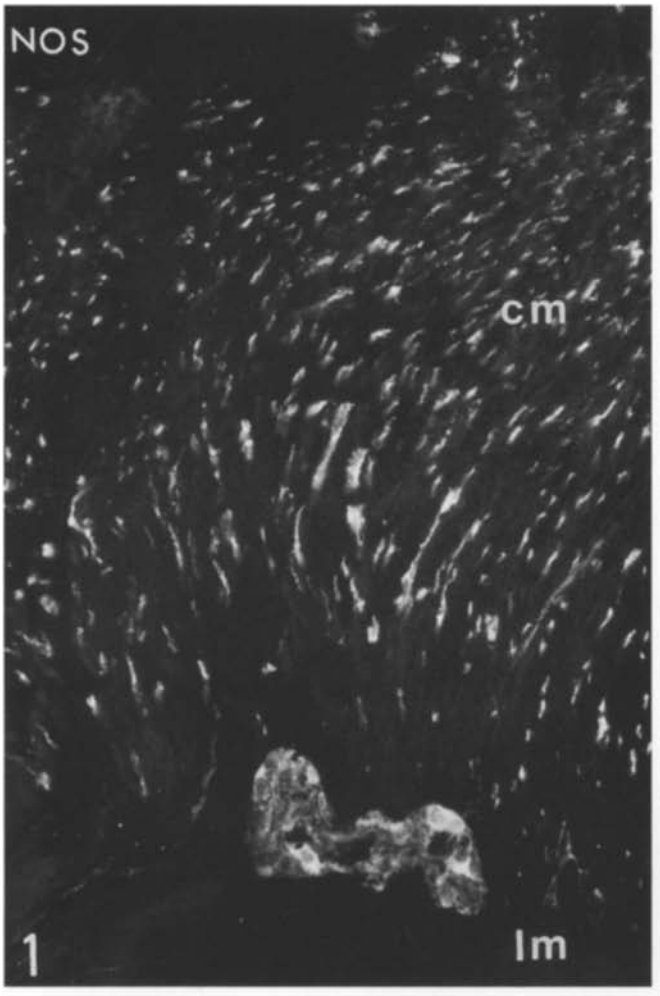
The distribution pattern of NOS-containing nerves in the normoganglionic segments was indistinguishable from that in controls. Numerous nerve cell bodies and fibres immunoreactive for NOS were localized in Auerbach's plexus, while they were much less numerous in the submucosal plexus. In the submucosal plexus, the positive cell bodies were mainly located in Schabadash's plexus (in the outer layer of submucosa) and infrequent in Meissner's plexus (in the inner layer of submucosa). NOS-positive nerve fibres were found exclusively in the circular muscle and were aligned parallel with muscle fibres. A scarce supply of NOS-positive nerve fibres was observed in the longitudinal muscle (Fig. 1). No NOS-

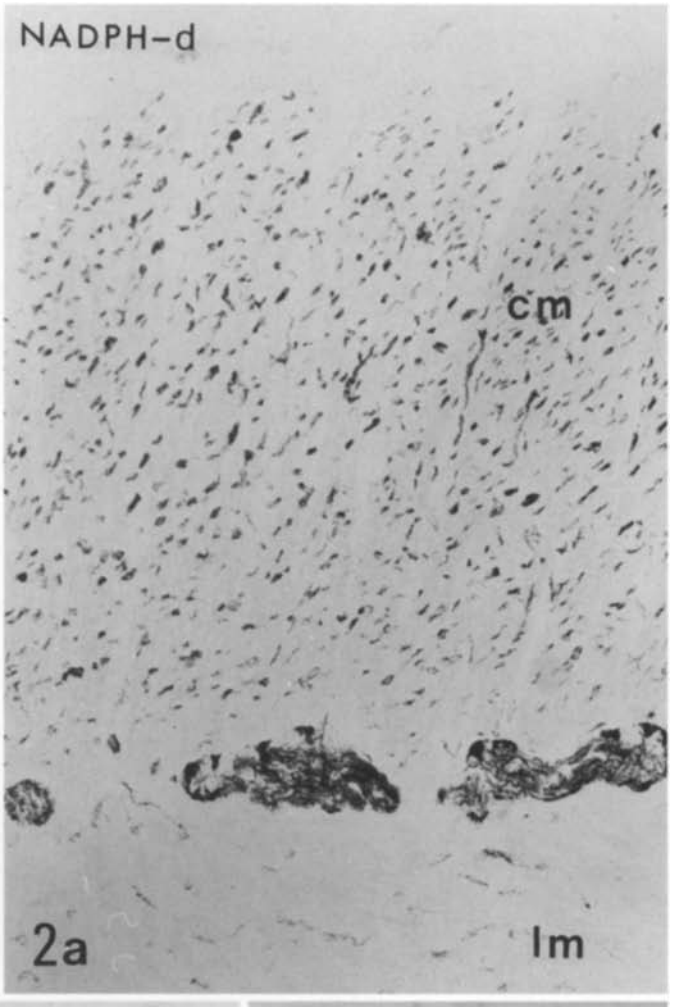
Table 1 Details of antisera used for single or double immunostaining

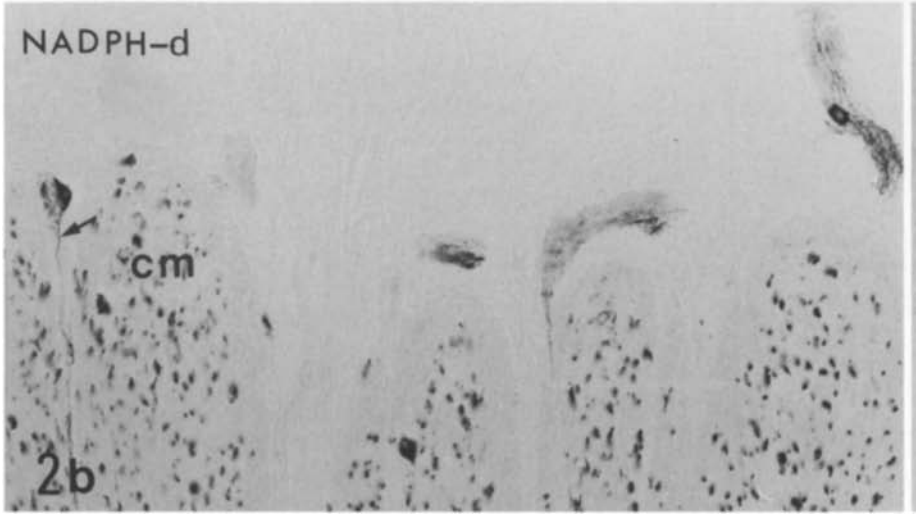
Antigen	Host species	Code number	Dilution	Source (reference)
Human brain nitric oxide synthase (bNOS)	Rabbit	N31020	1:100	Transduction Laboratories, USA
Vasoactive intestinal polypeptide (VIP)	Rabbit	R502	1:2000	Yanaihara et al. [46]
VIP	Guinea-pig	029301-2	1:1000	Peninsula Laboratories, USA
Protein gene product 9.5 (PGPg.5)	Rabbit	RA95101	1:4000	Ultraclone Limited, UK

Table 2 Localization of NOS (NADPH-diaphorase) and VIP in the colons of patients with Hirschsprung's disease (*MM* muscularis mucosae, *SP* submucosal plexus, *CM* circular muscle, *MP* myenteric plexus, *LM* longitudinal muscle, *e* extrinsic abnormal nerve fasciculi, *im* inner layer of circular muscle, – no fibres, ± few fibres, + moderate fibres, ++ numerous fibres)

	Normoganglionic		Oligoganglionic				Aganglionic	
			short type		long type			
	NOS	VIP	NOS	VIP	NOS	VIP	NOS	VIP
MM	–	+	±	+	–	–	±	±
SP	+	++	±	+	–	–	+ (e)	+ (e)
CM (im)	++	++	+(–)	+(±)	+(–)	+(–)	–	±
MP	++	++	+	+	+	+	+ (e)	+ (e)
LM	+	+	±	±	±	±	–	±







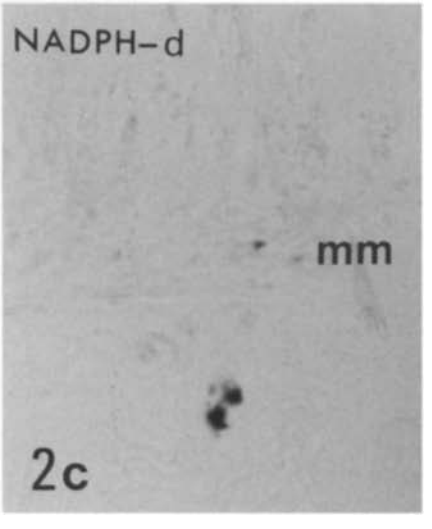
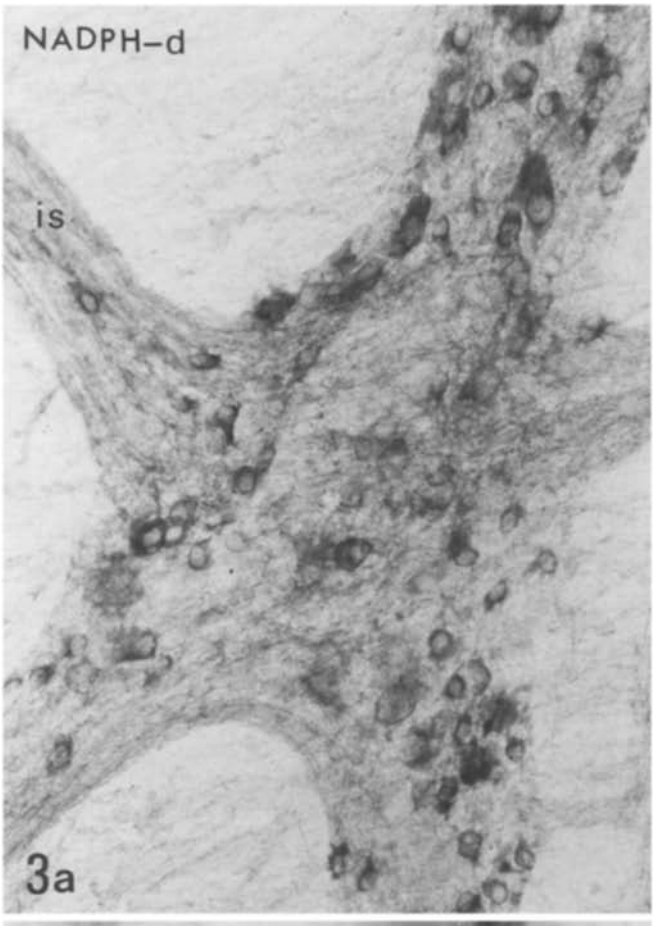


Fig. 1 Fluorescence micrograph of normoganglionic segment, showing nitric oxide synthase (NOS)-like immunoreactivity in nerve cells and fibres. Several nerve cells in Auerbach's plexus are found to be immunopositive for NOS. The circular muscle has a dense nerve supply of positive fibres throughout the entire thickness (*cm* circular muscle, *lm* longitudinal muscle). ×200

Fig. 2a–c Light micrographs of normoganglionic segment, showing nicotinamide adenine dinucleotide phosphate, reduced-diaphorase (NADPH-d) activity in enteric nerve cells and nerve fibres. **a** Positive nerve cells are located in Auerbach's plexus and numerous fibres are evenly distributed in the circular muscle. **b** Positive nerve cells in Schabadash's plexus. Note that an elongated axon process (*arrow*) is extending into the circular muscle. **c** Positive nerve cells in Meissner's plexus (*mm* muscularis mucosae). ×200



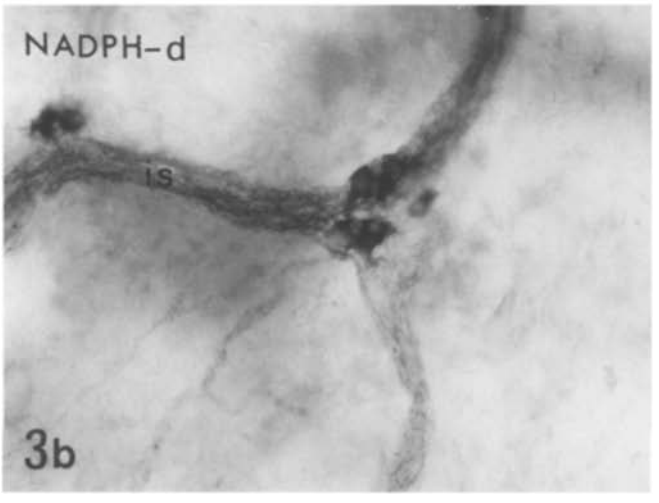
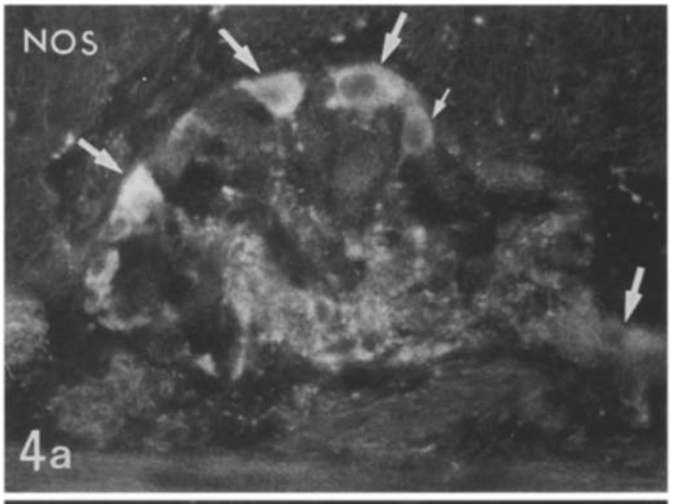


Fig. 3a, b Light micrographs of normoganglionic segment, showing NADPH-d staining in whole-mount preparations. **a** Myenteric plexus. **b** Submucosal plexus (*is* internodal strand). ×400



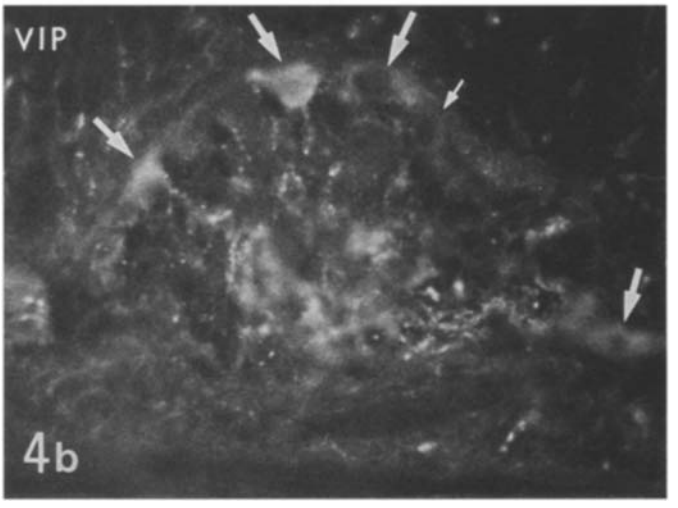
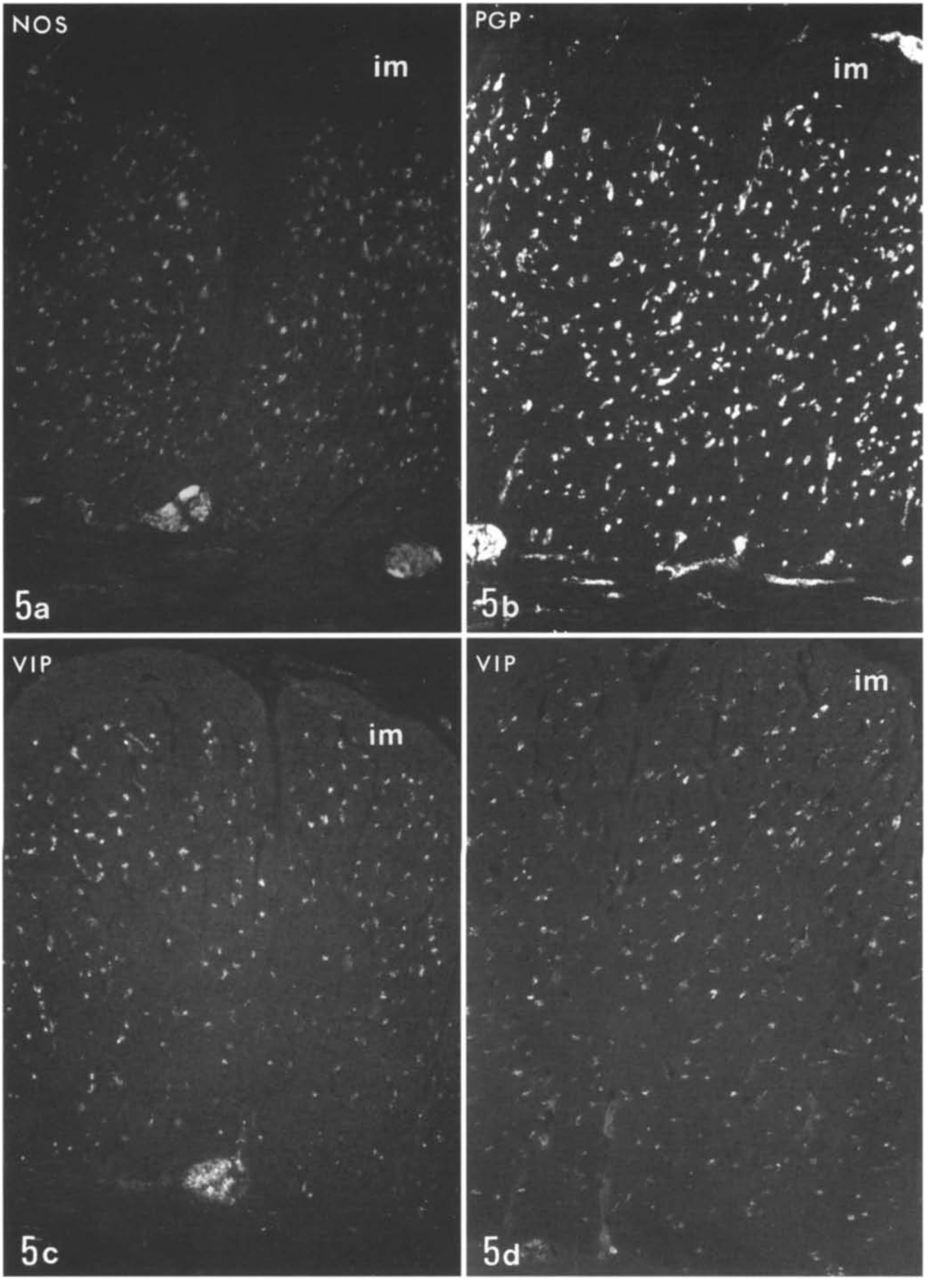
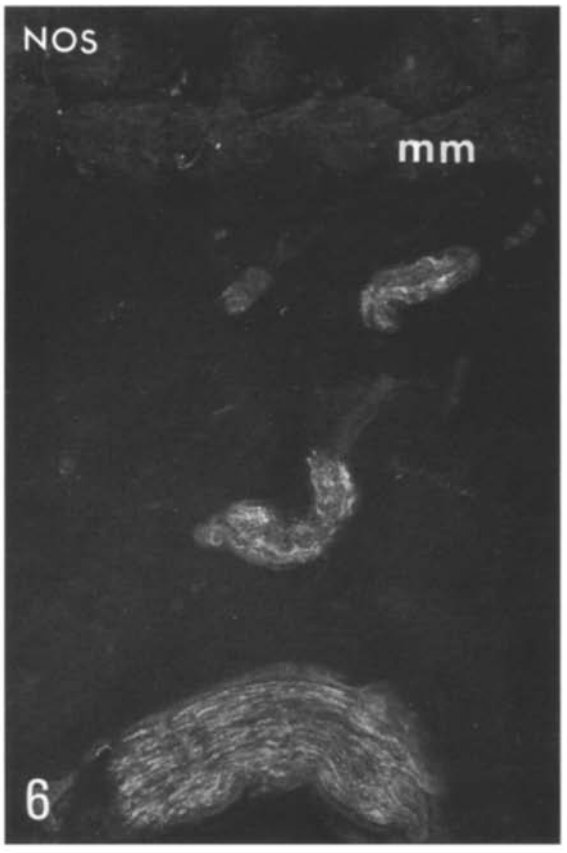


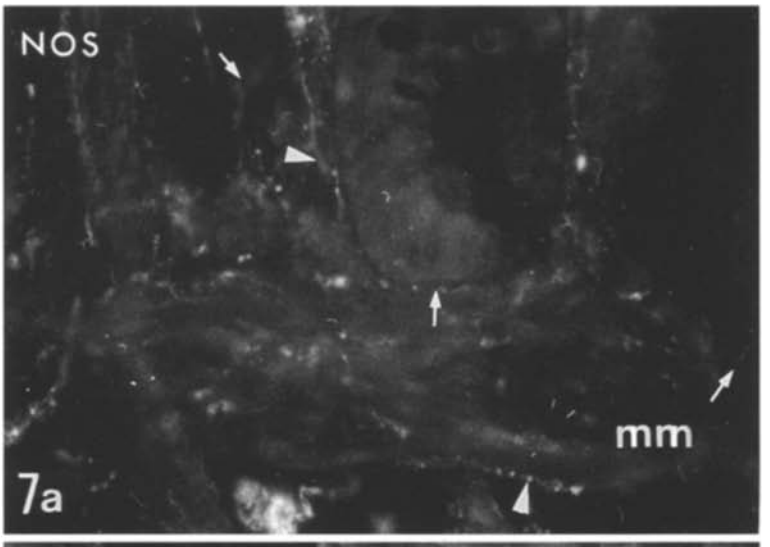
Fig. 4a, b Fluorescence micrographs of normoganglionic segment, showing double immunostaining for NOS and vasoactive intestinal polypeptide (VIP) in the myenteric plexus. **a** NOS-like immunoreactivity. **b** VIP-like immunoreactivity. Some nerve cells (*large arrows*) are immunoreactive for NOS and VIP, while others (*small arrows*) are positive for only NOS. ×400

positive fibres were demonstrable in either the muscularis mucosae or the lamina propria. NADPH-d staining revealed a distribution pattern similar to that of NOS immunostaining (Fig. 2a–c). The nerve cells and fibres positive for NADPH-d were identical to those positive for NOS, as shown by combined NOS and NADPH-d staining. In whole-mount preparations of the longitudinal muscle with Auerbach's plexus, NADPH-d activity occurred in perikarya and nerve fibres. The positive cells often appeared in clusters and had multiple, short lamellar processes, which are characteristic of the Dogiel type I. The internodal strands with varicosities also labelled for NADPH-d and were found to form meshworks (Fig. 3a). A small number of positive nerve cells were scattered in the submucosal plexus and formed smaller

Fig. 5a–d Fluorescence micrographs of the circular musculature of the oligoganglionic segment. **a** NOS-, **b** PGP 9.5- and **c** VIP-like immunoreactivites in long segment aganglionosis. **d** VIP-like immunoreactive nerve fibres in short segment aganglionosis. The three kinds of immunostaining fibres are different in density. Note that no nerve fibres are found in the inner layer of the circular muscle in long segment aganglionosis (*im* inner layer of the circular muscle). ×200


NOS
im
5a
PGP
im
5b
VIP
im
5c
VIP
im
5d





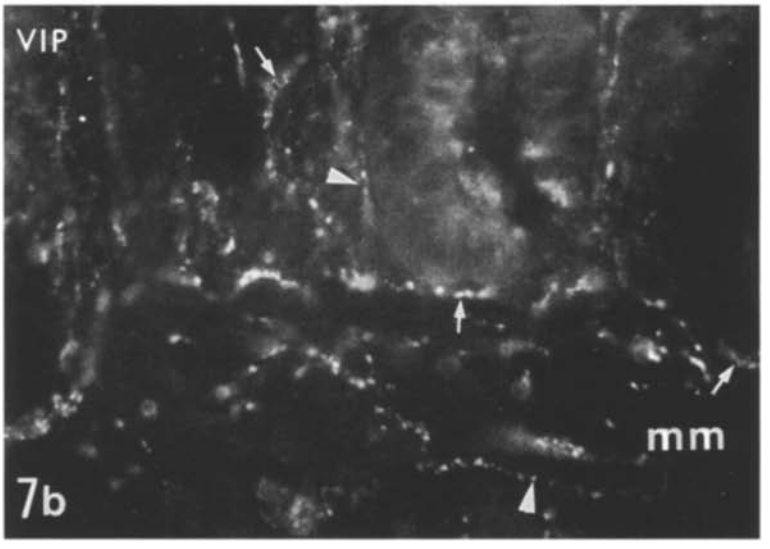


Fig. 6 Fluorescence micrograph of aganglionic segment showing NOS-like immunoreactivity in hypertrophic nerve bundles. Extrinsic nerve bundles in the submucosal connective tissue consist of a large number of NOS-like immunoreactive fibres. ×400

Fig. 7a, b Fluorescence micrographs of aganglionic segment showing double immunostaining for NOS and VIP in extrinsic small nerve bundles. **a** NOS-like immunoreactivity in the small nerve bundles of the lamina propria and muscularis mucosae. **b** VIP-like immunoreactivity in the same small nerve bundles. Some nerve fibres (*arrowheads*) are positive for both NOS and VIP and others (*arrows*) are reactive for only VIP. ×400

meshworks (Fig. 3b). These cells had the Dogiel type I morphology similar to those of Auerbach's plexus.

VIP-positive nerve cell bodies were found not only in Auerbach's plexus, but also in Schabadasch's and Meissner's plexuses of the submucosa. VIP-positive nerve fibres were numerous within the circular muscle, but sparse within the longitudinal muscle.

The co-localization of NOS- and VIP-immunoreactivity was confirmed by NOS/VIP double staining. In Auerbach's plexus, most of the NOS-positive nerve cells were found to contain VIP and only a few cells were immunopositive for either NOS or VIP (Fig. 4a, b). The vast majority of intramuscular nerve fibres were labelled by both NOS and VIP antisera. However, no doubly labelled cells were seen in the submucosal plexus.

In the oligoganglionic segment of colon the size of the myenteric and submucosal plexuses was smaller than that of control plexuses. The individual nerve cells were reduced in number. The number of NOS-positive nerve cells was apparently reduced in Auerbach's plexus, whereas they were hardly ever observed in Schabadash's or Meissner's plexuses. The intramuscular innervation was also decreased and disordered. The co-localization of NOS and VIP was found in a subpopulation of nerve cells of Auerbach's plexus and in the intramuscular nerve fibres. The co-localization patterns were comparable with that in the normoganglionic segment. In long segment aganglionosis, the inner layer of the circular muscle was devoid of nerve fibres positive for NOS, PGP 9.5 and VIP (Fig. 5a–c), while the other part of the circular muscle showed fewer of those nerve fibres. In short segment aganglionosis, the inner layer of the circular muscle had no NOS-positive fibres, but had a few nerve fibres positive for PGP 9.5 and VIP (Fig. 5d).

In the aganglionic segment of colon neither NOS nor VIP cell bodies were detected in the myenteric or submucosal plexuses. However, NOS and VIP immunoreactivity was seen within the extrinsic, hypertrophic nerve bundles and their rami, which are often situated in the intermuscular zone between the circular and longitudinal muscle layers. Small nerve bundles containing NOS and VIP were found consistently in the submucosal connective tissue and occasional fibres penetrated through the muscularis mucosae into the lamina propria (Fig. 6, 7a). NOS and VIP were found to co-exist to a lesser extent in a subpopulation of extrinsic nerve fibres (Fig. 7a, b). In addition, a few VIP-positive fibres were present within the muscularis propria. In contrast, there were no NOS-positive fibres within both the muscle layers.

In whole-mount preparations, the meshwork form of Auerbach's and submucosal plexuses was no longer detectable by NADPH-d staining. Instead, the extrinsic nerve bundles with a snake fence appearance were situated in the intermuscular and submucosal layers and were intensely stained by NADPH-d reaction.

Discussion

Motility of the gastrointestinal tract is directly controlled by the enteric inhibitory and excitatory motor neurons that innervate the smooth muscle layers. NANC nerves have been widely accepted to be the intrinsic inhibitory nerves regulating the motility of the gastrointestinal tract in several animals and man [16, 20, 40]. HD has been characterized by the functional obstruction of the bowel due to the defect of NANC inhibitory innervation in the aganglionic segment [14, 27, 29]. Besides VIP, recent works indicated that another putative NANC inhibitory neurotransmitter, NO, plays an important role in nerve-mediated relaxation of enteric smooth muscle [5, 34]. Physiological and histochemical studies have demonstrated that nitrergic nerves have a widespread distribution in the gut of mammalian species, including humans [17, 26]. It has also been shown that nitrergic nerves are densely present in the colon of the normoganglionic segment of HD, but almost completely lacking in the aganglionic segment [2, 32]. Therefore, it seems likely that the deficiency of nitrergic nerves causes the functional obstruction of the aganglionic segment.

Double staining for NOS immunohistochemistry and NADPH-d histochemistry showed that the two kinds of staining for nitrergic innervation were entirely superimposable in the human colon, as has been demonstrated in several animal experiments [3, 44, 47]. Therefore, the distribution of the intramural nitrergic nerves has been examined by using either NOS immunohistochemical or NADPH-d histochemical staining.

In the normoganglionic segment, the majority of nitrergic nerve cells were found in Auerbach's and Schabadasch's plexuses and occasionally in Meissner's plexus. The positive nerve fibres were more abundant in the circular muscle layer than in the longitudinal one. These findings consisted with those of previous reports [2, 32].

There has, as yet, been little information concerning the pattern of nitrergic innervation in the oligoganglionic segment [43]. NOS-containing nerve cells within Auerbach's and Schabadash's plexuses were reduced in number when compared with those of the normal colon. Moreover, the axons extending from nitrergic nerve cells were also decreased, and whole-mount preparations elucidated that the positive internodal strands were in a state of disorder. The density of nitrergic fibres was sparse in the circular muscle layer of the oligoganglionic segment. In the normal colon, the circular muscle layer is dually innervated by motor neurons derived from both Auerbach's and Schabadash's plexuses, and the nerve supply to the inner layer of the circular muscle mainly originates from Schabadasch's plexus [11, 18, 39, 40]. This characteristic innervation may imply that the inner layer of the circular muscle plays a key mediating role during peristalsis. It is noteworthy here that nitrergic fibres had totally disappeared in the inner layer of the circular muscle of the oligoganglionic segment. The deficient innervation in the inner layer of the circular muscle might account for the failure of circular muscle relaxation.

Several investigators have reported that the spontaneous oscillating contractile activity in the circular muscle is controlled in part by slow waves, which originate from a group of pacemaker cells situated along the submucosal surface of the circular muscle [22, 36]. This area is densely innervated by nitrergic nerve fibres [44]. The intersitial cells of Cajal (ICCs) have been suggested to be pacemaker cells in the intestinal musculature of several mammals, including humans [4, 12]. Ultrastructural observations also indicated that ICCs are present between the two smooth muscle layers as well as in the inner layer of the circular muscle, where they form a network and have a close contact with nerve terminals and smooth muscle cells [33, 48]. In the colon affected by HD, it is assumed that the lack of nitrergic nerve fibres in the inner layer of the circular muscle influences the activity of ICCs and/or the nerve-ICC junctions. In our unpublished immunohistochemical study ICCs positive for anti-human c-kit antiserum have disappeared from the inner layer of the circular muscle of the oligoganglionic segment. The absence of ICCs might be in relation with denervation of nitrergic fibres. Therefore, the functional obstruction of the colon in HD might occur in the oligoganglionic segment, although it has not yet been documented by pathophysiological examination.

NO is not the only substance involved in the nerve-mediated inhibition in the human enteric nervous system. VIP has been established as another putative NANC neurotransmitter [19]. Both NO and VIP have been believed to be important in the smooth muscle relaxation of gut. Grider et al. [20] stated that rat colonic relaxation may be caused by the combined effects of VIP and NO on smooth muscle cells, and that VIP release is enhanced by NO. In the guinea-pig gastric fundus, VIP release stimulates NO production in target smooth muscle cells [21]. Costa et al.

[8] revealed that NOS in the small and large intestines of the guinea-pig is completely co-localized with VIP. However, there is only limited co-localization of NOS with VIP in the intestines of the rat and toad [1, 30] and the degree of co-localization of NO and VIP in enteric nerves clearly varies. Bealer et al. [2] reported that an entire peptidergic-NANC inhibitory axis (VIP-NO) might be present in the normal human bowel. It seems plausible that the normal motility of the human colon is dependent on the interplay between VIP and NO [2]. It our study, double immunostaining for NOS and VIP showed that most NOS-positive nerve cells and fibres contained VIP, but a few VIP-positive cells did not contain NOS in control and normoganglionic colons. This means that the majority of intrinsic nitrergic nerve cell bodies and fibres are co-localized with VIP in the human colon.

Neither VIP-positive nor NOS-positive fibres were localized in the inner layer of the circular muscle of the oligoganglionic segment in cases of long segment aganglionosis. In contrast, only VIP-positive fibres were found in this layer in cases of short segment aganglionosis. The immunoreactivity for VIP was elucidated in the extrinsic nerve bundles and their rami, which are often found in the submucosal connective tissue [37]. Thus, it seems reasonable to consider that VIP-positive fibres within the inner layer of the circular muscle might originate from the extrinsic nerve bundles. Previous studies have indicated that the distribution of extrinsic nerves is different between short and long segment aganglionosis [38]. According to Kawana et al. [25], the extrinsic nerve bundles in short segment aganglionisis can reach the oligoganglionic parts of the colon, and their branches are distributed in the circular muscle layer of the proximal aganglionic and oligoganglionic parts. The extrinsic nerve bundles in long segment aganglionsis are not seen in the oligoganglionic part.

In the aganglionic segments of the colon, both NOS- and VIP-containing fibres were found in association with the extrinsic hypertrophic nerve fasciculi situated in the submucous connective tissue and the intermuscular zone, where NOS and VIP were co-localized in occasional fibres of the nerve fasciculi. A few NOS- and VIP-positive fibres were situated in the circular muscle layer of this segment. Kubota et al. [27] reported that the contraction of the aganglionic segment is induced by electrical stimulation, but is not followed by relaxation. Tsuto et al. [42] have shown that VIP-immunoreactive synaptic contacts with other neuronal components are lacking in the aganglionic segment. It seems reasonable to consider that extrinsic nitrergic and VIP-nergic nerves do not affect the motility of the dysfunctional bowel, even if the two transmitters substances are released.

Acknowledgements The authors would like to thank Drs. H. Ohgami, A. Nagasaki, K. Yamanaka and R. Hirose, Department of Surgery, Fukuoka Children's Hospital for the operative material and Drs. M. Kubota and T. Kamimura in the Department of Pediatric Surgery, Faculty of Medicine, Kyushu University for valuable advice. This work was supported in part by a Grant-in-Aid for General Scientific Research (C) and a Grant for Pediatric Research (6C-02) from the Ministry of Health and Welfare, Japan.

References

1. Aimi Y, Kimura H, Kinoshita T, Minami Y, Fujimura M, Vincent SR (1993) Histochemical localization of nitric oxide synthase in rat enteric nervous system. Neuroscience 53:553–560
2. Bealer JF, Natuzzi ES, Buscher C, Ursell PC, Flake AW, Adzick NS, Harrison MR (1994) Nitric oxide synthase is deficient in the aganglionic colon of patients with Hirschsprung's disease. Pediatrics 93:647–651
3. Belai A, Schmidt HHHW, Hoyle CHW, Hassall CJS, Saffrey MJ, Moss J, Förstermann U, Murad F, Burnstock G (1992) Colocalization of nitric oxide synthase and NADPH-diaphorase in the myenteric plexus of the rat gut. Neurosci Lett 143: 60–64
4. Berezin I, Huizinga JD, Daniel EE (1988) Interstitial cells of Cajal in the canine colon: A special communication network at the inner border of the circular muscle. J Comp Neurol 273: 42–51
5. Bult H, Boeckxstaens GE, Pelckmans PA, Jordaens FH, van Maercke Y, Herman AG (1990) Nitric oxide as an inhibitory non-adrenergic non-cholinergic neurotransmitter. Nature 345: 346–347
6. Burnstock G, Campbell G, Satchell D, Smythe A (1970) Evidence that adenosine triphosphate or a related nucleotide is the transmitter substance released by non-adrenergic inhibitory nerves in the gut. Br J Pharmacol Chemoter 40:668–688
7. Costa M, Buffa R, Furness JB, Solcia E (1980) Immunohistochemical localization of polypeptides in peripheral autonomic nerves using whole mount preparations. Histochemistry 65: 157–165
8. Costa M, Furness JB, Pompolo S, Brookes SJH, Bornstein JC, Bredt DS, Snyder SH (1992) Projections and chemical coding of neurons with immunoreactivity for nitric oxide synthase in the guinea-pig small intestine. Neurosci Lett 148:121–125
9. Cuffari C, Rubin SZ, Krantis A (1993) Routine use of the nitric oxide stain in the differential diagnosis of Hirschsprung's disease. J Pediatr Surg 28:1202–1204
10. Dawson TM, Bredt DS, Fotuhi M, Hwang PM, Snyder SH (1991) Nitric oxide synthase and neuronal NADPH diaphorase are identical in brain and peripheral tissues. Proc Natl Acad Sci USA 88:7797–7801
11. Domoto T, Bishop AE, Oki M, Polak JM (1990) An in vitro study of the projections of enteric vasoactive intestinal polypeptide-immunoreactive neurons in the human colon. Gastroenterology 98:819–827
12. Faussone-Pellegrini MS, Pantalone D, Cortesini C (1990) Smooth muscle cells, interstitial cells of Cajal and myenteric plexus interrelationships in the human colon. Acta Anat (Basel) 139:31–44
13. Faussone-Pellegrini MS, Bacci S, Pantalone D, Cortesini C, Mayer B (1994) Nitric oxide synthase immunoreactivity in the human ileocecal region. Neurosci Lett 170:261–265
14. Frigo GM, Del Tacca M, Lecchini S, Crema A (1973) Some observations on the intrinsic nervous mechanism in Hirschsprung's disease. Gut 14:35–40
15. Furness JB, Costa M (1982) Enteric inhibitory nerves and VIP. In: Said SI (ed) Vasoactive intestinal peptide. Raven Press, New York, pp 391–406
16. Furness JB, Costa M (1987) Influence of the enteric nervous system on motility. In: Furness JB, Costa M. The enteric nervous system. New York, Churchill Livingstone, pp 137–189
17. Furness JB, Li ZS, Young HM, Forstermann U (1994) Nitric oxide synthase in the enteric nervous system of the guinea-pig: A quantitative description. Cell Tissue Res 277:139–149
18. Gabella G (1979) Innervation of the muscularis externa. In: Bourne GH, Danielli JF (eds) Innervation of the gastrointestinal tract. Int Rev Cytol 59:129–193
19. Goyal RK, Rattan S, Said SI (1980) VIP as a possible neurotransmitter of non-cholinergic non-adrenergic inhibitory neurones. Nature 288:378–380
20. Grider JR (1993) Interplay of VIP and nitric oxide in regulation of the descending relaxation phase of peristalsis. Am J Physiol 264:G334–G340

21. Grider JR, Murthy KS, Jin JG, Makheouf GM (1992) Stimulation of nitric oxide from muscle cell by VIP: prejunctional enhancement of VIP release. Am J Physiol 262:G774–G778
22. Hara Y, Kubota M, Szurszewski JH (1986) Electrophysiology of smooth muscle of the small intestine of some mammals. J Physiol (Lond) 372:501–520
23. Howard ER (1973) Histochemistry in the diagnosis and investigation of congenital aganglionosis (Hirschsprung's disease). Am Surg 39:602–607
24. Kamijo K, Hiatt RB, Koelle GB (1953) Congenital megacolon. A comparison of the spastic and hypertrophied segments with respect to cholinesterase activities and sensitivities to acetylcholine, DFP and the Barium ion. Gastroenterology 24: 173–185
25. Kawana T, Nada O, Ikeda K, Goto S, Hirose R, Taguchi T, Kubota M, Toyohara T, Shono T (1989) Distribution and localization of glial fibrillary acidic protein in colons affected by Hirschsprung's disease. J Pediatr Surg 24:448–452
26. Keef KD, Du C, Ward SM, Mc Gregor DB, Sanders KM (1993) Enteric inhibitory neural regulation of human colonic circular muscle: Role of nitric oxide. Gastroenterology 105: 1009–1016
27. Kubota M, Ito Y, Ikeda K (1983) Membrane properties and innervation of smooth muscle cells in Hirschsprung's disease. Am J Physiol 244:G406–G415
28. Larsson LT, Malmfors G, Sundler F (1983) Peptidergic innervation in Hirschsprung's disease. Z Kinderchir 38:301–304
29. Larsson LT, Malmfors G, Wahlestedt C, Leander S, Håkanson R (1987) Hirschsprung's Disease: A comparison of the nervous control of ganglionic and aganglionic smooth muscle in vitro. J Pediatr Surg 22:431–435
30. Li ZS, Murphy S, Furness JB, Young HM, Campbell G (1993) Relationships between nitric oxide synthase, vasoactive intestinal peptide and substance P immunoreactivities in neurons of the amphibian intestine. J Auton Nerv Syst 44:197–206
31. Nakane PK, Barry Pierce G (1966) Enzyme-labeled antibodies: Preparation and application for the localization of antigen. J Histochem Cytochem 14:929–931
32. O'Kelly TJ, Davies JR, Tam PKH, Brading AF, Mortensen NJMC (1994) Abnormalities of nitric oxide producing neurons in Hirschsprung's disease: Morphology and implications. J Pediatr Surg 29:294–300
33. Rumessen JJ, Mikkelsen HB, Qvortrup K, Thuneberg L (1993) Ultrastructure of interstitial cells of Cajal in circular muscle of human small intestine. Gastroenterology 104:343–350
34. Sanders KM, Ward SM (1992) Nitric oxide as a mediator of nonadrenergic noncholinergic neurotransmission. Am J Physiol 262:G379-G392
35. Scherer-Singler U, Vincent SR, Kimura H, Mc Geer EG (1983) Demonstration of a unique population of neurons with NADPH-diaphorase histochemistry. J Neurosci Methods 9: 229–234
36. Smith TK, Brian Reed J, Sanders KM (1987) Origin and propagation of electrical slow waves in circular muscle of canine proximal colon. Am J Physiol 252:C215–C224
37. Taguchi T, Tanaka K, Ikeda K, Matsubayashi S, Yanaihara N (1983) Peptidergic innervation irregularities in Hirschsprung's disease. Virchows Arch 401:223–235
38. Taguchi T, Tanaka K, Ikeda K (1985) Immunohistochemical study of neuron specific enolase and S-100 protein in Hirschsprung's disease. Virchows Arch 405:399–409
39. Timmermans JP, Scheuermann DW, Stach W, Adriaensen D, De Groodt-Lasseel MHA (1990) Distinct distribution of CGRP-, enkephalin-, galanin-, neuromedin U-, neuropeptide Y-, somatostatin-, substance P-, VIP- and serotonin-containing neurons in the two submucosal ganglionic neural networks of the porcine small intestine. Cell Tissue Res 260: 367–379
40. Timmermans JP, Barbiers M, Scheuermann DW, Bogers JJ, Adriaensen D, Fekete E, Mayer B, Van Marck EA, De Groodt-Lasseel MHA (1994) Nitric oxide synthase immunoreactivity in the enteric nervous system of the developing human digestive tract. Cell Tissue Res 275:235–245
41. Tsuto T, Okamura H, Fukui K, Obata HL, Terubayashi H, Iwai N, Majima S, Yanaihara N, Ibata Y (1982) An immunohistochemical investigation of vasoactive intestinal polypeptide in the colon of patients with Hirschsprung's disease. Neurosci Lett 34:57–62
42. Tsuto T, Obata-Tsuto HL, Iwai N, Takahashi T, Ibata Y (1989) Fine structure of neurons synthesizing vasoactive intestinal peptide in the human colon from patients with Hirschsprung's disease. Histochemistry 93:1–8
43. Vanderwinden JM, De Laet MH, Schiffmann SN, Mailleux P, Lowenstein CJ, Snyder SH, Vanderhaeghen JJ (1993) Nitric synthase distribution in the enteric nervous system of Hirschsprung's disease. Gastroenterology 105:969–973
44. Ward SM, Xue C, Shuttleworth CW, Bredt DS, Synder SH, Sanders KM (1992) NADPH diaphorase and nitric oxide synthase colocalization in enteric neurons of canine proximal colon. Am J Physiol 263:G277–G284
45. Whitehouse FR, Kernohan JW (1948) Myenteric plexus in congenital megacolon. Study of eleven cases. Arch Intern Med 82: 75–111
46. Yanaihara N, Sakagami M, Sato H, Yamamoto K, Hashimoto T, Yanaihara C, Ito Z, Yamaguchi K, Abe K (1977) Immunological aspects of secretin, substance P and VIP. Gastroenterology 72:803–810
47. Young HM, Furness JB, Shuttleworth CWR, Bredt DS, Snyder SH (1992) Co-localization of nitric oxide synthase immunoreactivity and NADPH diaphorase staining in neurons of the guinea-pig intestine. Histochemistry 97:375–378
48. Zhou DS, Komuro T (1992) Interstitial cells associated with the deep muscular plexus of the guniea-pig small intestine, with special reference to the interstitial cells of Cajal. Cell Tissue Res 268:205–216