Immunohistochemical Studies

U. Rolle and P. Puri

15

15.1 Introduction

Several diagnostic methods are necessary in the examination of patients in whom Hirschsprung's disease (HD) is suspected. These are clinical examination, contrast enema, anorectal manometry and rectal biopsy. It has been shown that rectal suction biopsies (RSB) have the highest sensitivity (93%) and specificity (100%) rates in diagnosing HD [1].

Nevertheless the introduction of RSB, whilst making the procedure less traumatic for the patient, has made the diagnosis of HD more difficult for the pathologist. Many histopathologists are reluctant to make a positive diagnosis of HD on the basis of rectal biopsy results, using conventional hematoxylin-eosin stains. This reluctance is due to the doubt as to the amount of submucosa that must be scanned before the absence of ganglion cells can be confirmed as well as the relative difficulty of accurately identifying smaller and sparse submucosal ganglion cells by comparison with the more compact ganglion cells of the myenteric plexus.

The development of histochemical techniques for the detection of acetylcholinesterase (AChE) was a considerable advance in the investigation of HD [2, 3]. HD is histologically characterized by the association between the congenital absence of colonic ganglion cells and an increased AChE expression in the affected bowel. Although a high degree of histochemical accuracy exists in performing AChE histochemistry, results are not always uniform, and false-positive and false-negative results have been reported [4, 5]. Possible causes of false AChE tests may be variability in biopsy site, immaturity of the enzyme system and technical variations [1]. Moreover, in the very young age group investigated for HD, the ganglion cells of the submucosa could be immature and hyperplastic nerve fibers of the lamina propria and mus-

cularis mucosa are not always detectable. Furthermore, ganglion cells may be difficult to distinguish from endothelial or other submucosal cells. Other major factors are first that AChE histochemistry requires fresh-frozen tissue, and second that the interpretation of AChE histochemistry needs a certain level of expertise.

Therefore, alternative diagnostic neuronal markers have been sought to ensure the proper diagnosis of HD on rectal biopsies. These include various new immunohistochemical and histochemical neuronal markers for use in the investigation of bowel specimens, i.e. rectal biopsies and resected bowel.

Generally, immunohistochemistry is a powerful tool for investigation of various antigens using specific antibody–antigen reaction. The basic immunohistochemical methods are direct and indirect immunofluorescence or direct and indirect enzyme immunohistochemistry. Various immunohistochemical markers and special histochemical stains have also been used for research and clinical diagnosis of HD and allied gastrointestinal motility disorders in childhood. A list of neuronal markers discussed in this chapter, and a summary of their distribution and physiological role, are presented in Table 15.1.

Table 15.1 Neuronal markers

Marker	Distribution/physiological role
Cathepsin D	General marker
Neuron-specific enolase (NSE)	General marker: mature and immature neurons, their perikarya and axonal fibers
Protein gene product (PGP) 9.5	General marker: mature and immature neuron cells (enteric ganglia), and nerve fiber
Neurofilament	General marker
Peripherin	General marker of the peripheral nervous system; marker of neuronal differentiation
Microtubule associated proteins	General marker
Calretinin	General marker
Neural cell adhesion molecule	General marker
Nerve growth factor receptor	General marker
Ca-activated K channels	General marker
Neuropeptide Y	Neuropeptide; sympathetic ganglia in myenteric and submucosal
Vasoactive intestinal peptide (VIP)	Neuropeptide; marker of (inhibitory) NANC innervation
Substance P	Neuropeptide; marker of (excitatory) NANC innervation
Enkephalin/gastrin-releasing peptide	Markers of excitatory NANC innervation
Calcitonin gene-related peptide (CGRP)	Marker of intrinsic afferent neurons
Galanin	General marker
S-100 protein	Marker of neuronal supporting (glial) cells
Glial fibrillary acidic protein	Marker of glial cells
Choline acetyltransferase	Marker of cholinergic neurons
Vesicular acetylcholine transporter	Marker of cholinergic neurons
Dopamine β-hydroxylase	Marker of (nor)adrenergic nerve fibers
Tyrosine hydroxylase	Marker of (nor)adrenergic neurons
Synaptophysin	Synaptic marker
171B5	Synaptic marker
Nitric oxide synthase	Marker of inhibitory NANC innervation
Carbon monoxide	Marker of inhibitory NANC innervation
Capsaicin/purinergic receptors	Marker of sensory nerves

15.2 General Markers

15.2.1 Neuron-specific Enolase

Neuron-specific enolase (NSE) is exclusively localized within neurons of mammalian nervous tissue [6, 7]. NSE is supposed to be a selective marker of the degree of neuronal maturity since this molecule is expressed by neurons when they have initiated their specific metabolic and synaptic activities [8]. NSE immunohistochemistry leads to intense staining of ganglia which allows the recognition of small ganglion cells and the overall pattern of microinnervation since it also stains nerve fibers within the circular muscle of the bowel [9, 10]. Therefore NSE immunoreactivity has even been used for the diagnosis of hypoganglionosis and intestinal neuronal dysplasia (IND) on rectal biopsies [11]. On the other hand, it has been stated that immunohistochemical positivity of ganglion cells for NSE is lower than that for protein gene product 9.5 (PGP9.5) [12]. Different results have been reported regarding the usefulness of NSE immunohistochemistry in the detection of hypertrophic fibers in the lamina propria of HD specimens [11, 12]. A most recent study has revealed that NSE stains the increased network of coarse, thickened, and irregular nerve fibers within the affected aganglionic segments [10]. A comprehensive study of selected markers for the staining of the enteric nervous system (ENS) has revealed that NSE and S-100 are most suitable for clinical application [13].

15.2.2 Protein Gene Product 9.5

The brain-specific protein PGP9.5 is one of the most sensitive markers for identifying ganglion cells. Therefore PGP9.5 is a reliable marker for ganglion cells and nerve fibers of the mucosal and submucosal plexus in bowel biopsies [14]. PGP9.5 staining of the ganglion cell is more intense than NSE staining and PGP9.5 staining of nerve fibers is more intense than S-100 staining [12]. There are significantly reduced numbers of PGP9.5-positive fibers in the smooth muscle of HD as shown by a morphometric evaluation of PGP9.5-positive fibers in paraffin section immunohistochemistry [15]. On the other hand PGP9.5 stains the increased network of coarse, thickened, and irregular nerve fibers within the affected segments of HD [10]. PGP9.5 clearly stains the myenteric plexus in normal bowel and the hypertrophic fibers in HD (Fig. 15.1). PGP9.5 antibody was applied to whole-mount preparations of aganglionic bowel. This study revealed thick PGP9.5-immunoreactive nerve strands mixed with S-100 and neurofilament between the longitudinal and circular

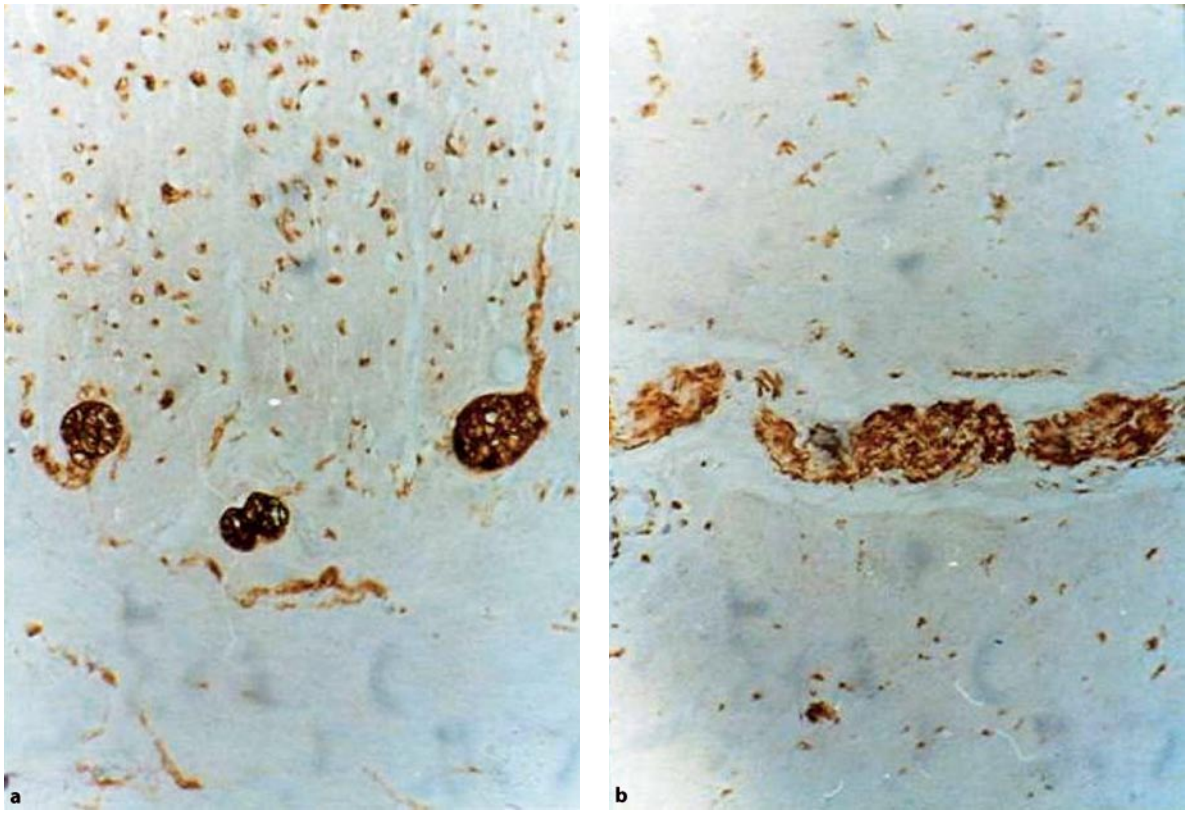

Fig. 15.1 PGP9.5 immunostaining: **a** myenteric ganglia in normal bowel; **b** hypertrophic fibers in HD bowel

muscle as well as within the submucosal layer [16]. The same study showed that immunohistochemical staining of whole-mount preparations enables the differentiation of oligoganglionic segments in HD and hypoganglionosis [16].

15.2.3 Cathepsin D

Cathepsin D is a member of a family of lysosomal acidic proteinases which play a major role in the intracellular catabolism of proteins [17]. Cathepsin D catabolizes neuropeptides such as substance P (SP), somatostatin, β-lipoprotein, and angiotensinogen. Mature and immature ganglion cell bodies within the submucosal and myenteric plexus of the human intestine showed intense granular cytoplasmatic immunoreactivity for cathepsin D [14]. No cathepsin D-immunoreactive cells were detected in aganglionic bowel [18]. Cathepsin D does not stain hypertrophic nerve fibers in aganglionic bowel [13]. Since cathepsin D stains exclusively ganglion cells (mature and immature) is has been suggested as a valuable tool in diagnosing HD.

15.2.4 Neurofilament Proteins

Low (NF-L, 68 kDa), medium (NF-M, 160 kDa) and high (NF-H, 200 kDa) molecular neurofilament proteins (NF) form the neurofilaments, which, together with neurotubules, constitute the cytoskeleton of the neurons [19]. Neurofilament cytoskeleton matures during development and shows an upregulation during late embryonic stages and after birth [20, 21]. NF-H immunoreactivity is not intense in ganglion cells. Nevertheless, antineurofilament antibodies have been used as one of the first immunohistochemical tests in the study and diagnosis of HD. Since some antibodies only recognize specific NF subunits different staining results have been achieved. Normal colon and ganglionic bowel of HD patients show partial staining of some axon bundles within the myenteric and submucosal plexus. In contrast heavily stained hyperplastic nerve bundles are evident in aganglionic bowel in HD [22]. NF-H and NF-M stain the increased network of coarse, thickened, and irregular nerve fibers within the mucosal and submucosal layers of aganglionic segments in HD [10, 23, 24].

15.2.5 Peripherin

The neuronal intermediate filament protein peripherin is expressed in developing and differentiated neurons from birth up to adulthood [25]. A comparative investigation using various antibodies revealed that peripherin is the best for the detection of human submucosal ganglion cells [14]. Peripherin was used to show histopathological differences between classical rectosigmoid HD and total colonic aganglionosis [26].

15.2.6 Microtubule-associated Proteins

Microtubules are major components of the neuronal cytoskeleton [27]. These microtubules are associated with proteins that control tubulin polymerization, regulate microtubule assembly and function and mediate cross-bridge formation with NFs [28]. Microtubule-associated protein 5 (MAP5) immunohistochemistry has revealed the features of the normal ENS [27].

MAP5 and microtubule-associated tau protein (tau) were excellent markers of the ENS since they were specifically located in nerve cell bodies and nervous processes of normal intestine as well as aganglionic segments [29]. MAP5 and tau expression was slightly reduced in aganglionic bowel and was evident in the hypertrophied nerve fibers of aganglionic bowel. MAP5 stained the increased network of coarse, thickened, and irregular nerve fibers within the affected segments of HD [10, 27].

15.2.7 Microtubule-associated Tau Protein

Anti-tau staining was achieved in normal ganglion cells of both myenteric and submucosal plexus and within intrinsic nerve fibers of normal controls. Intrinsic nerve fibers were positively stained by anti-tau also in oligoganglionic and aganglionic bowel of HD whereas the hypertrophic (extrinsic) intermuscular, submucosal and subserosal nerve fibers did not stain with anti-tau [24].

15.2.8 Calretinin

Calretinin is a calcium-binding protein which plays a an important role in the organization and functioning of the ENS [30]. Calcium-binding proteins (calretinin, calbindin) are involved in physiological calcium hemostasis. Ganglion cells and their projections express calretinin within the submucosal and myenteric plexus of normal bowel and ganglionic bowel of HD whereas in aganglionic segments of HD a lack of calretinin expression has been shown. The absence of calretinin immunostaining in the nerve fibers also represents a lack of calretinin in related nerve cells, which may serve as a diagnostic tool in the diagnosis of aganglionic segments [10].

15.2.9 Neural Cell Adhesion Molecule

Neural cell adhesion molecule (NCAM) is a cell-surface glycoprotein involved in cell–cell adhesion during devel-

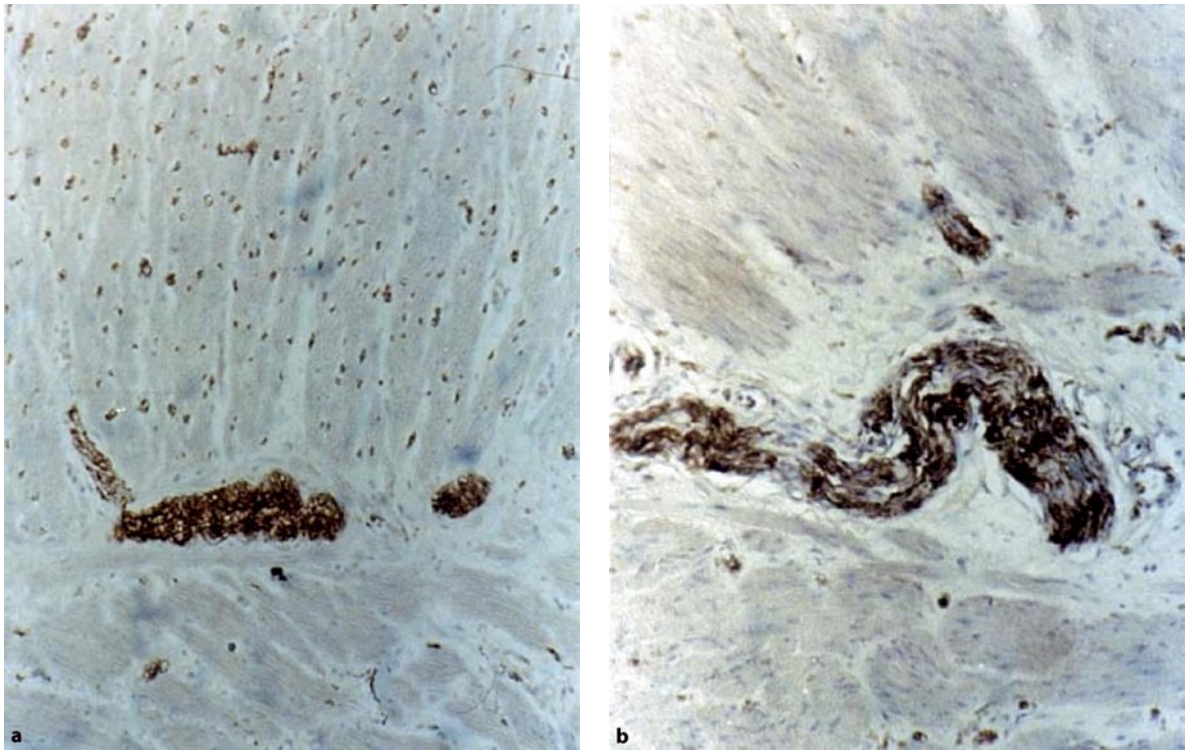

Fig. 15.2 NCAM immunostaining: **a** myenteric ganglia in normal bowel; **b** hypertrophic fibers in HD bowel

opment [31]. NCAM appears on early embryonic cells and is important in the formation of cell collectives and their boundaries at the sites of morphogenesis [32]. It is involved in adhesion between several types of neural cells and their processes and the formation of initial contacts between nerve and muscle.

Strong NCAM activity is found in normal and ganglionic bowel from HD patients, both in the submucous and myenteric nerve plexuses and also in the abundant nerve fibers within the longitudinal and circular muscle layers and in the internal sphincter (Fig. 15.2) [33, 34]. In contrast, in the aganglionic colon NCAM activity is either absent or markedly decreased within both the circular and longitudinal muscles. Hypertrophic nerve trunks express strong NCAM immunoreactivity. The lack of expression of NCAM on nerve fibers within the aganglionic smooth muscle suggests a developmental abnormality of the innervation of the muscle [15].

NCAM staining is a valuable general neuronal marker for the staining of submucous and myenteric plexus and we have found it particularly useful in the diagnosis of allied gastrointestinal motility disorders such as IND (Fig. 15.3) and hypoganglionosis [35]. Furthermore NCAM has been used to stain resected HD bowel specimens in order to discriminate between different staining results within short type, rectosigmoid type and long type HD [36].

15.2.10 Nerve Growth Factor Receptor

Nerve growth factor (NGF) is the best-characterized protein of a family of chemically related molecules (neurotrophins) that play an essential role in the development and function of neurons in the peripheral and central nervous systems [37, 38]. The effects of NGF are transmitted via receptors localized within the cholinergic neurons [39–41]. Nerve growth factor receptor (NGFR) is the transmembrane protein that binds NGF and brings it into the cell [42].

NGFR immunostaining of normal colon demonstrates numerous NGFR-positive nerve fibers in the circular and longitudinal muscle layers and strong NGFR staining of submucosal and myenteric ganglia. NGFR activity is absent or markedly reduced in the muscle layers of aganglionic colon, whereas the hypertrophic nerve trunks are surrounded by a thick NGFR-immunoreactive ring. The NGFR staining technique is useful for the diagnosis of HD and other innervation disorders (Figs. 15.4 and 15.5).

15.2.11 Ca^{2+}-activated K^+ Channels

Small conductance Ca^{2+}-activated K^+ (SK) channels play a fundamental role in all excitable cells. SK2 and SK3 are

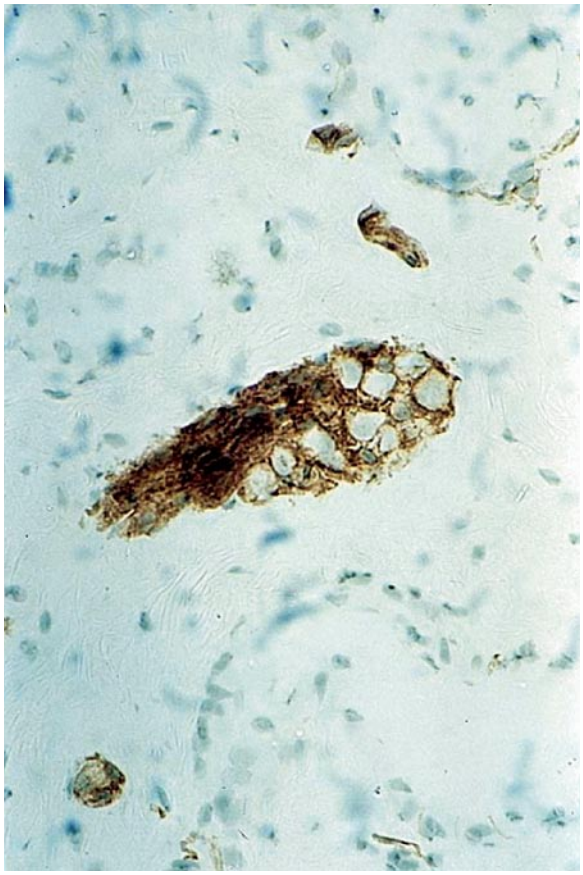

Fig. 15.3 NCAM immunostaining. Suction rectal biopsy with giant ganglion in IND

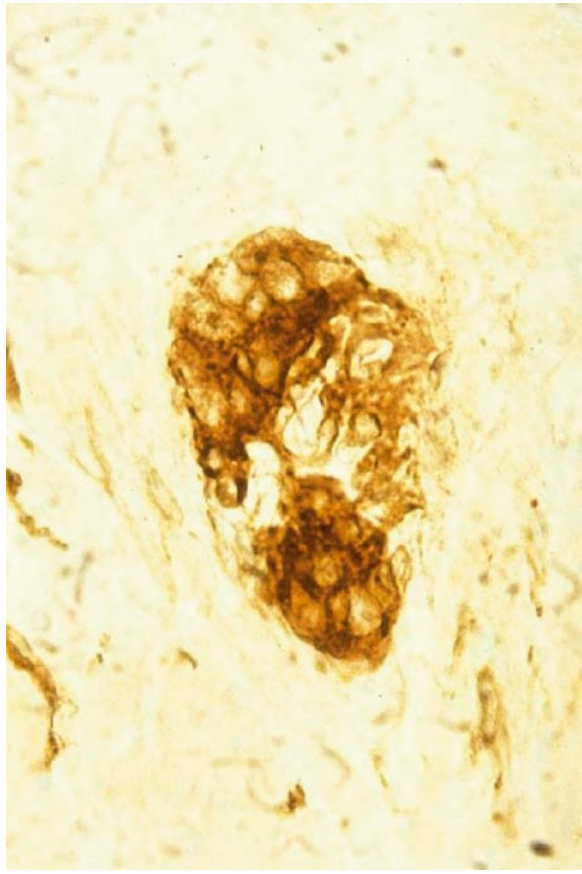

Fig. 15.4 NGFR immunostaining. Suction rectal biopsy with giant ganglion in IND

strongly expressed in normal bowel. Decreased expression of SK3 channels in the aganglionic bowel may contribute to motility dysfunction in HD [43].

15.2.12 Bcl_2

In colon biopsies of patients with different bowel dysmotility syndromes, Bcl_2 was found to be the best biomarker to discriminate immature small neurons in the diagnosis of hypoganglionosis and IND [44] since it was clearly expressed in immature small ganglion cells but did not stain, or only faintly stained, mature ganglion cells.

15.3 Cholinergic Markers

15.3.1 Choline Acetyltransferase and Peripheral Choline Acetyltransferase

Acetylcholine (Ach) is the major neurotransmitter in the ENS. Cholinergic nerves mediate increased gut activity, such as contraction [45], and are associated with mucosal ion transport [46]. AChE activity is the usual marker of cholinergic nerves and has become a widely accepted technique for diagnosis of HD since it stains the extrinsic fibers which penetrate the aganglionic segment in HD [27, 47, 48]. However it has been shown that AChE stains a variety of cholinergic and noncholinergic peripheral neurons [49, 50]. Choline acetyltransferase (ChAT) is a more specific and reliable marker of cholinergic nerves. ChAT is an enzyme which has been found in relatively small amounts in neural tissue [51]. To date immunocytochemistry for ChAT has been applied to frozen sections, whole-mounts and conventional formalin-fixed, paraffin-embedded human tissue sections [51–54]. Recently a splice variant, peripheral type of ChAT (pChAT) has been described and seems to be especially useful for studying the enteric cholinergic system [55].

Weakly stained ChAT-immunoreactive cells within the lamina propria as well as more strongly stained submucosal and myenteric ganglia are evident in normal human large bowel [54]. Aganglionic bowel sections have very strong ChAT-immunoreactive bundles in the

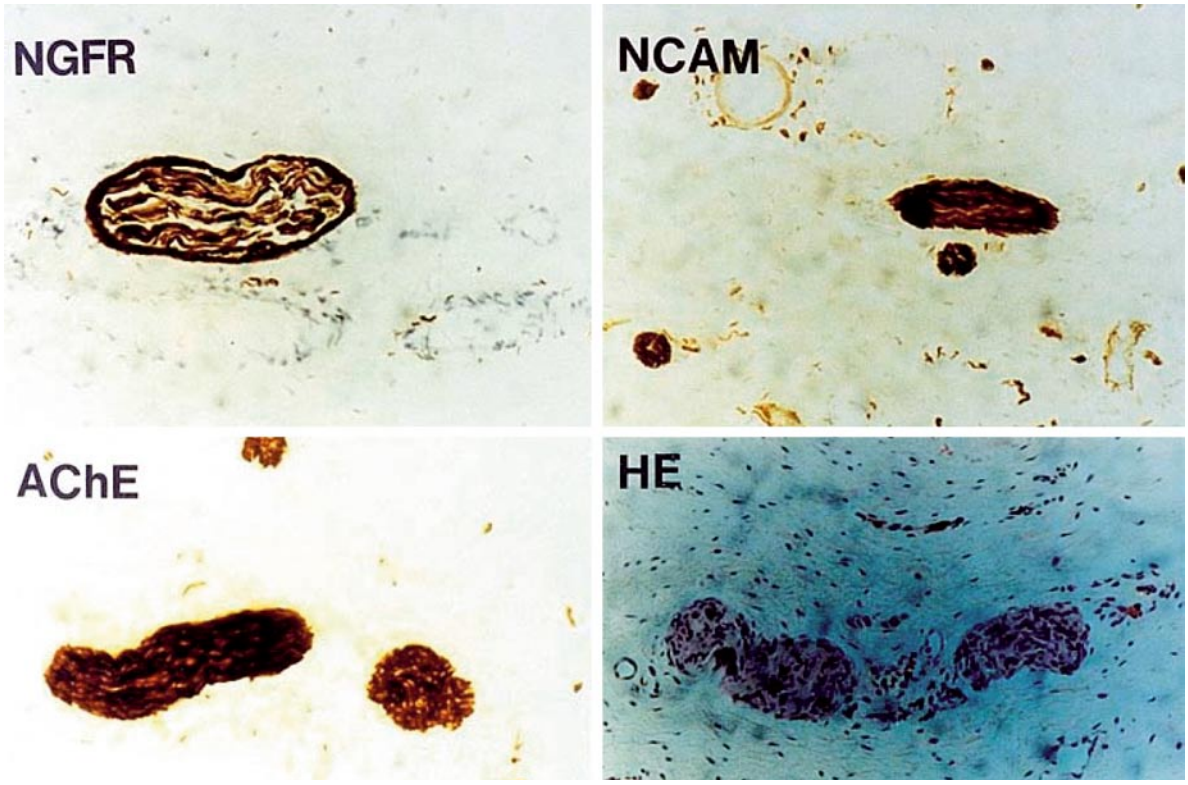

Fig. 15.5 Suction rectal biopsy. Staining with NGFR, NCAM, AChE and HE reveals submucous hypertrophic nerve trunk with perineurium only stained with NGFR

submucosal and muscularis externa, but mucosal fibers are not ChAT-immunoreactive [54]. This finding is surprising since the increased number of AChE-positive nerve fibers in the mucosal layers serves usually as a diagnostic marker in HD. A recent study using a rapid immunohistochemical technique has revealed that AChE and ChAT antibodies fail to determine cholinergic innervation [56].

15.3.2 Vesicular Acetylcholine Transporter

A very recent study has clearly shown that vesicular Ach transporter (VAChT) is a reliable marker of cholinergic neurons and nerve fibers within the ENS [57]. Furthermore, it has been shown that VAChT-positive cholinergic innervation is far more extensive than previously described in humans [57, 58]. VAChT offers the advantage of investigating cholinergic neurons of the ENS in paraffin-embedded tissue. So far no detailed study has been published using this antibody in the study of HD.

15.4 (Nor)Adrenergic markers (Tyrosine Hydroxylase/Dopamine β-Hydroxylase)

The (nor)adrenergic enzyme tyrosine hydroxylase (TH) has been shown to stain nerve fibers within normal bowel as well as HD bowel [59, 60]. Furthermore, abundant TH-positive hyperplastic fibers have been found in whole-mount preparations of aganglionic bowel [16]. A very recent study has revealed that TH stains normal perikarya of the human ENS whereas dopamine β-hydroxylase is absent from normal ganglion cells, but present in nerve fibers [57].

15.5 Non-adrenergic Non-cholinergic Markers

15.5.1 Nitric Oxide Synthase

Nitric oxide (NO) is the major inhibitory nonadrenergic noncholinergic (NANC) neurotransmitter in the gastrointestinal tract. NO is synthesized by the activation of neuronal NO synthase (NOS) [61]. NOS is abundant in

normal colon and ganglionic bowel of HD. Many nitrergic cells are localized in the myenteric plexus and within nerve fibers of the circular muscle. Submucosal nitrergic cells are mainly localized within the Schabadasch plexus [62]. NOS is colocalized with vasoactive intestinal polypeptide (VIP) in many of the ganglion cells of the myenteric plexus. In contrast, NOS is selectively absent from the plexus area and from the musculature of aganglionic bowel in HD, whereas moderate staining is observed in the hypertrophic nerve bundles in the submucosal layer [63]. These hypertrophic nerves also contain colocalized NOS/VIP-immunoreactive nerve fibers [62]. Numerous studies have shown the almost complete lack of neuronal NOS-immunoreactive nerve fibers in the aganglionic segment in patients with HD, which could prevent smooth muscle relaxation and might cause the lack of peristalsis in HD [61, 64–68].

15.5.2 Carbon Monoxide

Carbon monoxide (CO) is a neurotransmitter produced by heme oxygenase-2 (HO-2) in NANC neurons [69]. HO-2 immunoreactivities are found within the ganglion plexuses and intramuscular nerve fibers in normal bowel and normoganglionic HD bowel. HO-2-immunoreactive neurons have been specifically shown in the myenteric plexus. HO-2 is absent from the submucous and myenteric plexus of aganglionic bowel in HD which suggests that CO is involved in the pathophysiology of HD [70].

15.5.3 Pituitary Adenylyl-cyclase-activating Peptide

Pituitary adenylyl-cyclase-activating peptide (PACAP) acts via some of the VIP receptors [71]. PACAP-27 is capable of causing smooth muscle relaxation in the gut wall [72] and is a marker of NANC innervation.

15.5.4 Capsaicin and Purinergic Receptors

Capsaicin receptor has been isolated and named vanilloid receptor 1 (VR1). VR1 and the purinergic receptor (P2X3) are expressed by sensory neurons. Normal bowel contains VR1-immunoreactive fibers and nerve fascicles, but not cells. Hypertrophic nerves in HD display intense VR1-immunreactivity. P2X3-immunoreactive cell bodies have been detected in normal submucosal and myenteric plexus, whereas only weak P2X3 staining of hypertrophic nerves in HD has been found [73].

15.6 Neuropeptides

15.6.1 Vasoactive Intestinal Polypeptide

VIP is a NANC neurotransmitter [74]. Histological and physiological studies of the human colon have shown that VIP-positive nerve fibers in the circular and longitudinal muscle are inhibitory [75–78]. In a more detailed study, the population of VIP-immunoreactive fibers was 39% in the cecum and 63–65% in the transverse, descending, and sigmoid colon [79]. Further VIP-immunoreactive nerve cell bodies, nerve fibers and nerve endings are found throughout the ganglionic and oligoganglionic bowel in HD. The aganglionic segment of HD contains no VIP-immunoreactive nerve endings and the number of fibers is markedly reduced, and this might contribute to the constriction in the HD colon [80–83].

15.6.2 Substance P

Primary neurotransmitters of the motor neurons in the ENS are Ach and SP for excitatory, and VIP and NO for inhibitory functions [84]. SP has been identified as an excitatory neurotransmitter in human colon [85, 86]. The population of SP-immunoreactive fibers has been reported to be 15–21% throughout the human colon in humans [79]. SP seems to be absent from aganglionic bowel and reduced in IND [83, 87]. The defect of NANC innervation contributes to the motility disorder in HD and allied disorders.

15.6.3 Enkephalin and Gastrin-releasing Peptide

Enkephalin (Enk) and gastrin-releasing peptide (GRP) are part of the excitatory NANC neurotransmission [88]. These two neurotransmitters are moderately expressed in circular and longitudinal muscle of normal bowel. In contrast Enk and GRP are absent from aganglionic bowel and reduced in IND bowel [82, 83]. The reduced expression of NANC excitatory nerves may contribute to the disturbed muscle function in HD and IND.

15.6.4 Calcitonin Gene-related Peptide

The 37 amino acid neuropeptide calcitonin gene-related peptide (CGRP) plays a major role in many physiological and pathological regulatory functions of the ENS including the regulation of gastrointestinal smooth muscles and motility [89–92], sensory functions [93, 94], intestinal microcirculation [95, 96], secretion [97], amino acid absorption [98], lymphatic microcirculation and lymphocyte function [99, 100].

CGRP immunoreactivity is found in the ENS of sheep ileum, human small intestine and pig ileum in only one defined type of neuron, Dogiel type II cells, which are probably intrinsic primary afferent neurons [101]. There is moderate expression of CGRP-positive nerve fibers within normal bowel which does not differ substantially between ganglionic and aganglionic bowel [82].

15.6.5 Neuropeptide Y

The 36 amino acid peptide neuropeptide Y (NPY) is one of the major peptides in sympathetic neurotransmission [102, 103]. NPY-positive cells are observed in normal human submucosal and myenteric plexus, and a few additional NPY-positive fibers are found within the circular muscle. In contrast, much higher numbers of NPY-positive nerve fibers have been found in aganglionic bowel compared than in normal bowel, particularly in the circular muscle [82, 104]. Furthermore, in HD the concentration of NPY has been shown to be increased in both in the mucosa-submucosa and muscularis externa. These findings illustrate the hyperplasia of extrinsic NPY-positive aminergic fibers in HD [105].

15.6.6 Galanin

The neuropeptide galanin (GAL) is a 29 to 30 amino acid peptide which was originally isolated from porcine small intestine and is distributed within the central and peripheral nervous system [106–108]. In the ENS, GAL immunoreactivity is restricted to enteric nervous cells and nerve fibers [109, 110]. Galanin binds to specific receptors which subsequently causes relaxation and/or contraction [111–113] and regulation of intestinal fluid homeostasis [114, 115]. The expression of GAL-positive nerve fibers has been found to be not different or slightly reduced in HD bowel compared to normal bowel whereas a significant lack of GAL-positive structures has been observed in IND colon biopsies [82, 116, 117]. A recent study revealed an increased population of GAL receptor-positive, parasympathetic nerve fibers in the aganglionic segments of HD as compared to normal controls and IND [117]. This higher GAL receptor density especially in the submucosal layer of HD-affected segments seems to be due to increased parasympathetic activity.

15.7 Markers of Neuron-supporting Cells

15.7.1 S-100 Protein

S-100 proteins belong to a large subfamily of calcium-binding proteins which are evident in the cytoplasm and nucleus within several nervous and non-nervous tissues. As for many segments of the peripheral nervous system, the expression of S-100 proteins has been demonstrated mostly in the glial cells and Schwann cells of the enteric plexus [118]. Thus S-100 immunohistochemistry displays ganglion cells as prominent negatively stained cells surrounded by immunopositive Schwann cells (Fig. 15.6) [9, 10, 44]. S-100 antibody heterogeneously stains the whole hypertrophic nerve plexus in aganglionic bowel [119]. Although both S-100 and PGP9.5 antibodies detect nerve fibers in the mucosal layers of aganglionic bowel in HD, S-100 immunostaining appears to be more sensitive [12].

15.7.2 Glial Fibrillary Acidic Protein

Supportive cells of the ENS express glial fibrillary acidic protein (GFAP). GFAP immunoreactivity occurs predominantly in association with the myenteric plexus and to a lesser extent with the submucosal plexus of healthy colon. It has been suggested that the myenteric glia share the astroglial character of the central nervous system [44]. The extrinsic, hypertrophic nerve fasciculi of aganglionic bowel are selectively immunostained with GFAP. Therefore the demonstration of GFAP favors the diagnosis of HD [120].

15.8 Synaptic Markers

15.8.1 Synaptophysin

Synaptophysin is an integral membrane protein of the synaptic vesicles facing their cytoplasmatic surface [121]. This protein is an index of specific neuronal function such as storage and release of neurotransmitters. Synaptophysin is a marker of differentiating neuronal cells during prenatal life [19]. Synaptophysin stains submucosal ganglion cells [14].

There is markedly reduced immunoreactivity (i.e. a decreased number of SY-positive synapses) seen in the intestinal smooth muscle layers of transitional, aganglionic, and IND bowel segments, whereas immunoreactive synapses are abundant in the smooth muscle layers of ganglionic colon in HD. SY immunoreactivity also shows ganglion cells and hypertrophic nerve trunks clearly. Rapid SY staining is a simple and consistently reliable method for the intraoperative evaluation of the distribution of synapses in myenteric plexuses as well as smooth muscle layers [122].

Synaptophysin has also been used to study the intrinsic innervation in colonic dysganglionosis. This study showed a markedly decreased number of SY-immunoreactive nerve fibers within the aganglionic bowel and only weak staining of hypertrophic fibers with SY [35]. A later study also failed to detect synaptophysin immunoreactive hypertrophic fibers in aganglionic bowel of HD [119].

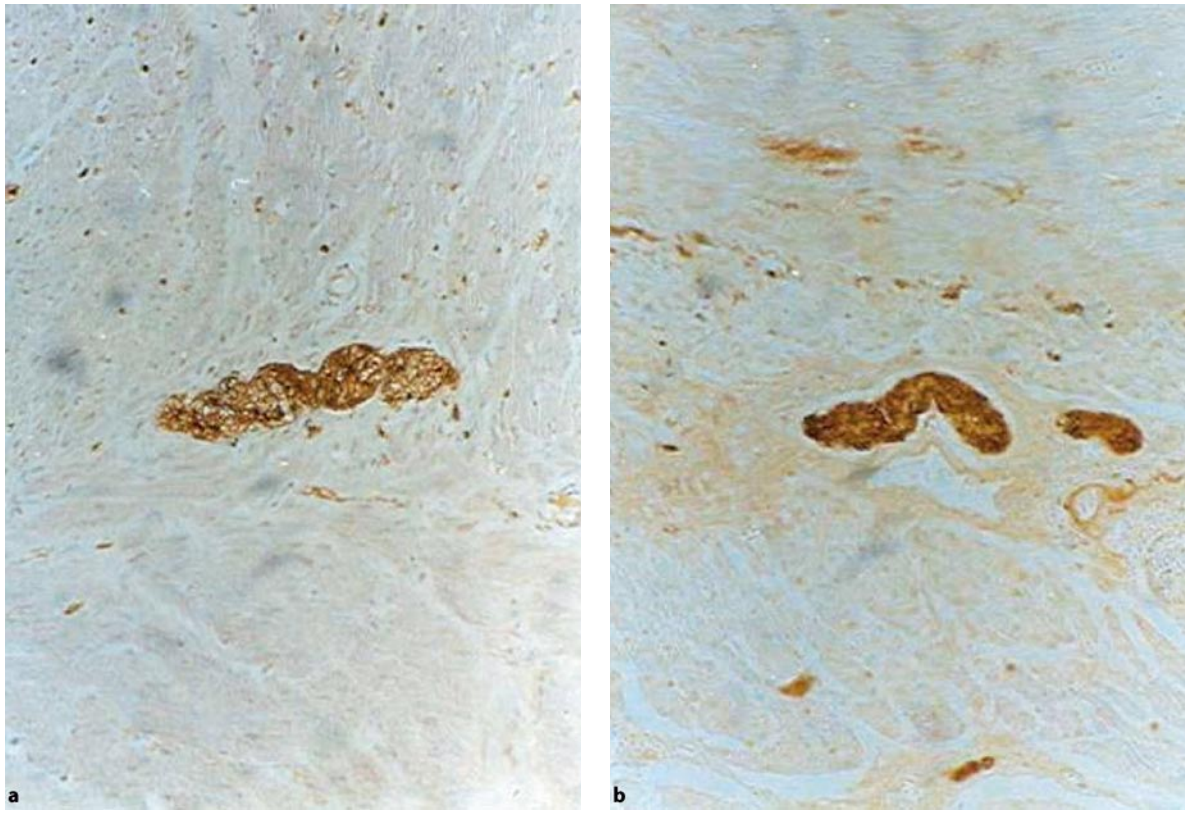

Fig. 15.6 S-100 immunostaining: **a** glial cells surrounding normal myenteric plexus; **b** glial cells around hypertrophic nerve fibers in HD

15.8.2 171B5 Protein

Synaptophysin and 171B5 proteins are specific membrane proteins of synaptic vesicles within synapses of the central and peripheral nervous system [121, 123]. Normal bowel shows a dense 171B5-immunoreactive innervation within the circular muscle and a rather weak innervation of nerve fibers within the longitudinal muscle [83]. In contrast, in aganglionic bowel 171B5 immunoreactivity can occasionally be demonstrated in synapses within the lamina propria but in none in the muscularis mucosae [124].

15.9 Specific Staining of Hypertrophic Nerve Fibers in HD

Enlarged submucosal nerve trunks are positively stained by VIP, galanin, NPY, and CGRP immunohistochemistry [125]. VR1 and P2X3 receptor antibodies stain a significant proportion of sensory nerves within the hypertrophic innervation of HD bowel [73].

15.10 Diagnostic and Clinical Use: Recommendations for Diagnosis

It seems to be important to discriminate between the use of immunohistochemistry in diagnosis and research into HD. The potential of immunohistochemistry in morphological and functional research of HD is almost unlimited. In contrast, the true value of immunohistochemistry in the diagnosis of HD seems to be limited. The major aspect of the histological diagnosis of HD is to display the defective innervation. For this reason a marker is needed which stains all existing ganglion cells, even immature and small cells. Furthermore, a reliable marker for hypertrophic extrinsic nerve fibers is necessary. Both of these markers are still missing.

The use of PGP9.5 and S-100 together has been recommended for immunohistochemical diagnosis of HD in formalin-fixed biopsies [12]. The combination of peripherin and S-100 staining has been recommended since peripherin reliably stains submucosal ganglia and S-100 enables the measurement of nerve fiber caliber [14]. Several antibodies, including neurofilament, synaptophysin, peripherin, neural cell adhesion molecule, positively stain ganglion cells [56].

A recent study has shown that the rapid immunohistochemical technique on frozen sections is not suitable for detection of ganglion cells or cholinergic innervation and is therefore not helpful in shortening the diagnosis time during surgery for HD [56]. VAChT antibodies have proved to be very effective in the staining of cholinergic ganglion cells and nerve fibers in paraffin sections. Therefore VAChT should be used in the diagnosis of HD if no frozen material is available.

References

1. De Lorjin F, Reitsma JB, Voskuijl WP, Aronson DC, Ten Kate FJ, Smets AMJB, Taminiau JAJM, Benninga MA (2005) Diagnosis of Hirschsprung's disease: a prospective, comparative accuracy study of common tests. J Pediatr 146:787–792
2. Karnovsky MJ, Roots L (1964) A "direct-coloring" thiocholine method for cholinesterase. J Histochem Cytochem 12:219–221
3. Lake BD, Puri P, Nixon HH, Claireaux AE (1978) Hirschsprung's disease. An appraisal of histochemically demonstrated acetylcholinesterase activity in suction rectal biopsy specimens as an aid to diagnosis. Arch Pathol Lab Med 102:244–247
4. Athow AC, Filipe MI, Drake DP (1990) Problems and advantages of acetylcholinesterase histochemistry of rectal suction biopsies in the diagnosis of Hirschsprung's disease. J Pediatr Surg 25:520–526
5. Moore SW, Johnson G (2005) Acetylcholinesterase in Hirschsprung's disease. Pediatr Surg Int 21:255–263
6. Marangos PJ, Zomzely-Neurath C, York C (1975) Immunological studies of a nerve specific protein. Arch Biochem Biophys 170:289–293
7. Pickel VM, Reis DJ, Marangos PJ, Zomzely-Neurath C (1976) Immunocytochemical localization of nervous system specific protein (NSP-R) in rat brain. Brain Res 105:184–187
8. Marangos PJ (1987) Neuron specific enolase, a clinically useful marker for neurons and neuroendocrine cells. Annu Rev Neurosci 10:269–295
9. Hall CL, Lampert PW (1985) Immunohistochemistry as an aid in the diagnosis of Hirschsprung's disease. Am J Clin Pathol 83:177–181
10. Barshack I, Fridman E, Goldberg I, Chowers Y, Kopolovic J (2004) The loss of calretinin expression indicates aganglionosis in Hirschsprung's disease. J Clin Pathol 57:712–716
11. Vinores SA, May E (1985) Neuron-specific enolase as an immunohistochemical tool for the diagnosis of Hirschsprung's disease. Am J Surg Pathol 9:281–285
12. Sams VR, Bobrow LG, Happerfield L, Keeling J (1992) Evaluation of PGP9.5 in the diagnosis of Hirschsprung's disease. J Pathol 168:55–58
13. Dzienis-Koronkiewicz E, Debek W, Sulkowska M, Chyczewski L (2002) Suitability of selected markers for identification of elements of the intestinal nervous system (INS). Eur J Pediatr Surg 12:397–401
14. Petchasuwan C, Pintong J (2000) Immunohistochemistry for intestinal ganglion cells and nerve fibres: aid in the diagnosis of Hirschsprung's disease. J Med Assoc Thai 83:1402–1409
15. Oh JT, Han A, Yang WI, Han SJ, Choi SH, Hwang EH (2002) Morphometric evaluation of PGP9.5 and NCAM expressing nerve fibres in colonic muscle of patients with Hirschsprung's disease. Yonsei Med J 43:31–36
16. Watanabe Y, Ito F, Ando H, Seo T, Kaneko K, Harada T, Iino S (1999) Morphological investigation of the enteric nervous system in Hirschsprung's disease and hypoganglionosis using whole-mount colon preparation. J Pediatr Surg 34:445–449
17. Kirschke H, Wiederanders B (1987) Lysosomal proteinases. Acta Histochem 82:2–4
18. Abu-Alfa AK, Kuan SF, West AB, Reyes-Mugica M (1997) Cathepsin D in intestinal ganglion cells: a potential aid to diagnosis in suspected Hirschsprung's disease. Am J Surg Pathol 21:201–205
19. Vannucchi MG, Midrio P, Zardo C, Faussone-Pellegrini (2004) Neurofilament formation and synaptic activity are delayed in the myenteric neurons of the rat fetus with gastroschisis. Neurosci Lett 364:81–85
20. Dahl D (1988) Early and late appearance of neurofilament phosphorylated epitopes in rat nervous system development: in vivo and in vitro study with monoclonal antibodies. J Neurosci Res 20:431–441
21. Tohyama T, Lee VMY, Rorke LB, et al (1991) Molecular milestones that signal axonal maturation and the commitment of human spinal cord precursor cells to the neuronal or glial phenotype in development. J Comp Neurol 310:1–15
22. Kluck P, van Muijen GN, van der Kamp AW, Tibboel D, van Hoorn WA, Warnaar SO, Molenaar JC (1984) Hirschsprung's disease studied with monoclonal antineurofilament antibodies on tissue sections. Lancet 24:642–654
23. Luider TM, van Dommelen MW, Tibboel D, Meijers JHC, Ten Kate FJW, Trojanowski JQ, et al (1992) Differences in phosphorylation state of neurofilament proteins in ganglionic and aganglionic bowel segments of children with Hirschsprung's disease. J Pediatr Surg 27:815–819
24. Deguchi E, Iwai N, Goto Y, Yanagihara J, Fushiki S (1993) An immunohistochemical study of neurofilament and microtubule-associated Tau protein in the enteric innervation in Hirschsprung's disease. J Pediatr Surg 28:886–890
25. Gorham JD, Baker H, Kegler D, Ziff EB (1990) The expression of the neuronal intermediate filament protein peripherin in the rat embryo. Dev Brain Res 57:235–248
26. Solari V, Piaseczna Piotrowska A, Puri P (2003) Histopathological differences between recto-sigmoid Hirschsprung's disease and total colonic aganglionosis. Pediatr Surg Int 19:349–354
27. Tam PKH, Boyd GP (1990) Origin, course, and endings of abnormal enteric nerve fibres in Hirschsprung's disease defined by whole-mount immunohistochemistry. J Pediatr Surg 25:457–461
28. Faussone-Pellegrini MS, Matini P, DeFelici M (1999) The cytoskeleton of the myenteric neurons during murine embryonic life. Anat Embryol 199:459–469
29. Tam PK, Owen G (1993) An Immunohistochemical study of neuronal microtubule-associated proteins in Hirschsprung's disease. Hum Pathol 24:424–431
30. Wattchow DA, Porter AJ, Brookes SJ, et al (1997) The polarity of neurochemically defined myenteric neurons in the human colon. Gastroenterology 113:487–506
31. Eledman GM (1985) Cell adhesion and the molecular processes of morphogenesis. Am Rev Biochem 54:135–169

32. Tosney KW, Watanabe M, Landmesser L, et al (1986) The distribution of NCAM in the chick hind limb during axon outgrowth and synaptogenesis. Dev Biol 114:437–452
33. Kobayashi H, O'Briain DS, Puri P (1994) Lack of expression of NADPH-diaphorase and neural cells adhesion molecule (NCAM) in colonic muscle of patients with Hirschsprung's disease. J Pediatr Surg 29:301–304
34. Kobayashi H, Hirikawa H, Puri P (1996) Abnormal internal anal sphincter innervation in patients with Hirschsprung's disease and allied disorders. J Pediatr Surg 31:794–799
35. Nogueira A, Campos M, Soares-Oliveira M, Estevao-Costa J, Silva P, Carneiro F, Carvalho JL (2001) Histochemical and immunohistochemical study of the intrinsic innervation in colonic dysganglionosis. Pediatr Surg Int 17:144–151
36. Doi T, Kobayashi H, Yamataka A, Lane GF, Miyano T (2005) Complete innervation profile of whole bowel resected at pull-through for Hirschsprung's disease. Unexpected findings. Pediatr Surg Int 21:889–898
37. Barde YA, Edgar D, Thoenen H (1980) Sensory neurons in culture: changing requirements for survival factors during development. Proc Natl Acad Sci U S A 77:1199–1204
38. Barde YA (1989) Trophic factors and neuronal survival. Neuron 2:1525–1534
39. Hefti F, Hartikka J, Salvatierra A, et al (1986) Localization of nerve growth factor receptors in cholinergic neurons of the human basal forebrain. Neurosci Lett 69:37–41
40. Kordower JH, Bartus RT, Bothwell M, et al (1988) Nerve growth factor receptor immunoreactivity in the nonhuman primate (Cebus apella): distribution, morphology, and colocalization with cholinergic enzymes. J Comp Neurol 277:465–486
41. Koliatsos VE, Clatterbuck RE, Nauta HW, et al (1991) Human nerve growth factor prevents degeneration of basal forebrain cholinergic neurons in primates. Ann Neurol 30:831–840
42. Thoenen H, Barde YA (1980) Physiology of nerve growth factor. Phys Rev 60:1284–1335
43. Piaseczna-Piotrowska A, Solari V, Puri P (2003) Distribution of Ca2+-activated K+ channels, SK2 and SK3, in the normal and Hirschsprung's disease bowel. J Pediatr Surg 36:978–983
44. Park SH, Min H, Chi JG, Park KW, Yang HR, Seo JK (2005) Immunohistochemical studies of pediatric intestinal pseudo-obstruction. Bcl2, a valuable biomarker to detect immature enteric ganglion cells. Am J Surg Pathol 29:1017–1024
45. Debas HT, Mulvihill SJ (1991) Neuroendocrine design of the gut. Am J Surg 161:243–249
46. Isaacs PET, Corbett CL, Riley AK, Hawker PC, Turnberg LA (1976) In vitro behaviour of acetyl choline ion transport. J Clin Invest 58:535–542
47. Mackenzie JM, Dixon MF (1987) An immunohistochemical study of the enteric neural plexi in Hirschsprung's disease. Histopathology 11:1055–1066
48. Costa M, Furness JB, Llewellyn-Smith IJ (1987) Histochemistry of the enteric nervous system. In: Johnson LR (ed) Physiology of the gastrointestinal tract. Raven Press, New York, pp 1–40
49. Bleys RLA, Groen GJ, Matthijssen MAH (1994) A method for identifying peripheral connections of perivascular nerves based on sensitive acetylcholinesterase staining via perfusion. J Histochem Cytochem 42:223–230
50. Schemann M, Sann H, Schaaf C, Mader M (1993) Identification of cholinergic neurons in enteric nervous system by antibodies against choline acetyltransferase. Am J Physiol 265:G1005–1009
51. Schemann M, Schaaf C, Mader M (1995) Neurochemical coding of enteric neurons in the guinea pig stomach. J Comp Neurol 353:161–178
52. Mann PT, Furness JB, Pompolo S, Mader M (1995) Chemical coding of neurons that project from different regions of intestine to the coeliac ganglion of the guinea pig. J Autonom Nerv Syst 56:15–25
53. Ratcliffe EM, deSa DJ, Dixon MF, Stead RH (1998) Choline acetyltransferase (ChAT) immunoreactivity in paraffin sections of normal and diseased intestines. J Histochem Cytochem 46:1223–1231
54. Nakajima K, Tooyama I, Yasuhara O, Aimi Y, Kimura H (2000) Immunohistochemical demonstration of choline acetyltransferase of a peripheral type (pChAT) in the enteric nervous system of rats. J Chem Neuroanat 18:31–40
55. Beschorner R, Mittelbronn M, Bekure K, Meyermann R (2004) Problems in fast intraoperative diagnosis in Hirschsprung's disease. Folia Neuropathol 42:191–195
56. Anlauf M, Schäfer MKH, Eiden L, Weihe E (2003) Chemical coding of the human gastrointestinal nervous system: cholinergic, VIPergic, and catecholaminergic phenotypes. J Comp Neurol 459:90–111
57. Porter AJ, Wattchow DA, Brookes SJ, Schemann M, Costa M (1996) Choline acetyltransferase immunoreactivity in the human small and large intestine. Gastroenterology 111:401–408
58. Larsson LT, Malmfors G, Ekblad E, Ekman R, Sundler F (1991) NPY hyperinnervation in Hirschsprung's disease: both adrenergic and nonadrenergic fibers contribute. J Pediatr Surg 26:1207–1214
59. Shen Z, Larsson LT, Malmfors G, Oberg K, Eriksson B, Sundler F (1994) Chromogranin A and B on neuronal elements in Hirschsprung's disease: an immunocytochemical and radioimmunoassay study. J Pediatr Surg 29:1293–1301
60. Takahashi T (2003) Pathophysiological significance of neuronal nitric oxide synthase in the gastrointestinal tract. J Gastroenterol 38:421–430
61. Guo R, Nada O, Suita S, Taguchi T, Masumoto K (1997) The distribution and co-localization of nitric oxide synthase and vasoactive intestinal polypeptide in nerves of the colons with Hirschsprung's disease. Virchows Arch 430:53–61
62. Vanderwinden JM, De Laet MH, Schiffmann SN, Mailleux P, Lowenstein CJ, Snyder SH, Vanderhaeghen JJ (1993) Nitric oxide synthase distribution in the enteric nervous system of Hirschsprung's disease. Gastroenterology 105:969–973
63. Bealer JF, Natuzzi ES, Flake AW, Adzick NS, Harrison MR (1994) Effect of nitric oxide on the colonic smooth muscle of patients with Hirschsprung's disease. J Pediatr Surg 29:1025–1029
64. Hanani M, Louton V, Udassin R, Freund HR, Karmeli F, Rachmilewitz D (1995) Nitric oxide-containing nerves in bowel segments of patients with Hirschsprung's disease. J Pediatr Surg 30:818–822
65. Tomita R, Munakata K, Kurosu Y, Tanjoh K (1995) A role of nitric oxide in Hirschsprung's disease. J Pediatr Surg 30:437–440

66. Larsson LT, Shen Z, Ekblad E, Sundler F, Alm P, Andersson KE (1995) Lack of neuronal nitric oxide synthase in nerve fibers of aganglionic intestine: a clue to Hirschsprung's disease. J Pediatr Gastroenterol Nutr 20:49–53
67. Teromata M, Domoto T, Tanigawa K, Yasui Y, Tamura K (1996) Distribution of nitric oxide synthase-containing nerves in the aganglionic intestine of mutant rats: a histochemical study. J Gastroenterol 31:214–223
68. Zakhary R, Poss KD, Jaffrey SR, et al (1997) Targeted gene deletion of heme oxygenase 2 reveals neural role for carbon monoxide. Proc Natl Acad Sci U S A 94:14848–14853
69. Chen Y, Lui VCH, Sham MH, Tam PKH (2002) Distribution of carbon monoxide-producing neurons in human colon and on Hirschsprung's disease patients. Hum Pathol 33:1030–1036
70. Masuo Y, Ohtaki T, Masuda Y, Tsuda M, Fujino M (1992) Binding sites for pituitary adenylate cyclase activating polypeptide (PACAP): comparison with vasoactive intestinal polypeptide (VIP) binding site localization in rat brain sections. Brain Res 575:113–123
71. Mungan Z, Arimura A, Ertan A, Rossowski WJ, Coy DH (1992) Pituitary adenylate cyclase-activating polypeptide relaxes rat gastrointestinal smooth muscle. Scand J Gastroenterol 27:375–380
72. Facer P, Knowles CH, Tam PKH, Ford N, Dyer N, Baecker PA, Anand P (2001) Novel capsaicin (VR1) and purinergic (P2X3) receptors in Hirschsprung's intestine. J Pediatr Surg 36:1679–1684
73. Grider JR, Makhlouf GM (1986) Colonic peristaltic reflex: identification of vasoactive intestinal peptide as mediator of descending relaxation. Am J Physiol 251:G40–G45
74. Domoto T, Bishop AE, Oki M, et al (1990) An in vitro study of the projections of enteric vasoactive intestinal polypeptide-immunoreactive neurons in the human colon. Gastroenterology 98:819–827
75. Faussone-Pellegrini MS, Bacci S, Pantalone D, et al (1993) Distribution of VIP-immunoreactive nerve cells and fibers in the human ileocoecal region. Neurosci Lett 157:135–139
76. Ferri G, Adrian TE, Ghatei MA, et al (1983) Tissue localization and relative distribution of regulatory peptides in separated layers from the human bowel. Gastroenterology 84:777–786
77. Wattchow DA, Brookes SJH, Costa M (1995) The morphology and projections of retrograde labelled myenteric neurons in the human intestine. Gastroenterology 109:866–875
78. Uemura S, Hurley MR, Hutson JM, Chow CW (1998) Distributions of substance P- and VIP-immunoreactive nerve fibres in the colonic circular muscle in children. Pediatr Surg Int 14:66–70
79. 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
80. Tsuto T, Okamura H, Fukui K, Obata-Tsuto HL, Terubayashi H, Yanagihara J, et al (1985) Immunohistochemical investigations of gut hormones in the colon of patients with Hirschsprung's disease. J Pediatr Surg 20:266–270
81. Larsson LT, Malmfors G, Sundler F (1988) Neuropeptide Y (NPY), calcitonin gene-related peptide (CGRP) and galanin in Hirschsprung's disease – an immunocytochemical study. J Pediatr Surg 23:342–345
82. Munakata K, Tomita R, Kurosu Y (1997) Preliminary Immunohistochemical new findings in the myenteric plexus of patients with intestinal neuronal dysplasia type B. Eur J Pediatr Surg 7:21–29
83. Furness JB, Bornstein JC, Pompolo S, et al (1995) Plurichemical transmission and chemical coding of neurons in the digestive tract. Gastroenterology 108:554–563
84. Grider JR (1989) Identification of neurotransmitters regulating intestinal peristaltic reflex in humans. Gastroenterology 97:1414–1419
85. Wattchow DA, Furness JB, Costa M (1988) Distribution and coexistence of peptides in nerve fibres of external muscle of the human gastrointestinal tract. Gastroenterology 95:32–41
86. Larsson LT, Sundler F (1990) Neuronal markers in Hirschsprung's disease with special reference to neuropeptides. Acta Histochem Suppl 38:115–125
87. Furness JB, Costa M (1987) The enteric nervous system. Churchill Livingstone, Edinburgh
88. Palmer JM, Schemann M, Tamura K, Wood JD (1986) Calcitonin gene-related peptide excites myenteric neurons. Eur J Pharmacol 132:163–170
89. Bartho L, Lembeck F, Holzer P (1987) Calcitonin gene-related peptide is a potent relaxant of intestinal muscle. Eur J Pharmacol 135:449–451
90. Rasmussen TN, Gregersen H, Harling H, Holst JJ (1992) Calcitonin gene-related peptide: effect on contractile activity and luminal cross-sectional area in the isolated, perfused porcine ileum. Scand J Gastroenterol 27:787–792
91. Grider JR (1994) CGRP as a transmitter in the sensory pathway mediating peristaltic reflex. Am J Physiol 266: G1139–1145
92. Sternini C (1991) Tachykinin and calcitonin gene-related peptide immunoreactivities and mRNAs in the mammalian enteric system and sensory ganglia. Adv Exp Med Biol 298:39–51
93. Rasmussen TN, Schmidt P, Poulsen SS, Holst JJ (2001) Localisation and neural control of the release of calcitonin gene-related peptide (CGRP) from the isolated perfused porcine ileum. Regul Pept 98:137–143
94. Vanner S (1994) Co-release of neuropeptides from capsaicin-sensitive afferents dilates submucosal arterioles in the guinea-pig ileum. Am J Physiol 267:G223–G230
95. Kawasaki H (2002) Regulation of vascular function by perivascular calcitonin gene-related peptide-containing nerves. Jpn J Pharmacol 88:39–43
96. Tache Y (1992) Inhibition of gastric acid secretion and ulcers by calcitonin gene-related peptide. Ann N Y Acad Sci 657:240–247
97. Barada KA, Saade NE, Atweh SF, Khoury CI, Nassar CF (2000) Calcitonin gene-related peptide regulates amino acid absorption across rat jejunum. Regul Pept 90:39–45
98. Ichikawa S, Shiozawa M, Iwanaga T, Uchino S (1991) Immunohistochemical demonstration of peptidergic nerve fibers associated with the central lacteal lymphatics in the duodenal villi of dogs. Arch Histol Cytol 54:241–248

99. Ichikawa S, Dreedharan SP, Goetzl EJ, Owen RL (1994) Immunohistochemical localization of peptidergic receptors in Peyer's patches of the cat ileum. Regul Pept 54:385–395
100. Chiocchetti R, Grandis A, Bombardi C, Lucchi ML, Dal Lago DT, Bortolami R, Furness JB (2006) Extrinsic and intrinsic sources of calcitonin gene-related peptide immunoreactivity in the lamb ileum: a morphometric and neurochemical investigation. Cell Tissue Res 323:183–196
101. Tatemoto K, Carquist M, Mutt M (1982) Neuropeptide Y – novel brain peptide with structural similarities to peptide YY and pancreatic polypeptide. Nature 296:659–660
102. Lundberg JM, Terenius L, Hökfelt T, Goldstein M (1983) High level of neuropeptide Y in peripheral noradrenergic neurons in various mammals including man. Neurosci Lett 42:167–172
103. Hamada Y, Bishop AE, Federici G, Rivosecchi M, Talbot IC, Polak JM (1987) Increased neuropeptide Y immunoreactive innervation of aganglionic bowel in Hirschsprung's disease. Virchows Arch A 411:369–377
104. Koch TR, Roddy DR, Carney JA, Telander RL, Go VL (1988) Distribution, quantitation, and origin of immunoreactive neuropeptide Y in the human gastrointestinal tract. Regul Pept 21:309–319
105. Tatemoto K, Rokaeus A, Jornvall H, McDonald TJ, Mutt V (1983) Galanin – a novel biologically active peptide from porcine intestine. FEBS Lett 164:124–128
106. Melander T, Hokfelt T, Rokaeus A, Fahrenkrug J, Tatemoto K, Mutt V (1985) Distribution of galanin-like immunoreactivity in the gastro-intestinal tract of several mammalian species. Cell Tissue Res 239:253–260
107. Hoyle CH, Burnstock G (1989) Galanin-like immunoreactivity in enteric neurons of the human colon. J Anat 166:23–33
108. Bauer FE, Adrian TE, Christofides ND, Ferri GL, Yanaihara N, Polak JM, Bloom SR (1986) Distribution and molecular heterogeneity of galanin in human, pig, guinea pig, and rat gastrointestinal tracts. Gastroenterology 91:877–883
109. Melander T, Hokfelt T, Rokaeus A (1986) Distribution of galanin-like immunoreactivity in the rat central nervous system. J Comp Neurol 248:475–517
110. Bauer FE, Zintel A, Kenny MJ, Calder D, Ghatei MA, Bloom SR (1989) Inhibitory effect of galanin on postprandial gastrointestinal motility and gut hormone release in humans. Gastroenterology 97:260–264
111. Katsoulis S, Clemens A, Morys-Wortmann C, Schworer H, Schaube H, Klomp HJ, Folsch UR, Schmidt WE (1996) Human galanin modulates human colonic motility in vitro. Characterization of structural requirements. Scand J Gastroenterol 31:446–451
111. King SC, Slater P, Turnberg LA (1989) Autoradiographic localization of binding sites for galanin and VIP in small intestine. Peptides 10:313–317
113. Benya RV, Matkowskyi KA, Danikovich A, Hecht G (1998) Galanin causes Cl-secretion in the human colon. Potential significance of inflammation-associated NF-kappa B activation on galanin-1 receptor expression and function. Ann N Y Acad Sci 863:64–77
114. Homaidan FR, Tang SH, Donowitz M, Sharp GW (1994) Effects of galanin on short circuit current and electrolyte transport in rabbit ileum. Peptides 15:1431–1436
115. Larsson LT (1994) Hirschsprung's disease – immunohistochemical findings. Histol Histopathol 9:615–629
116. Berger A, Kofler B, Santic R, Zipperer E, Sperl W, Hauser-Kronberger C (2003) 125I-labeled galanin bindings sites in congenital innervation defects of the distal colon. Acta Neuropathol 105:43–48
117. Gonzalez-Martinez T, Perez-Pinera P, Diaz-Esnal B, Vega JA (2003) S-100 proteins in the human peripheral nervous system. Microsc Res Tech 60:633–638
118. Alpy F, Ritie L, Jaubert F, Becmeur F, Mechine-Neuville A, Lefebvre O, Arnold C, Sorokin L, Kedinger M, Simon-Assmann P (2005) The expression pattern of laminin isoforms in Hirschsprung's disease reveals a distal peripheral nerve differentiation. Hum Pathol 36:1055–1065
119. Kawana T, Nada O, Ikeda K (1988) An immunohistochemical study of glial fibrillary acidic (GFA) protein and S-100 protein in the colon affected by Hirschsprung's disease. Acta Neuropathol 76:159–165
120. Wiedenmann B, Franke WW (1985) Identification and localization of synaptophysin, an integral membrane glycoprotein of Mr 38,000 characteristic of pre-synaptic vesicles. Cell 41:1017–1028
121. Kobayashi H, Miyano T, Yamataka A, Lane GJ, Fujimoto T, Puri P (1997) Use of synaptophysin polyclonal antibody for the rapid intraoperative immunohistochemical evaluation of functional bowel disorders. J Pediatr Surg 32:38–40
122. Obata K, Kojima N, Nishiye H, Inoue H, Shirao T, Fujita SC, et al (1987) Four synaptic vesicle-specific proteins: identification by monoclonal antibodies and distribution in the nervous tissue and the adrenal medulla. Brain Res 404:169–179
123. Yamataka A, Miyano T, Urano M, Nishiye H (1992) Hirschsprung's disease: diagnosis using monoclonal antibody 171B5. J Pediatr Surg 27:820–822
124. Romanska HM, Bishop AE, Brereton RJ, Spitz L, Polak JM (1993) Immunocytochemistry for neuronal markers shows deficiencies in conventional histology in the treatment of Hirschsprung's disease. J Pediatr Surg 28:1059–1062