Histol Histopath (1994) 9: 615-629

Histology and Histopathology

Invited Review

Hirschsprung's disease - immunohistochemical findings

L.T. Larsson
Department of Pediatric Surgery, University of Lund, Lund, Sweden

Summary. Hirschsprung's disease (HSCR) is characterized by a non-propulsive distal intestinal segment (usually colon) leading to a functional obstruction. An absence of ganglia in the affected segment explains the synonymous term «aganglionosis coli». The lack of peristalsis is partly due to a deficient intestinal smooth muscle relaxation based on an absence of non-adrenergic, non-cholinergic (NANC) inhibitory innervation. Morphological studies using conventional microscopy, immunohistochemistry and immunochemistry against general neuronal markers and neuropeptides have been used to characterize the disturbed NANC innervation in HSCR.

An increased cholinergic and adrenergic innervation is registered in the aganglionic segment in spite of the lack of neuronal cell bodies: Neuropeptides like vasoactive intestinal peptide (VIP), pituitary adenylate cyclase-activating polypeptide (PACAP), gastrin-releasing peptide (GRP), calcitonin gene-related peptide (CGRP), substance P (SP), enkephalins and galanin immunoreactive nerve fibres are all reduced in number in the aganglionic segment. In contrast, neuropeptide Y (NPY)-containing nerve fibres are increased in number in the diseased segment, probably reflecting the adrenergic hyperinnervation. General neuronal markers including chromogranins have been used to map the neuronal network in the HSCR intestine and also to investigate the endocrine cell system in the intestinal mucosa. Nitric oxide is a potent component of the NANC inhibitory innervation and its synthesizing enzyme, nitric oxide synthase (NOS), is shown to be almost absent in the neuronal system in aganglionic intestine.

Key words: Hirschsprung's disease, Immunocytochemistry, Gut innervation, Neuropeptides

Offprint requests to: Ass. Prof. L.T. Larsson, M.D., Ph.D., Department of Pediatric Surgery, University Hospital, S-221 85 Lund, Sweden

Historical background

Harald Hirschsprung lived from 1830 to 1916 and worked as a pediatrician in Copenhagen between 1870 and 1904. In 1886 he reported on a disease in two boys characterized by an impaired intestinal motor function, progressive abdominal distention and fatal outcome, whose autopsy showed no signs of mechanical obstruction in spite of grossly distended large intestines. He named the disease congenital dilatation of the colon or megacolon congenitium (Madsen, 1964). Early reports on the histology of the large intestine from patients who died as a consequence of Hirschsprung's disease have indicated absence of ganglion cells in the distal colon (Tittel, 1901; Dalla Valle, 1920), but it was not until 1948 that Whitehouse and Kernohan described the typical histological appearance in Hirschsprung's disease (Whitehouse and Kernohan, 1948). Their examination revealed an absence of neurons and ganglia in the distal narrow part of the bowel. The transitional zone between the narrow and dilated part of the colon was found to contain a few scattered neurons. Thick bundles of nerve fibres were seen running between the two muscle layers and in the submucosal layer, in positions normally occupied by the myenteric and submucous ganglia (Whitehouse and Kernohan, 1948). The term aganglionosis coli was thus introduced as an alternative to the terms megacolon congenitum and Hirschsprung's disease. The aganglionic segment usually extends from the anus and a variable distance orally. Most often the rectum and lower sigmoid are involved, but occasionally the entire large intestine is aganglionic. The first internationally recognized report on a successful operation of Hirschsprung's disease was published in 1948 (Swenson and Bill, 1948). It described the resection of the aganglionic intestine and a colon-anal anastomosis. However, already in 1898 the famous English surgeon Frederick Treves reported on a pediatric case, where he successfully excised the narrow distal part of the colon and performed a colon-anal anastomosis (Treves, 1898). Today, several different

operative procedures are used, all including a resection of the aganglionic segment but varying in the way the anal anastomosis is performed. The overall results of these procedures are fairly good.

Pathophysiological background

The intestinal dysfunction in Hirschsprung's disease with a narrow, non-propulsive bowel without peristaltic waves and with a defective recto-sphincteric inhibition reflex leads to a functional obstruction (Schärli, 1982). The finding of normal receptor mechanisms and a normal resting membrane potential in muscle cells from aganglionic intestine, indicates that the cause of the bowel constriction is neurogenic rather than myogenic (Wright and Sheperd, 1965; Kubota et al., 1983). Denervation hypersensitivity has been suggested to cause the constriction (Ehrenpreis et al., 1968). However, several observations contradict this view, including the finding of neuro-muscular relationships and an increased adrenergic innervation in the diseased bowel (Howard and Garrett, 1970; Toulokian et al., 1973). The prominent nerve bundles in aganglionic intestine are strongly acetylcholine esterase-positive and the existence of such nerve fibres is typical in Hirschsprung's disease (Meier-Ruge et al., 1972). The finding of an increased acetylcholine esterase activity has led to the hypothesis that a parasympathetic hyperactivity might lead to the spastic contraction seen in the aganglionic segment (Meier-Ruge et al., 1972; Ikawa et al., 1980; Patrick et al., 1980). Already in 1898 Langley observed an atropine-resistant relaxation of the stomach after vagal nerve stimulation (Langley, 1898). Numerous subsequent reports have suggested the existence of nervous mechanisms besides the adrenergic and cholinergic ones involved in the control of intestinal motility (Burnstock et al., 1964; Crema et al., 1968). In aganglionic intestine these non-adrenergic, non-cholinergic (NANC) inhibitory mechanisms have been shown to be absent (Wright and Sheperd, 1965; Nirasawa et al., 1968; Frigo et al., 1973; Kubota et al., 1983; Larsson et al., 1987). Several peptides have been proposed as neurotransmitter candidates and the term peptidergic has been used to describe this NANC part of the intestinal nervous system (Polak et al., 1979; Sundler et al., 1980; Bishop et al., 1981; Furness et al., 1982).

Classical neurotransmitters in Hirschsprung's disease

An increased activity of acetylcholine esterase (AChE) is found in the aganglionic intestine and AChE histochemistry is widely used in the diagnosis of Hirschsprung's disease (Meier-Ruge et al., 1972; Lake et al., 1978; Meier-Ruge and Schärli, 1986). Adrenergic hyperinnnervation of the aganglionic bowel has also been demonstrated in Hirschsprung's disease (Bennet et

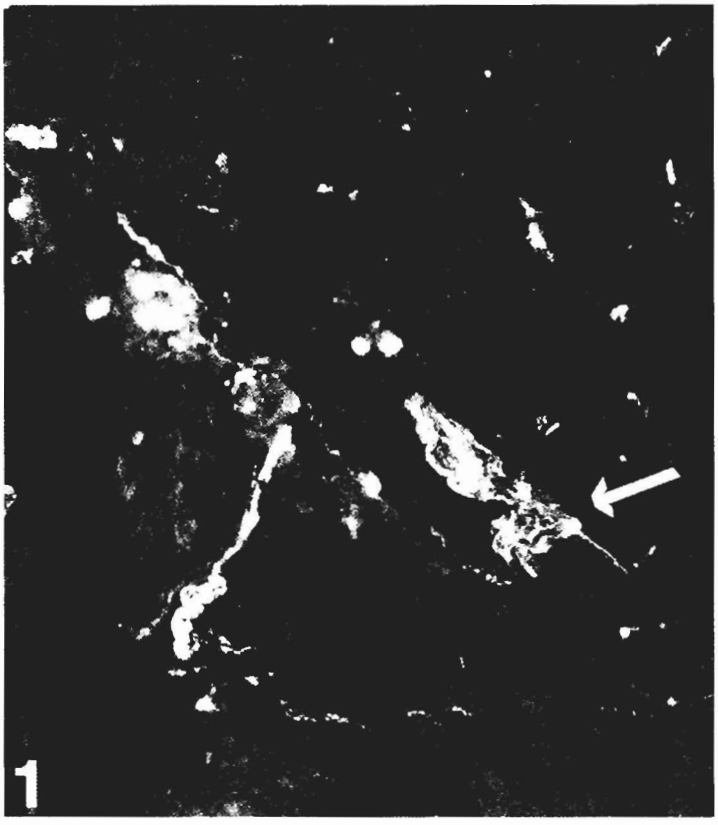


Fig. 1. Immunostaining for tyrosine hydroxylase in aganglionic bowel. Large nerve bundle in the intermuscular space (arrow). x 150

al., 1968; Howard and Garrett, 1970) using the catecholamine histofluorescence technique (Falck et al., 1962). Tyrosin hydroxylase (TH) has also been used as a marker for noradrenergic neurons in histochemical studies on the adrenergic innervation in Hirschsprung's disease (Fig. 1) thereby confirming earlier findings

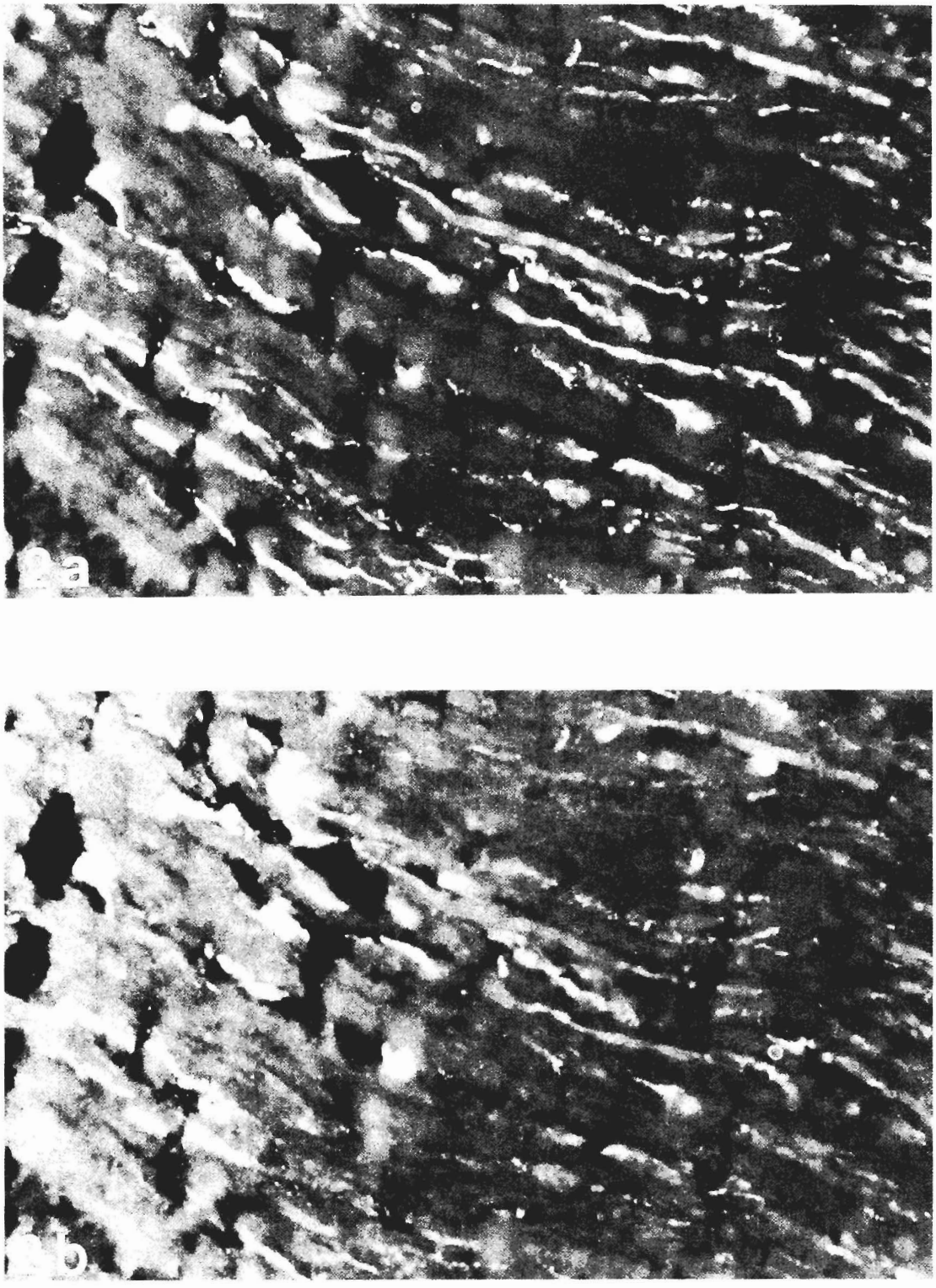


Fig. 2. Immunostaining for VIP **(a)** in nerve fibres in ganglionic smooth muscle showing coexistence with PACAP **(b)**. x 250

(Larsson et al., 1991).

Neuropeptides in Hirschsprung's disease

Vasoactive intestinal polypeptide (VIP), peptide histidine isoleucine (PHI)

VIP is a 28 aminoacid peptide isolated from the porcine intestinal wall and distributed throughout the human body (Said and Mutt, 1970). The intestinal tract, especially the sphincteric regions, is richly supplied with VIP-containing nerve fibres (Fig. 2A), which are intrinsic in origin (Alumets et al., 1979; Malmfors et al., 1981; Håkanson et al., 1982; Sjölund et al., 1983a). VIP is known to relax intestinal smooth muscle and has been proposed as a relaxatory neurotransmitter (Leander et al., 1981; Furness and Costa, 1982). In aganglionic bowel, VIP-immunoreactive nerve fibres are markedly reduced (Fig. 3) (Dupont et al., 1980; Bishop et al., 1981a,b; Freund et al., 1981; Tsuto et al., 1982; Larsson et al., 1983; Taguchi et al., 1983). However, quite a lot of VIP-immunoreactive nerve fibres are found in the large nerve trunks characteristic of aganglionic intestine. The mucosal VIP innervation also seems to be much less affected than in the smooth muscle layers (Fig. 4) (Larsson et al., 1988a). Nerve fibres displaying immunoreactivity for PHI are uniformly distributed in the human intestine and coexist with VIP in peripheral neurons (Christofides et al., 1983; Yanaihara et al., 1983; Sundler et al., 1985a,b). Results of nucleotide sequencing indicates that PHI is a part of the VIP precursor molecule: pre-pro VIP (Itoh et al., 1983). Generally, PHI and VIP-immunoreactive nerve fibres have a similar distribution and the two peptides actually coexist in nerve fibres both in ganglionic and aganglionic intestine (Larsson et al., 1988a). In patients with a very long aganglionic segment including the distal ileum, immunohistochemistry indicates a more profound reduction of VIP-containing nerve fibres in the aganglionic ileum when compared to aganglionic colon (my own observations).

Pituitary adenylate cyclase-activating polypeptide (PACAP) in aganglionic intestine

PACAP is a hypothalamic peptide which is also present in the gut and the respiratory tract (Miyata et al., 1989; Uddman et al., 1991; Sundler et al., 1992). Two different variants of PACAP are known: PACAP-38 and PACAP-27. PACAP shows homology with VIP and is considered as a new member of the VIP peptide family (Miyata et al., 1989, 1990; Gourler et al., 1990; Kimura et al., 1990; Uddman et al., 1991; Sundler et al., 1992). Like VIP, PACAP has been shown to exert relaxant effects on mammalian intestinal smooth muscle, but the adenylate cyclase stimulating activity is greater than for VIP and PHI (Miyata et al., 1989; Schmidt et al., 1990; Mungan et al., 1991). In human gut, PACAP immunoreactive nerve fibres and cell bodies are present, but a reduced number of PACAP-containing nerve fibres is found in the aganglionic bowel (Shen et al., 1992). Coexistence of PACAP and VIP could be demonstrated

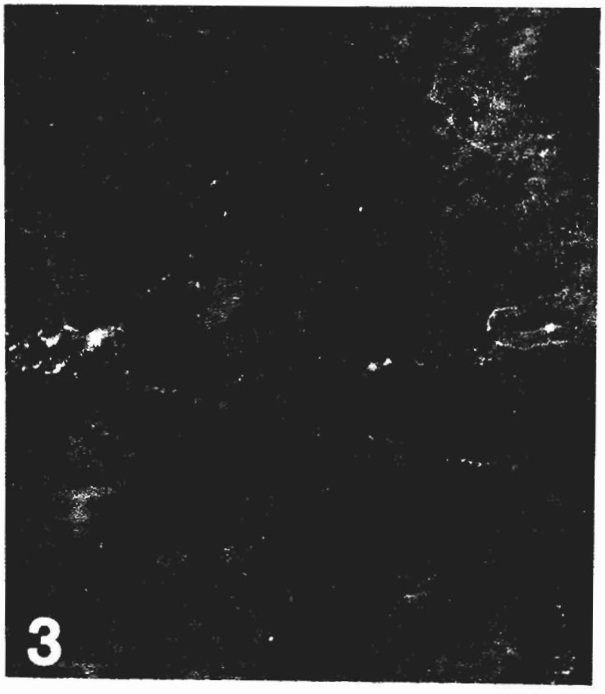


Fig. 3. Very sparse immunoreactivity for VIP in nerve fibres in aganglionic intestine. x 150

Fig. 4. VIP immunoreactivity in aganglionic bowel. Fairly dense innervation in the muscularies mucosa and mucosa layers. x 150

in nervous elements in both ganglionic and aganglionic segments of the bowel (Fig. 2A,B) (Shen et al., 1992), and the reduction of PACAP and VIP in the aganglionic portion of the intestine may contribute to the defective intestinal motor control in patients with Hirschsprung's disease.

Gastrin releasing peptide (GRP)

GRP-containing nerve fibres are found predominantly in myenteric ganglia in the human gut wall (Bishop et al., 1981a,b; Larsson et al., 1983). In the human pyloric region, however, GRP-immunoreactive nerve fibres are fairly numerous in the smooth muscle layers (Malmfors and Sundler, 1986). In smooth muscle specimens from guinea-pig taenia coli GRP has been shown to evoke a contractile response (Leander et al., 1984). In the aganglionic intestine from patients with Hirschsprung's disease, GRP-immunoreactive nerve fibres are completely absent (Larsson et al., 1983).

Substance P (SP), calcitonin gene-related peptide (CGRP)

Nerve fibres showing SP-immunoreactivity are numerous throughout the intestinal tract, in all layers of the gut wall, and SP-immunoreactive nerve cell bodies are found in both myenteric and submucous ganglia (Fig. 5) (Pearse and Polak, 1975; Brodin et al., 1981). An intrinsic origin of the vast majority of SP-immunoreactive nerve fibres has been suggested (Malmfors et al., 1981). SP has been shown to contract intestinal smooth muscle specimens by a direct effect on smooth muscle receptors (Leander et al., 1981; Folkers et al., 1984; Larsson et al., 1987). Apart from this, SP is known to be a mediator of inflammatory responses in the airways (Bynke et al., 1983; Lundberg et al., 1985a,b). A reduction of the amount of SP and a reduced density of SP-immunoreactive nerve fibres is observed in the aganglionic segment in Hirschsprung's disease (Ehrenpreis and Pernow, 1953; Tafuri et al., 1974; Larsson et al., 1983; Taguchi et al., 1983; Tsuto et al., 1985; Tam, 1986). However, a certain amount of SP-immunoreactive nerve fibres occur in the smooth muscle and in the pathological nerve trunks observed between the muscle layers (Fig. 6A). Also, the SP-innervation of the mucosa seems to be much less affected than that of the muscle layers (Larsson et al., 1988a).

Colocalization between SP and CGRP has been demonstrated in sensory neurons in different species and released together with SP from sensory neurons (Gibbins et al., 1985; Lee et al., 1985; Lundberg et al., 1985a,b; Sundler et al., 1985a,b). CGRP-containing nerve fibres are few to moderate in number in the normal intestine and the CGRP nerve fibre density does not differ overtly when comparing with aganglionic intestine (Larsson et al., 1988b). A population of nerve fibres containing both CGRP and SP has been found in the submucosa both in ganglionic and aganglionic intestine suggesting a relatively intact sensory innervation in the aganglionic

Fig. 5. Substance P immunoreactivity in smooth muscle and myenteric ganglia in ganglionic bowel. x 200

intestine (Fig. 6A,B) (own observation).

Enkephalins

Enkephalins are present in nerve fibres in the human gut, localized in the myenteric and submucous ganglia and in the smooth muscle layers (Fig. 8) (Alumets et al., 1978; Larsson et al., 1983). The enkephalins, which are a family of opiate receptor agonists including leucin-enkephalin, methionine-enkephalin, dynorphine etc., are believed to mediate non-cholinergic colonic contractions (Sjöqvist et al., 1984). Enkephalin containing nerve fibres are fairly numerous in ganglionic intestine but seem to be absent in the aganglionic intestine (Larsson et

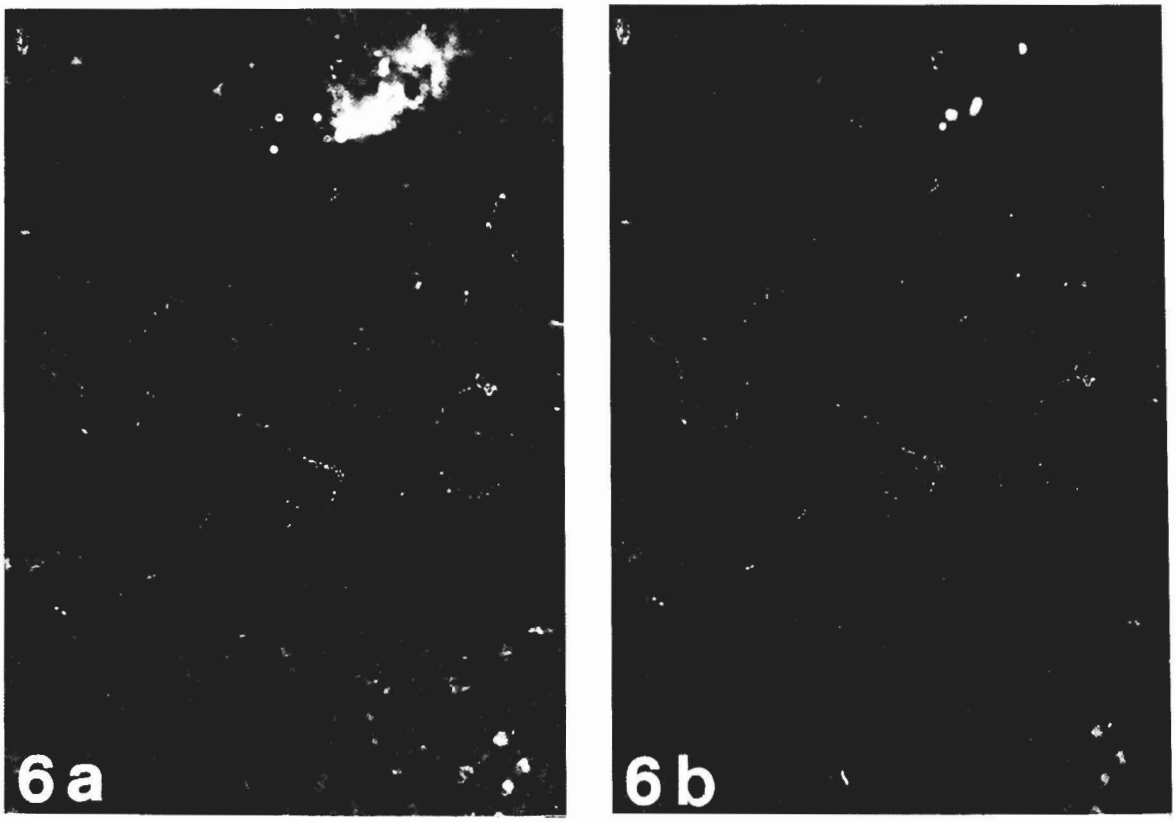


Fig. 6. Co-existence between SP **(a)** and CGRP **(b)** in the submucous layer in aganglionic intestine. x 200

Fig. 7. CGRP immunoreactivity in a pathological nerve bundle situated between the longitudinal and circular muscle layer in aganglionic intestine. x 150

al., 1983; Tsuto et al., 1985).

Galanin

Galanin immunoreactivity occurs in cell bodies in myenteric and submucous ganglia and in nerve fibres which are richly distributed in the intestine of several species, including man (Ekblad et al., 1985; Melander et al., 1985; Larsson et al., 1988b). Galanin is known to contract intestinal smooth muscle preparations from several species (Ekblad et al., 1985). The density of galanin containing nerve fibres in aganglionic intestine is roughly the same as in ganglionic intestine, and nerve fibres displaying galanin immunoreactivity are observed in the hypertrophied nerve trunks in aganglionic gut (Larsson et al., 1988b).

Neuropeptide Y (NPY)

NPY is a 36-aminoacid peptide known to be present in the peripheral (including the enteric-) nervous system (Furness et al., 1983; Lundberg et al., 1983; Sundler et al., 1983). The mammalian gut harbours two NPY-containing nerve fibre populations, one of extrinsic origin that is identical with adrenergic fibres and another non-adrenergic emanating from the intramural ganglia (Lundberg et al., 1983; Ekblad et al., 1984; Sundler et al., 1986). In aganglionic intestine a striking hyper-innervation of NPY-containing nerve fibres is found (Figs. 9, 10) (Hamada et al., 1987; Larsson et al., 1988b,

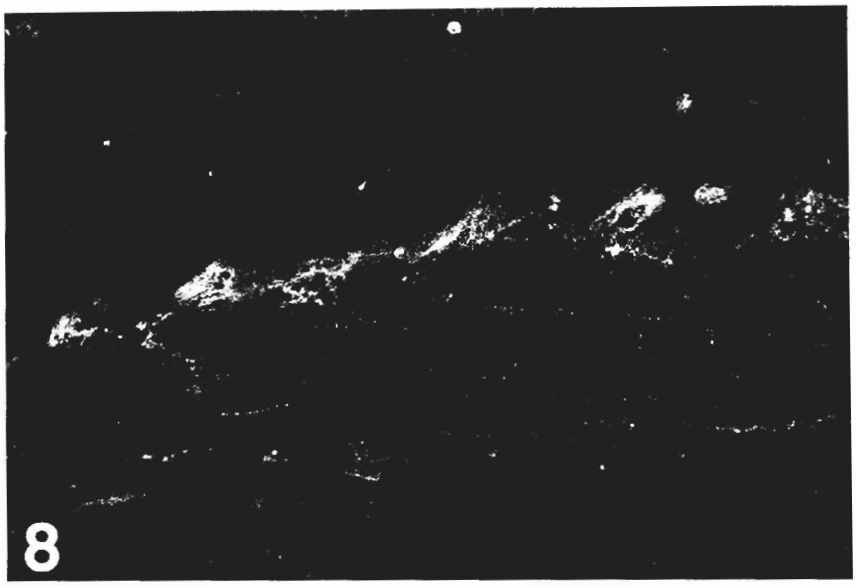


Fig. 8. Immunoreactivity for leu-enkephalin in myenteric ganglia and smooth muscle layer in normal intestine. x 150

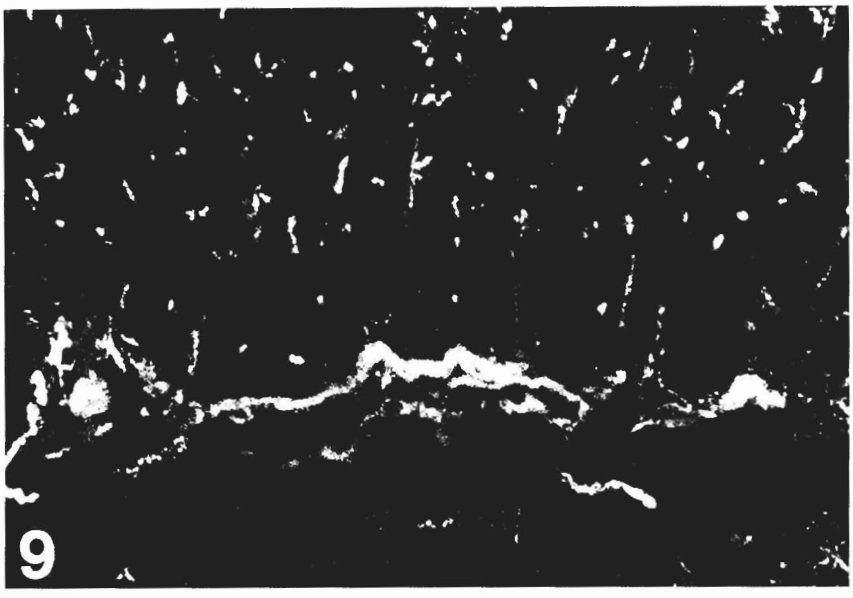


Fig. 9. NPY immunoreactivity in aganglionic intestinal smooth muscle. x 200

1991). Coexistence studies using the adrenergic marker TH and VIP show a population of adrenergic NPY/TH-containing nerve fibres situated around blood vessels, and another population of NPY/VIP-containing nerve fibres, lacking TH, with a distribution in all layers of the ganglionic intestinal wall (Larsson et al., 1991). NPY is considered to be mainly inhibitory with respect to gastrointestinal motility (Hellström et al., 1985) and it is not inconceivable that NPY and VIP act in conjunction in view of their coexistence in a population of non-adrenergic nerve fibres. In the aganglionic segment a marked hyperinnervation of both adrenergic NPY/TH-containing nerve fibres but also of NPY/VIP-containing nerve fibres and of nerve fibres containing NPY alone is found in all intestinal layers (Larsson et al., 1991). The presence of an NPY hyperinnervation has been suggested as an additional tool for the immunohistochemical diagnosis of Hirschsprung's disease.

General neuronal markers

Apart from AChE and markers for adrenergic neurons, general neuronal markers including neuron specific enolase (NSE) (Tam, 1986), neurofilaments (Fig. 11) (Klück et al., 1986), protein gene product (PGP) (Hamada et al., 1987), and chromogranins (Shen et al., 1994) have been used to demonstrate the large nerve bundles seen throughout the aganglionic bowel wall and the intestinal endocrine cells (Figs. 12-14) (Larsson et al., 1990).

The mucosal endocrine-paracrine cells are part of the diffuse neuroendocrine system in the gut and denervation procedures have been shown to affect endocrine cell activity and density (Ahlman et al., 1984; Axelsson et al., 1988). The human colorectal mucosa harbours cells displaying immunoreactivity for serotonin, somatostatin, glucagon/glicentin and peptide YY (PYY) (Lehy et al., 1981; Ferri et al., 1982; Sjölund et al., 1983b; Böttcher et al., 1986; Dolk et al., 1987). Chromogranins which are a family of a closely-related acidic proteins including chromogranin A and B are widely distributed in neuroendocrine cells (Grube et al., 1986; Rindi et al., 1986; Lloyd et al., 1988; Buffa et al., 1989). Chromogranin A is thought to participate in the intragranular storage of amines and peptide hormones and may function intracellularly as a calcium-regulatory protein involved in secretory mechanisms (Grube et al., 1986; Reiffen and Gratzl, 1986). In the aganglionic bowel no reduction in the density of endocrine cells can be seen (Larsson et al., 1990). Rather, cells storing chromogranin A display an increase in cell density in aganglionic intestine when compared to ganglionic

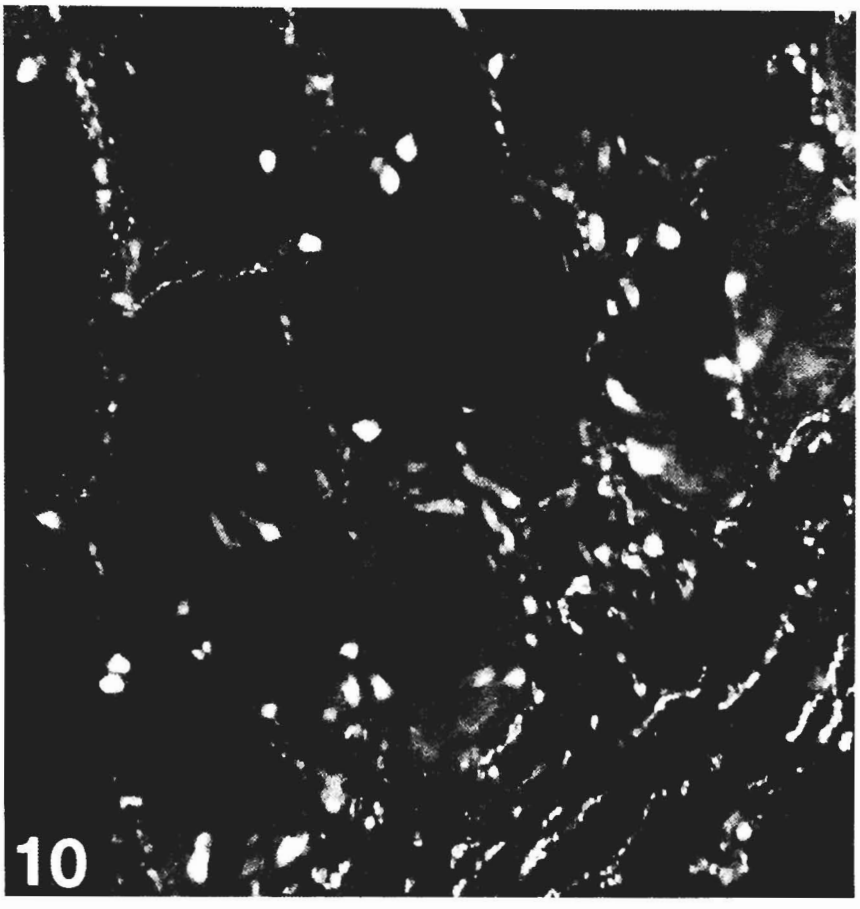


Fig. 10. Very dense population of NPY-immunoreactive nerve fibres in the muscularies mucosa and mucosal layer in aganglionic intestine. x 250

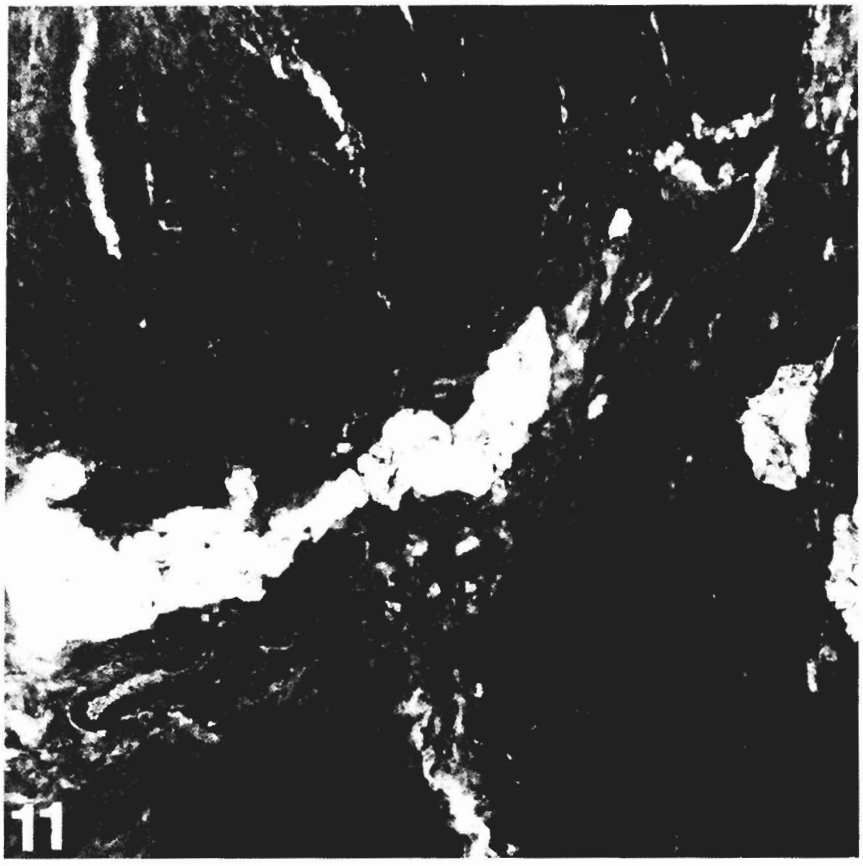


Fig. 11. Pathological nerve trunks in the intermuscular space in aganglionic intestine stained for neurofilaments. x 250

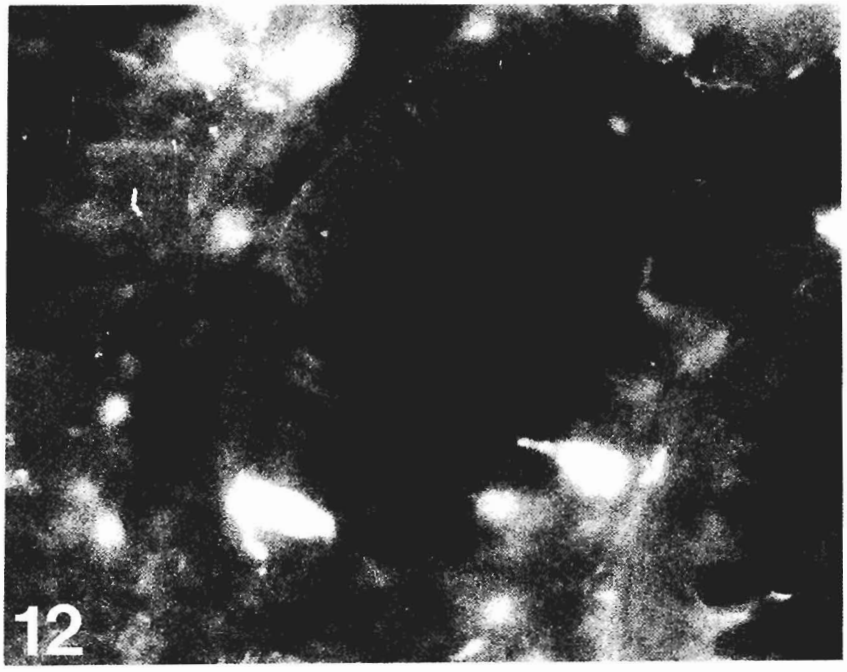


Fig. 12. Chromogranin immunoreactivity in endocrine cells in mucosa from aganglionic bowel. x 350

(Figs. 12-14). This supports the finding of a modest change in the mucosal innervation of aganglionic intestine despite the lack of submucous ganglia (Taguchi et al., 1983; Larsson et al., 1988a, 1990).

Chromogranin immunoreactive neuronal elements are present in the human intestine and a population of these nerve fibres is found to store also TH both in ganglionic and aganglionic intestine (Shen et al., 1994). In aganglionic bowel the density of chromogranin immunoreactive nerve fibres is markedly increased in comparison with the ganglionic intestine (Shen et al., 1994). Apart from the existence of chromogranins in adrenergic neurons, coexistence with NPY, galanin and VIP is also found in the aganglionic intestine (Shen et al., 1994).

Nitric oxide (NO)

Recently, NO has been recognised as a neuro-

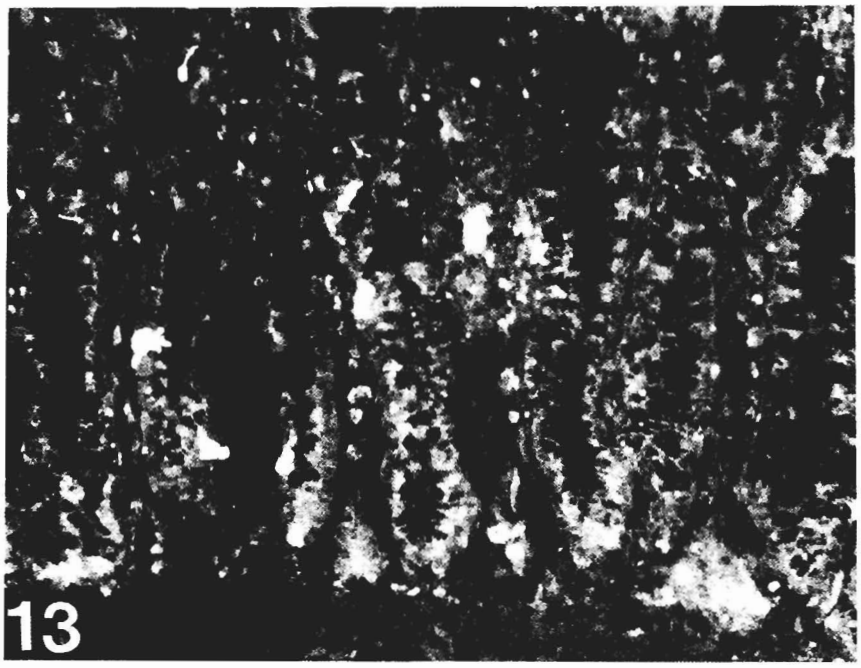


Fig. 13. Chromogranin immunoreactivity in mucosal endocrine cells in ganglionic intestine. x 250

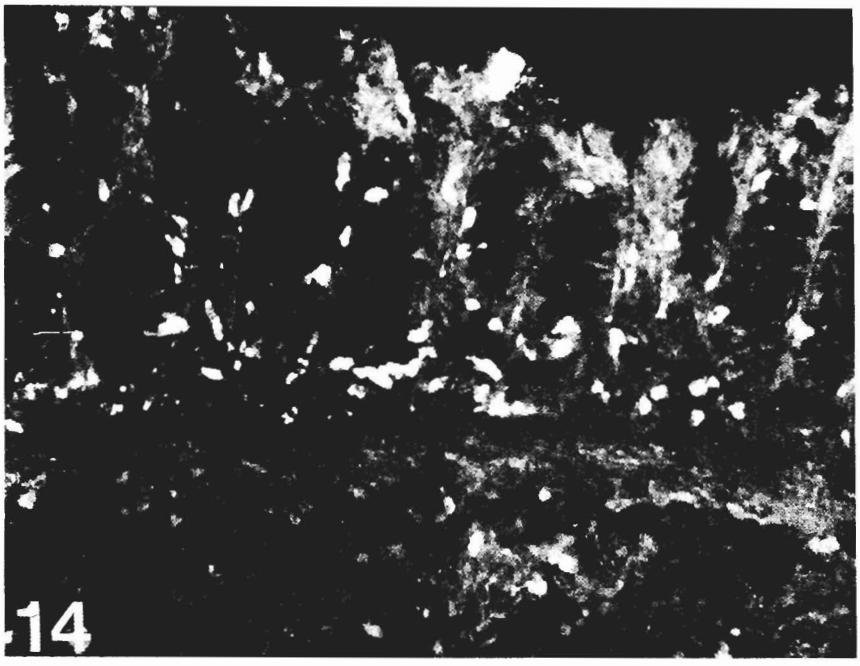
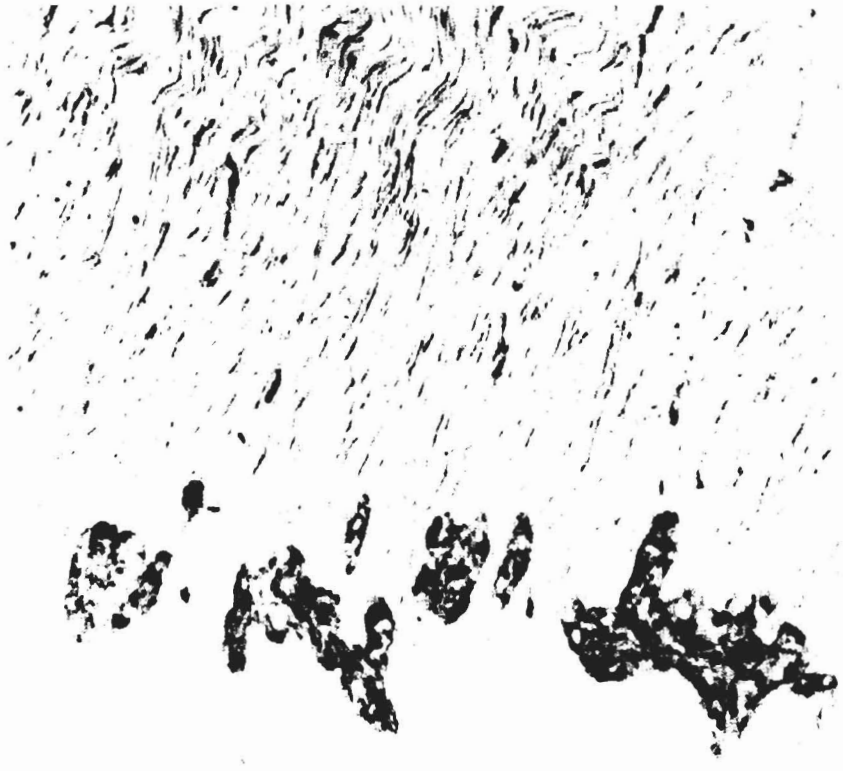


Fig. 14. Chromogranin immunoreactivity in mucosal endocrine cells from aganglionic intestine. x 250

transmitter and a potent NANC smooth muscle inhibitor, produced by the enzyme NO synthase (NOS) (Costa et al., 1991; Dawson et al., 1991; Maggi et al., 1991; Snyder and Bredt, 1991; Stark and Szurszewski, 1992). Nicotine amide adenine dinucleotide phosphate (NADPH) diaphorase activity and immunoreactivity for NOS has been shown to be identical and present in nervous elements in the human intestine (Fig. 15) (Dawson et al., 1991; Hope et al., 1991; Young et al., 1992; Larsson et al., submitted). NO is a potent smooth muscle inhibitor and NOS has been shown to be associated with inhibitory motor neurons throughout the gastrointestinal tract, sometimes colocalized with VIP and an important component of peristalsis (Brookes et al., 1991; Bredt and Snyder, 1992; Stark and Szurszewski, 1992). In the aganglionic intestine from patients with Hirschsprung's disease an almost complete lack of fibres with NOS immunoreactivity or NADPH diaphorase activity is seen (Fig. 16) (Larsson et al., submitted). The lack of NOS and VIP in enteric nerve fibres may contribute to the inability of the aganglionic smooth muscle to relax and thereby possess peristaltic waves. In the ganglionic intestine a large proportion of ganglion cells in the myenteric ganglia are NOS immunoreactive whereas only occasional NOS immunoreactive nerve cell bodies can be seen in the submucous ganglia (Larsson et al., submitted). Recently, a multicentre study has been initiated in order to study the proportion of NOS immunoreactive cell bodies in myenteric ganglia from patients operated on because of Hirschsprung's disease. This study is based on the clinical impression that some patients with Hirschsprung's disease in spite of a primarily successful operation show signs of an intestinal dysfunction emanating from the remaining ganglionic intestine. Preliminary results show that these patients have a lower rate of NOS-immunoreactive cell bodies in their myenteric ganglia when compared to patient with a normal postoperative course (own observations).

Concluding remarks

Hirschsprung's disease could be considered to be the only well-defined model for intestinal neuronal dysfunction. Since 1948 it has been surgically treated successfully, but the pathophysiological mechanisms are not yet clear. Typical for the disease is a lack of non-adrenergic, non-cholinergic (NANC)-inhibitory innervation leading to a defective smooth muscle relaxation and thereby lack of peristalsis. This functional



15

Fig. 15. NADPH diaphorase staining in nerve fibres and nerve cell bodies in myenteric ganglia and intestinal smooth muscle from ganglionic bowel. x 200

16

Fig. 16. Weak staining for NADPH diaphorase in aganglionic bowel. A pathological nerve bundle is seen running between the circular and longitudinal intestinal muscle layers. x 200

obstruction left untreated, leads to a severe constipation or even neonatal ileus. During more than a decade now, several neuropeptides have been found to act as neurotransmitters in the NANC part of the enteric nervous system. Severe derangements of the density of different neuropeptide-containing nerve fibres are found in the diseased aganglionic gut. The nerve fibres in the submucosal and mucosal layers including the density of endocrine cells are not deranged to such a great extent as in the smooth muscle innervation. The different neuropeptides that comprise a part of the NANC intestinal innervation are all known to have effect on the intestinal smooth muscle motility. However, the derangement of these peptide-containing nerve fibres in aganglionic intestine cannot fully explain the pathophysiology of the disease. Very recently, NO has been put forward as an important smooth muscle relaxant, also in the human intestine. An almost complete lack of NOS immunoreactive nerve fibres is seen in the aganglionic intestine. In order to evaluate the pathophysiological significance of this finding with respect to Hirschsprung's disease, however, demands additional functional studies. Recently, also another atypical neuronal messenger: namely carbonmonoxide (CO) (Marks et al., 1991) has given the research society in the field of Hirschsprung's disease another tool for trying to solve the enigmatic pathophysiology.

References

Ahlman H., Grönstad K., Nilsson O. and Dahlström A. (1984). Biochemical and morphological studies on the secretion of 5-HT into the gut lumen of the rat. Biogenic Amines 1, 63-73.

Alumets J., Håkanson R., Sundler F. and Shang K.J. (1978). Leu-enkephalin-like material in nerves and enterochromaffin cells in the gut. Histochemistry 56, 187-196.

Alumets J., Fahrenkrung J., Håkanson R., Schaffalitsky de Muckadell O., Sundler F. and Uddman R. (1979). A rich VIP nerve supply is characteristic of sphincters. Nature 288, 155-156.

Axelsson J., Ekelund M., Håkason R. and Sundler F. (1988). Gastrin and the vagus interact in the throphic control of the rat oxyntic mucosa. Regul. Pept. 22, 237-243.

Bennet A., Garret J. and Howard E. (1968). Adrenergic myenteric nerves in Hirschsprung's disease. Br. Med. J. 1, 487-489.

Bishop A.E., Ferri G.L., Probert L., Bloom S.R. and Polak J.M. (1981a). Peptidergic nerves. Scand. J. Gastroenterol. 17, 43-59.

Bishop A.E., Polak J.M., Lake B.D., Bryant M.G. and Bloom S.R. (1981b). Abnormalities of the colonic regulatory peptides in Hirschsprung's disease. Histopathology 5, 679-688.

Böttcher G., Alumets J., Håkanson R. and Sundler F. (1986).

Coexistence of glicentin and peptide YY in colorectal L-cells n at and man. An electron microscopic study. Regul. Pept. 13, 283-291.

Bredt D.S. and Snyder S.H. (1992). Nitrix oxide, a novel neuronal messenger. Neuron 8, 3-11.

Brodin E., Aluments J., Håkanson R., Leander S. and Sundler F. (1981). Immunoreactive substance P in chicken gut: distribution, development and possible functional significance. Cell Tissue Res. 216, 455-469.

Brookes S.J.H., Steele P.A.S. and Costa M. (1991). Calcretinin immunoreactivity in cholinergic motor neurons, interneurons and vasomotor neurons in the guinea-pig ileum. Cell Tissue Res. 263, 471-481.

Buffa R., Gini A., Pelagi M., Siccardi A.G., Bisiani C., Zanini A. and Colcia E. (1989). Immunoreactivity of hormonally-characterized human endocrine cells against three novel anti-human chromogranin B (B11 and B13) and chromogranin A (A11) monoclonal antibodies. Arch. Histol. Cytol. 52, 99-105.

Burnstock G., Campbell G., Bennet H. and Holman M.E. (1964). Innervation of the guinea pig tenaea coli: Are there intrinsic inhibitory nerves which are distinct from sympathetic nerves? Int. J. Neuropharmacol. 3, 163-166.

Bynke G., Håkanson R., Hörig J. and Leander S. (1983). Bradykinin contracts the pupillary sphincter and induces occular inflammation through release of neuronal substance P. Eur. J. Pharmacol. 91, 469-475.

Christofides N., Yiangou Y., Arons E., Ferri G., Tatemoto K., Polak J.M. and Bloom S.R. (1983). Radioimmunoassay and intramural distribution of PHI-IR in human intestine. Dig. Dis. Sci. 28, 507-512.

Costa M., Furness J.B., Brooks S.J.H., Bredt D.S. and Snyder S.H. (1991). Presence and chemical coding of neurons with nitric oxide synthase immunoreactivity in the guinea pig small intestine. Proc. Aust. Physiol. Pharmacol. Soc. 22, 97.

Crema A., Deltacca M., Frigo G.M. and Lecchini S. (1968). Presence of a non-adrenergic inhibitory system in the human colon. Gut 9, 633-637.

Dalla Valle A. (1920). Ericerche istoloiche su di un caso di megacolon congenito. Pediatria 29, 740-752.

Dawson T.M., Bredt D.S., Fotuhim Hwang P.M. and Snyder S.H. (1991). Niitric oxide synthase and neuronal NADPH diaphorase are identical in brain and peripheral tissues. Proc. Natl. Acad. Sci. USA 88, 7797-7801.

Dolk A., Broden G., Holmström B., Elwin C.E., Böttcher G., Sundler F. and Nilsson G. (1987). Endocrine cells in the human colorectal mucosa: immunocytochemical observations on patients with prolapse or internal procidentia of the rectum. Int. J. Colorect. Dis. 2, 77-81.

Dupont C., Navarro J., Chenut B. and Rosselin G. (1980). Modification of VIP intestinal continent associated with abnormal nervous myenteric plexus: A biological feature of chronic intestinal obstruction. J. Pediatr. 96, 1037-1039.

Ehrenspreis T. and Pernow B. (1953). On the occurrence of substance P in the recto-sigmoid in Hirschsprung's disease. Acta Physiol. Scand. 27, 380-388.

Ehrenpreis T.H., Norberg K.A. and Wirsén C. (1968). Sympathetic innervation of the colon in Hirschprung's disease. A histochemical study. J. Pediatr. Surg. 3, 43-48.

Ekblad E., Edwinsson L., Wahlestedt C., Uddman R., Håkanson R. and Sundler F. (1984). Neuropeptide Y coexists and cooperates with noradrenalin in perivascular nerve fibers. Regul. Pept. 8, 225-235.

Ekblad E., Håkanson R., Sundler F. and Wahlestedt C. (1985). Galanin, neuromodulatory and direct contractile effects on smooth muscle preparations. Br. J. Pharmacol. 86, 241-246.

Falck B., Hillarp N.A., Thieme G. and Thorp A. (1962). Fluorescence of catecholamines and related compounds condensed with formaldehyde. J. Histochem. Cytochem. 10, 348-252.

Ferri G.L., Botti P.L., Vezzadini P., Biliotti G., Bloom S.R. and Polak J.M. (1982). Peptide-containing innervation of the human intestinal mucosa. Histochemistry 76, 413-420.

Folkers K., Håkanson R., Hörig J., Jiecheng X. and Leander S. (1984). Biological evaluation of substance P antagonists. Br. J. Pharmacol. 83, 449-456.

Freund H., Humphrey C. and Fischer J. (1981). Reduced tissue content of vasoactive intestinal peptide in aganglionic oolon of Hirschsprung's disease. Am. J. Surg. 141, 243-244.

Frigo G.M., Deltacca M., Lecchini S. and Crema A. (1973). Some observations on the intrinsic nervous mechanisms in Hirschsprung's disease. Gut 14, 35-40.

Furness J.B. and Costa M. (1982). Enteric inhibitory nerves and VIP. In: Vasoactive intestinal peptide. Said S.I. (ed). Raven Press. New York. pp 391-406.

Furness J.B., Costa M., Murphy R., Beardsley A.M., Oliver J.R., Llewellyn-Smith I.J., Geshai R.L., Shulkess A.A., Moody T.W. and Meiyer D.K. (1982). Detection and characterization of neurotransmitters, particularly peptides, in the gastrointestinal tract. Scand. J. Gastroenterol. 17, 61-70.

Furness J.B., Costa M., Emson P.C., Håkanson R., Moghimzadeh E., Sundler F., Taylor I.L. and Chance R.E. (1983). Distribution, pathways and reactions to drug treatment of nerves with neuropeptide Y and pancreatic polypeptide-like immunoreactivity in the guinea-pig digestive tract. Cell Tissue Res. 234, 71-92.

Gibbins I.L., Furness J.B., Costa M., McIntyre I., Hillyard C.J. and Girgis S. (1985). Colocalization of calcitonin gene-related peptide-like immunoreactivity with substance P in cutaneous vascular and visceral sensory neurons of guinea-pigs. Neurosci. Lett. 57, 125-130.

Gourler P., Woussen M.C., van der Meers A., van der Meers-Peiret M.C., Robberecht P. and Christophe J. (1990). Pharmacological characterization of specific PACAP receptors in the rat pancreatic acinar cell line AR 4-2J. Digestion 46, 38.

Grube D., Aunis D., Bader F., Cetin Y., Jörns A. and Yoshie S. (1986). Chromogranin A (CGA) in the gastroentero-pancreatic (GEP) endocrine system. Histochemistry 85, 441.

Hamada Y., Bishop A.E., Federici G., Rivosecci M., Talbot I.C. and Polak J.M. (1987). Increased neuropeptide Y-immunoreactive innervation of aganglionic bowel in Hirschsprung's disease. Virchows Arch. (A) 411, 369-377.

Hellström P.M., Olerup O. and Tatemoto K. (1985). Neuropeptide Y may mediate effects of symphathetic nerve stimulations on colonic motility and blood flow in the cat. Acta Physiol. Scand 124, 613-624.

Hope B.T., Michel G.J., Knigge K.M. and Wincent S.R. (1991). Neuronal NADPH diaphorase is a nitric oxide synthase. Proc. Natl. Acad. Sci. USA 88, 2811-2814.

Howard E.R. and Garrett J.R. (1970). Electron microscopy of myenteric nerves in Hirschsprung's disease and in normal bowel. Gut 11, 1007-1014.

Håkanson R., Sundler F. and Uddman R. (1982). Distribution and topography of peripheral VIP nerve fibers: Functional implications. In: Vasoactive intestinal peptide. Said S.I. (ed). Raven Press. New York. pp 121-144.

Ikawa H., Yokoyama J., Morikawa Y., Hagashi A. and Katsumata K. (1980). A quantitative study of acetylcholine in Hirschsprung's disease. J. Pediatr. Surg. 15, 48-51.

Itoh N., Obata K., Yanaihara N. and Okamoto H. (1983). Human prepro-vasoactive intestinal polypeptide contains a novel PHI 27-like peptide, PHM-27. Nature 304, 547-549.

Kimura C., Ohkubo S., Ogi K., Hosoya M., Itoh Y., Onda H., Miyata A., Jiang L., Dahl R., Stibbs H.H., Arimura A. and Fujino M. (1990). A novel peptide which stimulates adenylate cyclase: Molecular cloning and characterization of the ovine and human cDNAs. Biochem. Biophys. Res. Commun. 166, 81-89.

Klück P., Kate F.J.V., van der Kamp A.W.M., Tibboel D. and Molenar J.C. (1986). Pathologic explanation for postoperative obstipation in Hirschsprung's disease revealed with monoclonal antibody staining. Am. J. Clin. Pathol. 86, 419-492.

Kubota M., Ito Y. and Ikeda K. (1983). Membrane properties and innervation of smooth muscle cells in Hirschsprung's disease. Am. J. Physiol. 255, 406-415.

Lake B.B., Puri P., Nixon H. and Claireaux A.E. (1978). Hirschsprung's disease, and appraisal of histochemically demonstrated acetyl-choline esterase activity in suction rectal biopsies specimens as an aid to diagnosis. Arch. Pathol. Lab. Med. 102, 244-247.

Langley J.N. (1898). On inhibitory fibers in the vagus to the end of the oesophagus and stomach. J. Physiol. London 23, 407-414.

Larsson L.T., Malmfors G., Wahlestedt C., Leander S. and 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.

Larsson L.T., Malmfors G. and Sundler F. (1988a) Neuropeptide Y (NPY) calcitonin gene-related peptide (CGRP) and galanin in Hirschsprung's disease - An immunocytochemical study. J. Pediatr. Surg. 23, 342-345.

Larsson L.T., Malmfors G. and Sundler F. (1988b). Defects in peptidergic innervation inHirschprung's disease. Pediatr. Surg. Int. 3, 147-155.

Larsson L.T., Sundler F. and Ekman R. (1990). Intestinal endocrine cells in Hirschsprung's disease. Int. J. Colorect. Dis. 5, 155-160.

Larsson L.T., Malmfors G., Ekblad E., Ekman R. and Sundler F. (1991). NPY hyperinnervation in Hirschsprung's disease: Both adrenergic and non-adrenergic fibers contribute. J. Pediatr. Surg. 26, 1207-1214.

Larsson L.T., Malmfors G. and Sundler F. (1983). Peptidergic innervation in Hirschsprung's disease. Z. Kinderchir. 38, 301-304.

Larsson L.T., Shen Z., Ekblad E., Sundler F., Alm P. and Andersson K.E. (1994). Lack of nitric oxide production in ganglionic intestine - A clue to Hirschsprung's disease. (submitted).

Leander S., Håkanson R. and Sundler F. (1981). Nerves containing substance P, vasoactive intestinal polypeptide, enkephalin or somatostatin in the guinea-pig taenea coli. Cell Tissue Res. 215, 21-39.

Leander S., Ekman R., Uddman R., Sunder F. and Håkanson R. (1984). Neuronal cholecystokinin, gastrin releasing peptide, neurotensine and beta-endorphin in guinea-pig intestine. Distribution and possible motor function. Cell Tissue Res. 235, 521-531.

Lee Y., Takani K., Kawai Y., Girgis S., Hillyard C., MacIntyre I., Emson P.C. and Tohyama M. (1985). Distribution of calcitonin gene-related peptide in the rat peripheral nervous system with reference to its coexistence with substance P. Neuroscience 15, 1227-1237.

Lehy T., Pranzi G. and Christina M.L. (1981). Correlative immunocyto-chemical and electron microscopic studies: Identification of glucagon-, somatostatin- and pancreatic polypeptide-like-containing cells in human colon. Histochemistry 71, 67-80.

Lloyd R.V., Cano M., Rosa P., Hille A. and Huttner W.B. (1988). Distribution of chromogranin A and secretogranin (chromogranin B) in neuroendocrine cells and tumour. Am. J. Pathol. 130, 296-304.

Lundberg J.M., Terenius L., Hökfelt T. and Goldstein M. (1983). High levels of neuropeptide Y in peripheral noradrenergic neurons in various mammals including man. Neurosci. Lett. 42, 167-172.

Lundberg J.M., Saria A., Teodorsson-Norheim E., Brodin E., Hua X., Marling C.R., Gamse R. and Hökfelt T. (1985a). Multiple tachykinins in capsaicin-sensitive afferents: Occurrence, release and biological effects with special reference to irritation of the airways. In: Tachykinin antagonists. Håkanson R. and Sundler F. (eds). Elsevier Amsterdam. pp 159-169.

Lundberg J.M., Franco-Cereseda A., Hua X., Hökfelt T. and Fischer J.A. (1985b). Coexistence of substance P and calcitonin gene-releated peptide-like immunoreactivities in sensory nerves in relation to cardio-vascular and bronchoconstrictor effects of capsaicin. Eur. J. Pharmacol. 108, 315-319.

Madsen C.M. (1964). Hirschsprung'disease. Acad. Diss. Copnehagen.

Maggi C.A., Barbanti G., Turini D. and Giuliani S. (1991). Effect of NG-monomethyl L-arginine and NG-nitro L-arginine on non-adrenergic non-cholinergic relaxation in the circular muscle of the human ileum. Br. J. Pharmacol. 103, 1970-1972.

Malmfors G. and Sundler F. (1986). Peptidergic innervation in infantile hypertrophic pyloric stenosis. J. Pediatr. Surg. 21, 303-306.

Malmfors G., Leander S., Brodin E., Håkanson R., Holmin T. and Sundler F. (1981). Peptide-containing neurons intrinsic to the gut wall. Cell Tissue Res. 215, 225-238.

Marks G.S., Brien J.F., Nakatsu K. and McLaughlin B.E. (1991). Does carbonmonoxide have a physiological function? Trends Pharmacol. Sci. 12, 185-188.

Meier Ruge W. and Schärli A.F. (1986). The epidemiology and enzyme histotopochemcial characterization of ultrashort-segment Hirschsprung's disease. Pediatr. Surg. Int. 1, 37-42.

Meier-Ruge W., Lutterbeck P.M., Herzog B., Morger R., Moser R. and Schärli A. (1972). Acetylcholine esterase activity in suction biopsies of the rectum in the diagnosis of Hirschsprung's disease. J. Pediatr. Surg. 7, 11-17.

Melander T., Hökfelt T., Rökaeus A., Fahrenkrug J., Tatemoto K. and Mutt V. (1985). Distribution of galanin-like immunoreactivity in gastrointestinal tract of several mammalian species. Cell Tissue Res. 239, 253-270.

Miyata A., Arimura A., Dahl R.R., Minamino N., Uehara A., Jiang L., Culler M.D. and Coy D.H. (1989). Isolation of a novel 38 residue-hypothalamic polypeptide which stimulates adenylate cyclase in pituitary. Biochem. Biophys. Res. Commun. 164, 567-574.

Miyata A., Jiang L., Dahl R.R., Kitada C., Kubo K., Fujino M., Minamino N. and Arimura A. (1990). Isolation of neuropeptide corresponding to the N-terminal 27 residues of the pituitary adenylate cyclase activating polypeptide with 38 residues (PACAP 387). Biochem. Biophys. Res. Commun. 170, 643-648.

Mungan Z., Rossowski W.J., Ertan A., Coy D.H. and Arimura A. (1991). Relaxant effect of pituitary adenylate cyclase activating polypeptide (PACAP) on rat intestinal smooth muscle. Gastroenterology 100, A474.

Nirasawa Y., Yokoyama J., Ikawa H., Morikawa Y. and Katsumata K. (1968). Hirschsprung's disease: Catecholamine content, alpha-

adrenoreceptors, and the effect of electrical stimulation in aganglionic colon. J. Pediatr. Surg. 21, 136-142.

Patrick W.J.A., Beasley G.T.M. and Smith I.I. (1980). Histochemical diagnosis of Hirschsprung's disease and a comparison of the histochemical and biochemical activity of acetylcholine esterase in rectal mucosal biopsies. J. Clin. Pathol. 33, 336-343.

Pearse A.G.E. and Polak J.M. (1975). Immunocytochemical localization of substance P in mammalian intestine. Histochemistry 41, 373-375.

Polak J.M., Path M. and Bloom S.R. (1979). Neuropeptides of the gut: a newly discovered major control system. World J. Surg. 3, 393-406.

Reiffen S.F.U. and Gratzl M. (1986). Chromogranins, widespread in endocrine and nervous tissue bined Ca^{2+}. FEBS Lett. 795, 327-330.

Rindi G., Buffa R., Sessa F., Tortora O. and Solci E. (1986). Chromogranin A, B and C immunoreactivities of mammalian endocrine cells. Histochemistry 85, 19-28.

Said S.I. and Mutt V. (1970). Polypeptide with broad biological activity: Isolation from small intestine. Science 169, 1217-1218.

Schmidt W.E., Höcker F., Katsoulis S., Wallwitz B., Stuber E., Schwarzhoff R., Schäfer F., Eckart K., Fölsch U.R. and Creutzfeldt W. (1990). PACAP 20-27 and PACAP 38 synthesis and biological activity of two novel regulatory brain-gut peptides. Digestion 46, 101.

Schärli A. (1982). Pathophysiology of Hirschsprung's disease. In: Hirschprung's disease. Holschneider A.M. (ed). Hippokrates Stuttgart and Thieme-Stratton. New York. pp 23-40.

Shen Z., Larsson L.T., Malmfors G., Absood A., Håkanson R. and Sundler F. (1992). A novel neuropeptide, pituitary adenylate cyclase-activating polypeptide (PACAP), in human intestine: Evidence for reduced content in Hirschsprung's disease. Cell Tissue Res. 269, 369-374.

Shen Z., Larsson L.T., Malmfors G., Oberg K., Eriksson B. and Sundler F. (1994). Chromogranin A and B in neuronal elements in Hirschsprung's disease: An immunocytochemical and radioimmunoassay study. J. Ped. Surg. (in press).

Sjölund K., Schaffalitzky de Muckadell O.B., Fahrenkrug J., Håkanson R., Petersson B.G. and Sundler F. (1983a). Peptide-containing nerve fibers in the gut wall in Crohn's disease. Gut 24, 724-733.

Sjölund K., Sandén G., Håkanson R. and Sundler F. (1983b). Endocrine cells in human intestine: An immunocytochemical study. Gastroenterology 85, 1120-1130.

Sjöqvist A., Hellström P., Jodal M. and Lundgren O. (1984). Neurotransmitters involved in the colonic contractions and vasodilatations elicited by activation of the pelvic nerves in the cat. Gastroenterology 86, 1481-1487.

Snyder S.H. and Bredt D.S. (1991). Nitric oxide as a neuronal messenger. Trends Pharmacol. Sci. 12, 125-128.

Stark M.E. and Szurszewski J.H. (1992). Role of nitric oxide in gastrointestinal and hepatic function and disease. Gastroenterology 103, 1928-1949.

Sundler F., Håkanson R. and Leander S. (1980). Peptidergic nervous system in the gut. In: Clinics in Gastroenterology. Creutzfeldt W. (ed). WB Saunders Ltd. London, Philadelphia, Toronto. pp 517-543.

Sundler F., Moghimzadeh E., Håkanson R., Ekelund M. and Emson P.C. (1983). Nerve fibers in the gut and pancreas of the rat, displaying neuropeptide Y immunoreactivity. Cell Tissue Res. 230, 487-493.

Sundler F., Alumets J., Ekblad E., Böttcher G. and Håkanson R. (1985a). Coexistence of peptides in the neuroendocrine system. In: Biogenetics and neurohormonal peptides. Håkanson R. and Thorell J. (eds). Academic Press. London. pp 213-243.

Sundler F., Brodin E., Ekblad E., Håkanson R. and Uddman R. (1985b). Sensory nerve fibers: distribution of substance P, neurokinin A and calcitonin gene-related peptide. In: Tachykinin antagonists. Håkanson R. and Sundler F. (eds). Academic Press. Amsterdam. pp 3-14.

Sundler F., Håkanson R., Ekblad E., Uddman R. and Wahlestedt C. (1986). Neuropeptide Y in the peripheral adrenergic and enteric nervous system. Int. Rev. Cytol. 102, 243-269.

Sundler F., Ekblad E., Absood A., Håkanson R., Köves K. and Arimura A. (1992). Pituitary adenylate cyclase activating peptides (PACAP), a novel VIP-like gut neuropeptide. Neuroscience 46, 439-454.

Swenson O. and Bill A.H. (1948). Resection of rectum and rectosigmoid with preservation of the sphincter for benign spastic lesions producting megacolon. Surgery 24, 212-220.

Tafuri W.L. Maria T.A., Pitella J.E. and Boglioli L. (1974). An electron microscopical study of the Auerbachs plexus and determination of substance P of the colon in Hirschsprung's disease. Virchows Arch. 341-350.

Taguchi T., Tanaka K., Ikeda K., Matsubayashi F. and Yanaihara N. (1983). Peptidergic innervation irregularities in Hirschsprung's disease. Virschows Arch. 401, 223-235.

Tam P. (1986). An immunochemical study with neuron specific enolase and substance P of human enteric innervation. The normal developmental pattern and abnormal deviations in Hirschsprung's disease and pyloric stenosis. J. Pediatr. Surg. 21, 227-232.

Tittel K. (1901). Uber eine angeborene Missbildung des Dicdarmes. Win. Klin. Wchnschr. 14, 903-907.

Toulokian R.J., Aghajahian G. and Roth R.H. (1973). Adrenergic hyperactivity of the aganglionic colon. J. Pediatr. Surg. 8, 191-195.

Treves F. (1898). Idiopathic dilatation of the colon. Lancet 29, 276-279.

Tsuto T., Okamura H., Fukui K., Obata H., Hirofumi T., Iwai N., Majima S., Yanaihara M. and Ibata Y. (1982). An immunohistochemical investigation of vasoactive intestinal polypeptide in the colon of patients with Hirschsprung's disease. Neurosci. Lett. 34, 57-62.

Tsuto T., Okamura H., Fukui K., Obata H., Terubayashi H., Yanaihara J., Iwai N., Majima S., Yanaihara N. and Ibata Y. (1985). Immunohistochemical investigations of gut hormones in the colon of patients with Hirschsprung's disease. J. Pediatr. Surg. 20, 266-270.

Uddman R., Luts A., Arimura A. and Sundler F. (1991). Pituitary adenylate cyclase-activating peptide (PACAP), a new vasoactive intestinal peptide (VIP)-like peptide in the respiratory tract. Cell Tissue Res. 265, 197-201.

Whitehouse F.R. and Kernohan J.W. (1948). Myenteric plexus in congenital megacolon. Arch. Int. Med. 82, 75-111.

Wright P.G. and Sheperd J.J. (1965). Response to drugs of isolated human colonic muscle from a case of Hirschsprung's disease. Lancet 2, 1161-1164.

Yanaihara N., Nokihara K., Yanaihara C., Iwanaga T. and Fujita T. (1983). Immunocytochemical demonstration of PHI and its coexistence with VIP in intestinal nerves of the rat and pig. Arch. Histol. Jpn. 46, 575-581.

Young H.M., Furness J.B., Shuttleworth C.W.R., Bredt D.S. and Snyder S.H. (1992). Co-localization of nitric oxide synthase immunoreactivity and NADPH diaphorase staining in neurons of the guinea-pig intestine. Histochemistry 97, 375-378.