Alexander M. Holschneider
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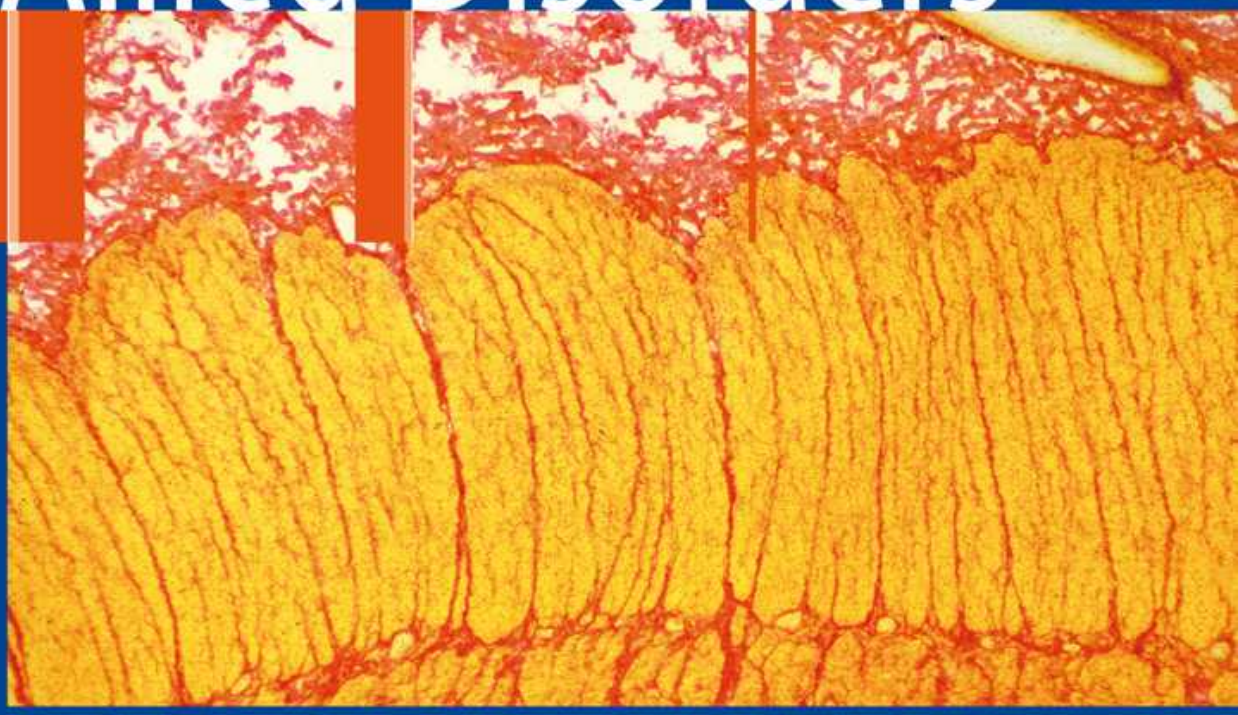
Editors

Hirschsprung's Disease and Allied Disorders



Third Edition

Springer

A. M. Holschneider · P. Puri (Eds.)

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Third Edition

With 318 Figures and 49 Tables

Springer

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Foreword

Drs. Holschneider and Puri have again given me the honor of writing the foreword to this magnificent new edition of their book.

This book will continue to be recognized as the most comprehensive and well-documented text ever written on this subject. This new edition expands the horizons of our knowledge of difficult and challenging conditions such as Hirschsprung's disease.

Dr. Grosfeld, a prestigious professor of pediatric surgery, was invited to write on the historical perspective of Hirschsprung's disease, and he has done so with a characteristically masterful style.

The chapter on the pathophysiology of Hirschsprung's disease is now written by Dr. Puri and Dr. Montedonico.

Dr. Moore has written a very interesting chapter on congenital anomalies and genetic associations in Hirschsprung's disease. The chapter on radiological diagnosis is now written by Dr. Kelleher.

This edition of the book characteristically continues to expand upon the genetic basis of the condition. Dr. Puri has been working in this particular area in the laboratory for many years, and we all grateful for his efforts and his contribution.

The chapter on immunohistochemical studies written by Dr. Rolle and and Dr. Puri summarizes the very exciting advances in this type of diagnosis.

An additional chapter by Dr. Milla on adynamic bowel syndrome expands our knowledge on the spectrum of motility disorders of the bowel and urinary tract.

Finally, Dr. Somme and Dr. Langer have written an additional chapter on the transanal pull-through procedure for the treatment of Hirschsprung's disease. There is no question that this new therapeutic approach represents a very important contribution to the treatment of this condition.

Again, we applaud the efforts of the editors in selecting a group of talented experts and innovators to contribute to what is still the best book on the subject.

Alberto Peña, MD

Preface

Hirschsprung's disease is one of the most important and most fascinating diseases in paediatric surgery. Our understanding of Hirschsprung's disease is developing rapidly, not only in relation to its pathophysiology and the development of new surgical techniques, but especially in relation to new genetic findings. A first comprehensive description of the pathophysiology, clinical symptoms, diagnosis and therapy of Hirschsprung's disease was outlined in 1970 by Theodor Ehrenpreis, Professor of Pediatric Surgery at the Karolinska Institute, Stockholm, Sweden, in a booklet entitled "Hirschsprung's Disease". The booklet of 176 pages was dedicated to Harald Hirschsprung (1830–1916) of Copenhagen, Denmark, and to Ovar Swenson of Chicago, Illinois, USA, the two pioneers in the study of Hirschsprung's disease. Harald Hirschsprung was a paediatrician, and Ovar Swenson a paediatric surgeon, who performed the first successful resection of an aganglionic bowel segment. That first book, published by Yearbook Medical Publishers, mainly discussed questions of postoperative continence based on the results of a large series of patients treated successfully at the Karolinska Institute.

In 1978 Ehrenpreis permitted one of the editors of the present edition to prepare an update of his internationally recognized book. Therefore, in 1982, a new book on Hirschsprung's disease by Alexander Holschneider was published by Hippokrates (Thieme-Stratton) with a foreword by Th. Ehrenpreis. It was a multiauthored textbook with particular prominence given to the results of an international clinical research study of the postoperative results in Hirschsprung's disease, undertaken from 1976 to 1978 by the author himself and a technical assistant, with special regard to the underlying surgical techniques. The follow-up studies were performed with the help of the Volkswagen Foundation in 16 paediatric surgical departments in Europe and the United States over a period of 3 years. The most interesting and unique aspect of this study was the fact that all clinical and electromanometrical investigations were performed by the same research team, independent of the staff of the individual hospital. As a result of this study concept, a most objective comparison of the results of Swenson's, Soave's, Duhamel's and Rehbein's techniques was achieved.

However, as our understanding of Hirschsprung's disease and associated motility disorders of the gut increased, a second edition of this book was published in 2000, this time by Harwood Academic Publishers, part of the Gordon and Breach Publishing Group. The title of this new book was changed to "Hirschsprung's Disease and Allied Disorders", because we included other enteric plexus disorders and smooth muscle disorders of the gut. The editors of this again multiauthored edition were Alexander Holschneider and Prem Puri. The book was divided into three parts: Physiology and Pathophysiology, Clinical Aspects, and Treatment and Results. As well as discussion of normal colonic motor function and the pathophysiology of classical Hirschsprung's disease, the book included special chapters on the development of the enteric nervous system, the functional anatomy of the enteric nervous system, animal models of aganglionosis, the molecular genetics of Hirschsprung's disease and the RET protein in human fetal development and in Hirschsprung's disease. New areas of special interest included intestinal neuronal dysplasia, particular forms of intestinal neuronal malformations, enterocolitis, megacystis-microcolon-intestinal hypoperistalsis syndrome, degenerative hollow visceral myopathy mimicking Hirschsprung's disease, and newer diagnostic techniques such as special neuronal markers, electron microscopy and anal sphincter achalasia. This second edition was the most comprehensive book ever published on Hirschsprung's disease and allied disorders.

With the passage of time, our understanding of enteric plexus disorders has exploded. Ehrenpreis in his preface of 1970 cited the President of the Swedish Nobel Prize Committee who stated that there are more scientists living today than during all past centuries. After having reviewed the recent literature on Hirschsprung's disease and allied disorders we are convinced that this is even more relevant today. Therefore, a new edition of Hirschsprung's disease and allied disorders was realized with the help of Springer. The previous chapters

"Clinical Generalities of Hirschsprung's Disease", "Disorders and Congenital Malformations associated with Hirschsprung's Disease", "Megacystis-Microcolon-Intestinal Hypoperistalsis Syndrome", "Degenerative Hollow Visceral Myopathy Mimicking Hirschsprung's Disease" and "Diagnosis of Hirschsprung's Disease and Allied Disorders" have been updated. A new separate chapter on "NAPDH-Diaphorase Histochemistry" has been introduced in the part "Diagnosis", next to the updated chapters "Histopathological Diagnosis and Differential Diagnosis of Hirschsprung's Disease", "Immunohistochemical Studies" and "Electron Microscopic Studies of Hirschsprung's Disease". For reasons of clarity, previously separated chapters such as the former chapters 5 and 6 "Molecular Genetics of Hirschsprung's Disease" and "Ret-Protein in Human Foetal Development and in Hirschsprung's Disease" have been brought together and concentrated in a new chapter. Chapter 3 "Functional Anatomy of the Enteric Nervous System" by M.D. Gershon and chapter 6 "Normal Colonic Motor Function and Relevant Structure" by J. Christensen have been reproduced. Chapter 12 "Particular Forms of Intestinal Neuronal Malformations" and chapter 14 "Megacolon in Adults" have become part of the new chapter 8 "Hirschsprung's Disease: Clinical Features" and chapter 18 "Neurocristopathies and Particular Associations with Hirschsprung's Disease". Chapter 17 "Intestinal Obstructions Mimicking Hirschsprung's Disease" has become chapter 21 "Adynamic Bowel Syndrome".

The chapters referring to the different surgical techniques have been updated too, but the concept of the previous editions, to compare the detailed description of one of the pioneer surgeons with the experience of a second author with the same technique, was given up. In the third edition of the book both parts of each chapter dealing with a specific surgical technique have been brought together to create new contributions for each of the different surgical approaches. The chapter "Laparoscopically Assisted Anorectal Pull-through" has been updated and a new chapter "Transanal Pull-through for Hirschsprung's Disease" has been introduced. Finally, the previous chapters dealing with early and late complications have also been brought together and the contribution of Teitelbaum and Coran on long-term results and quality of life has been updated.

The new edition is again a multiauthored book, and we have to thank all the internationally well-known authors and coauthors for their excellent and sophisticated contributions. It is their interest, help and effort that has again made possible the drawing together in one volume of the collective wisdom of many of the leading experts in Hirschsprung's disease and related disorders. Their contributions to this volume again provide a step forward in the elucidation of the genetic basis, and the correct diagnosis and treatment of this interesting disease and its allied disorders.

Besides the authors and coauthors, we would like to thank Mrs. Elisabeth Herschel of the Children's Hospital of Cologne, and the Children's Medical and Research Foundation, Our Lady's Children's Hospital, Dublin, for their support. Finally, we wish to thank the editorial staff of Springer, Heidelberg, Germany, particularly Ms. Gabriele Schroeder, for their interest and encouragement to publish a third edition of this book on a most important subject in paediatric surgery.

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1 Hirschsprung's Disease: A Historical Perspective — 1691–2005

J. L. Grosfeld

Hirschsprung's disease is a common cause of neonatal intestinal obstruction that is of great interest to pediatric surgeons throughout the world. Prior reports concerning the historical origins ascribe the initial description of this condition to Fredericus Ruysch, a Dutch anatomist in Amsterdam in 1691 [20, 33, 91, 137]. He described a 5-year-old girl with abdominal pain who did not respond to the "usual treatment of the day to relieve pain, pass wind and kill worms". She eventually died. The information regarding the patient was incomplete in regard to the events that occurred at the time of her birth and except for enormous dilatation of the colon, the autopsy findings were not clearly described. Although this may have represented a case of Hirschsprung's disease there was inadequate evidence to be sure of the actual diagnosis [33]. Similarly, Domenico Battini in Italy in 1800 described a child whom he followed for 10 years with severe constipation who eventually died and demonstrated severe colonic dilatation at autopsy consistent with, but not pathognomonic of, megacolon [39]. An additional report by Ebers in 1836 noted a 17-year-old boy with a history of constipation "since early youth" who died [33]. In 1869, Jacobi was the first to describe two newborn infants with intestinal obstruction that may have been attributable to congenital megacolon. One recovered after the administration of enemas; the other required a colostomy, that completely resolved the symptoms, but died of subsequent peritonitis [73]. No obstruction was found at autopsy and the colonic dilatation had disappeared.

Scattered reports concerning the autopsy findings in anecdotal cases of constipation in older children and adults that started at birth or early youth and progressed to intestinal obstruction appeared in the literature during the next 15 years [20, 33]. In 1884, Gee (as reported by Cass [20]) considered it possible, based on the findings of an autopsy of a 4-year-old child, that the condition was related to the presence of "spasm" of the sigmoid colon since the rectum was not involved in the typical dilatation and hypertrophy noted in his patient. In 1885, Bristowe described the course of an 8-year-old girl who died of intestinal obstruction after longstanding constipation. Her autopsy demonstrated dilatation of the colon and upper rectum that ceased abruptly 2 inches from the anus. No anal stricture or stenosis was observed [14]. This may have represented an instance of low segment Hirschsprung's disease.

While a number of other physicians reported instances of severe constipation and colon dilatation in children that eventually led to their demise, Harald Hirschsprung, a Danish pediatrician from Queen Louise Children's Hospital, Copenhagen, presented the most telling and concise description of congenital megacolon at the Society of Pediatrics in Berlin in 1886. His treatise was entitled "Constipation in newborns due to dilatation and hypertrophy of the colon" [33, 56]. At the time, he was unaware of the previous reports concerning the subject [33]. He presented the pathologic colon specimens and case reports of two infant boys who had symptoms of constipation soon after birth and who eventually died at 11 and 8 months, respectively. The first patient failed to pass stool at birth and required repeated enemas to relieve his obstruction. Constipation continued in the ensuing months despite breast feeding and was managed by laxatives. He was hospitalized for a 2-month period when he was 8 months old. Spontaneous bowel motions never occurred and the boy's abdomen was enormously distended. After a bowel motion was provoked, the distension decreased. Following discharge from the hospital he developed abdominal distension and frequent loose stools. He experienced rapid weight loss and was readmitted to the hospital and died the same day at 11 months of age. At autopsy, the sigmoid and transverse colon was enormously dilated and the muscle wall of the bowel was hypertrophied. The rectum was described as not being dilated and there was no site of narrowing. The second patient basically had the same presenting history of constipation from birth. He died at 8 months of age following the onset of severe abdominal distension and diarrhea (probably enterocolitis). At autopsy, the colon appeared similar to that of the first patient, but the appearance of the rectum was not described, although it was noted that the rectum was empty on digital examina-

tion. Hirschsprung's presentation was published in 1888 [56]. He neither offered a method of treatment nor proposed an etiology for this condition.

In 1898, Treves described a patient with idiopathic dilatation of the colon. He treated the patient with colon irrigations and performed a rectosigmoid resection and colostomy [171]. He documented the presence of a "narrow distal rectum" and presumed that this was the cause of the obstruction (a fact that went unrecognized for many years) [171]. A year later (1899), Griffith published a collective review of 55 similar cases in the literature [48]. In 1900, Fenwick attributed the findings in infants with hypertrophy and dilatation of the colon to "spasm of the anal sphincters" [38]. The same year, Lennander was the first to suggest a neurogenic origin for this condition. He observed megasigmoid in the absence of mechanical obstruction in a 4-year-old boy and interpreted the findings as due to "deficient innervation" and treated the boy successfully with faradic (electric) enemas [92]. In 1901, Tittel in Austria is credited with the first histologic study suggestive of Hirschsprung's disease noting sparse development of plexuses throughout the colon, but normal findings in the ileum [169]. Brentano corroborated these findings in a patient three years later [13].

In 1904 Hirschsprung described his personal experience with ten patients with this condition that he now referred to as "congenital dilatation of the colon". Nine of the ten patients were boys and five had died at the time of his report between 2 and 11 months of age. The other patients continued to have significant problems with constipation. The bowel was dilated and hypertrophied in each of the patients autopsied. There was no evidence of mechanical obstruction. The mucosa of the colon showed inflammatory changes and ulceration that Hirschsprung interpreted as the result of fecal retention. While he now considered the condition to be congenital in nature, he continued his fixation on the abnormally dilated and hypertrophied colon and still did not speculate on the etiology nor offer specific treatment. Hirschsprung's observations were published in 1904 as the first textbook chapter devoted to congenital dilatation of the colon in *Traite des maladies de l'enfance* (2nd edition) edited by Grancher and Comby. Shortly after, Hirschsprung retired from active practice because of cerebral stenosis and ultimately died in 1916 at 86 years of age.

Ehrenpreis indicated that Mya had actually originated the term megacolon congenita in 1894, and some years later the term Hirschsprung's disease was brought into use to describe the condition that Harald Hirschsprung so carefully described and brought into focus [33]. Although Hirschsprung was not a pediatric surgeon, in addition to his acclaim regarding congenital megacolon, he made other important contributions to the field of children's surgery in the areas of esophageal and intestinal atresia, pyloric stenosis and the non-operative management of intussusception [57, 58, 125, 170]. Interested readers are referred to additional publications concerning this unusual personality [12, 20, 40, 75, 93, 125, 134, 170].

With the world now more aware of this common condition, additional reports describing similar clinical findings began to appear in the literature. Many of these reports concerned adult patients with a short history of constipation and atypical or inadequate autopsy studies that likely had other diagnoses. In regard to surgical interventions, Perthes described transanal resection of the rectal folds and valves in 1905, and Finney in 1908 and Barington-Ward in 1915 reported "temporary success" following resection of the dilated bowel [6, 20, 33]. Patients continued to do poorly and the etiology of this condition remained elusive. In 1920, Dalla Valla shed new light on the subject when he reported the absence of ganglion cells in the sigmoid colon in two brothers who had normal ganglion cells in the proximal colon [24]. These observations were corroborated by Cameron 8 years later [15]. In 1923, Ishikawa noted the absence of parasympathetic nerves in the pelvic colon in a 4-year-old girl and he and others induced experimental megacolon in laboratory animals by resecting the parasympathetic nerves to the distal colon [1, 33, 70]. In 1927, Wade and Royle performed a lumbar sympathectomy to reduce sympathetic tone in the affected bowel in a patient who relapsed after a sigmoid resection [177]. Other reports appeared documenting the use of sympathectomy for this condition [2, 76, 126]. In the 1930s spinal anesthesia was also employed to treat the sympathetic hyperfunction that was presumed to be the cause of symptoms in patients with megacolon with some improvement noted [53]. In 1931, Irwin provided a careful description of Auerbach's plexus [69]. In the late 1930s and early 1940s clinical reports described some improvement in symptoms after administration of parasympathomimetic drugs to patients with megacolon [80]. In 1940, Tiffin and associates described local absence of ganglion cells in the myenteric plexus in a patient with congenital megacolon with ganglia present above and below the area in question [168].

Despite these observations, many authors including Ehrenpreis, refuted the evidence regarding sympathetic hyperfunction and for that matter any neurogenic disturbance as the cause of the disease [1, 32]. In 1943, Whitehouse et al. suggested that both medical and surgical attempts to ablate sympathetic tone were equally unsuccessful and recommended segmental resection of the dilated intestine as the most appropriate therapy [183]. In 1945, Grimson and colleagues similarly recommended a one-stage resection for "obstinate megacolon and ileosigmoidostomy" [49]. Ehrenpreis considered the loss of ganglion cells reported by others as a secondary event resulting from persistent colonic dilatation and stasis and in 1946, he defined Hirschsprung's disease as "a dysfunction of evacuation of the colon of as yet unknown origin,

occurring in the absence of morphological and mechanical causations giving rise secondarily to a characteristic dilatation of the colon" [32, 33].

Following the end of World War II in 1945, further light was shed on the subject that would dramatically change the course for children with Hirschsprung's disease. In 1948, Drs. Swenson, Neuhauser (a radiologist) and Pickett in Boston using a barium enema and fluoroscopy, recognized an area of spasm in the rectum or rectosigmoid that defined the site of obstruction in patients with congenital megacolon [155]. This established the barium enema as a useful diagnostic tool in Hirschsprung's disease. In six patients, Swenson and Bill performed a life-saving proximal colostomy that relieved obstructive symptoms. This improvement following colostomy was similar to the observations made by Jacobi in 1869 and Treves in 1898 [73, 154, 158, 171]. Closure of the colostomy in three of the infants resulted in recurrence of obstructive symptoms. These astute clinical observations led to the decision to resect the colon from a point proximal to the abnormal area of obstruction identified on the barium studies and the narrow distal rectum (now recognized as the site of physiologic obstruction) and perform a coloanal anastomosis above the dentate line to preserve continence. This was a historic landmark event, the first successful operative procedure for Hirschsprung's disease—the Swenson procedure [154]. The procedure was initially developed in the experimental surgical laboratory at Boston Children's Hospital and then applied in the clinical setting. The operation was undertaken based on careful clinical observations and thoughtful deduction ignoring the controversy at the time regarding the influence of bowel innervation and the presence or absence of ganglion cells in this disorder [155, 158, 159].

That same year, Zuelzer and Wilson described the autopsy findings in 11 infants who died of Hirschsprung's disease [193]. No mechanical cause of obstruction was noted. All 11 had absence of ganglion cells in the distal segment with six having a recognizable definitive level of obstruction. They suggested that Hirschsprung's disease was a functional intestinal obstruction that had a congenital neurogenic basis and that an enterostomy should be considered [193]. Also in 1948, Whitehouse and Kernohan described the autopsy findings in 11 children who died of megacolon [184]. None had ganglion cells present and nonmyelinated nerve trunks between the longitudinal and circular muscle layers were identified in the distal bowel. They noted variations in the length of the transition zone between the aganglionic distal rectum and when normal ganglion cells were noted proximally [184].

In 1949, Bodian et al. reviewed 73 patients who presented with findings consistent with congenital megacolon [7]. In 39 patients he confirmed the diagnosis of Hirschsprung's disease by recognizing the presence of a spastic segment in the rectosigmoid and noting absence of ganglion cells in the spastic distal segment. The 34 patients who did not fit these criteria were labeled as "idiopathic cases" [7]. These findings may explain the controversy noted in early reports concerning the presence or absence of ganglion cells, and finally separated patients with Hirschsprung's disease from those with other motility disturbances and causes of colonic dilatation. In 1951, Bodian reported the first instance of aganglionosis affecting the entire bowel from the duodenum to the rectum [8]. All of these studies reaffirmed the importance of Dalla Valla's original report in 1920 describing absence of ganglion cells [24]. In 1951, Hiatt performed manometric studies in patients with Hirschsprung's disease and confirmed that the abnormal distal segment was the area of obstruction. The rectum lacked peristaltic activity but showed mass contraction and there was loss of anorectal relaxation of the internal anal sphincter [55].

Although Swenson's operation now provided surgeons with a satisfactory method to treat Hirschsprung's disease, some considered this a tedious operation and the results were not quite as good in other people's hands. Alternative procedures were sought. In 1952, State (Minneapolis, Minnesota) described the use of a low anterior resection to manage this condition [151]. The operation left considerable residual aganglionic tissue in place frequently causing recurrence of symptoms and was ultimately abandoned. In 1953, Sandegard in Sweden reported the first successful operation in a patient with total colonic aganglionosis (TCA) by performing a total colectomy and an ileoanal anastomosis [138]. In 1956, Bernard Duhamel of St Denis, France, described the retrorectal transanal pull-though procedure for the treatment of Hirschsprung's disease [30]. This concept was developed to preserve the nerves to the bladder and nervi erigente and left the aganglionic rectum in place. The normal proximal bowel was brought down to the perineum through an incision 1.0 cm above the dentate line in the posterior rectal wall. Since that time numerous modifications have been employed to alter the location of the anal incision to preserve part of the internal anal sphincter to avoid incontinence and to ablate the residual blind aganglionic rectal pouch to avoid the development of an obstructing fecaloma.

In 1960, Grob in Zurich, Switzerland, used a different location for the posterior incision. He made the incision 2.0–2.5 cm above the pectinate line, but this resulted in constipation [50]. Pagès in Paris made the rectal incision 1.5 cm above the pectinate line to avoid incontinence and constipation [116]. A variety of clamps and subsequently stapling devices were employed to divide the colorectal spur comprising the posterior wall of the aganglionic rectal stump and the anterior wall of the normally innervated pull-through segment by Martin, Ikeda, Soper and Miller and Steichen et al. [67, 100, 101, 150, 152]. In 1958, Rehbein of Bremen, Germany, reported his experience with low anterior resection taking the anastomosis

down to 3–4 cm above the pectinate line [128]. This procedure was associated with an increased anastomotic leak rate and significant constipation, but is still used in some German-speaking countries.

In 1963, Soave of Genoa, Italy, described the endorectal pull-through procedure bringing the innervated bowel down to the perineum through a muscular sleeve of the aganglionic rectum [149]. Performing the mucosal stripping dissection within the muscle wall reduced the risk of injury to the nerves to the bladder and nervi erigentes. The original Soave procedure left the pulled through bowel segment extending from the anal opening. After a period to allow adherence of the bowel to the anal tissues, the protruding segment was resected [149]. The preservation of the muscular sleeve was not an original technique as it had been described by Hochenegg in Austria in 1898, and was used by Ravitch in an adult patient with a benign colonic conditions in 1948 [59, 127]. Similarly, Kiesewetter used the concept during repair of high anorectal malformations [78]. Pellerin in France (1962) and Cutait in Brazil (1965) modified the endorectal technique by performing a delayed anastomosis, and in 1964 Boley (New York) further modified the procedure by performing a primary anastomosis at the time of the pull-through procedure [10, 23, 119].

Recognizing that the barium enema was not always diagnostic particularly in the neonate, in 1959 Swenson et al. described the full-thickness rectal biopsy to obtain material for a tissue diagnosis [156]. Shandling reported his experience with a simple punch biopsy to obtain tissue in 1960 [144]. That same year, Gherardi noted that the level of aganglionosis was similar in the submucosal and myenteric plexuses [45]. Bodian was the first to use a submucosal biopsy for the diagnosis of Hirschsprung's disease [9]. In 1965 Dobbins and Bill employed a suction rectal biopsy instrument to obtain tissue for diagnosis [29]. This was successfully employed by Campbell and Noblett in 1969, and was modified by Noblett later that year using a special suction biopsy tube [16, 114]. In 1968, Meir-Ruge confirmed the effective use of submucosal rectal biopsy in Europe [103]. In the current era suction rectal biopsy remains the preferred technique used to diagnose Hirschsprung's disease particularly in neonates and infants [165].

During the same period other investigators evaluated the diagnostic efficacy of anorectal manometrics in infants with Hirschsprung's disease [90, 142, 143]. The techniques measures resting anal canal pressures and determines if the normal anorectal reflex resulting in relaxation of the sphincter is present when the rectum is distended. Loss of the anorectal response is interpreted as being consistent with Hirschsprung's disease [113]. These studies were inconsistent in premature infants and some neonates because of perceived immaturity of the anorectal response and limitations in equipment sensitivity in this age group [63, 71, 94]. However, additional studies using advanced semiconductor technology and miniature probes have demonstrated a normal anorectal reflex in premature and full-term neonates [162].

Despite the ability of clinicians to histologically diagnose Hirschsprung's disease by confirming the absence of ganglion cells on rectal biopsy, there remained a significant number of children with conditions that resembled aganglionic megacolon but who had ganglion cells present on their specimens. This was the condition that Bodian referred to as "idiopathic megacolon" in his observations on the histology of Hirschsprung's disease in 1949 [7]. In 1971, Meier-Ruge in Switzerland published his classic article describing colonic neuronal dysplasia [103, 104]. The following year he described the benefit of acetylcholinesterase staining of the hypertrophied nerve fibers in the lamina propria and muscularis in the diagnosis of Hirschsprung's disease [105]. Special staining techniques that were employed to identify instances of hypoganglionosis, immaturity of the submucosal and myenteric plexuses and anorectal achalasia became commonplace in evaluating conditions that mimicked Hirschsprung's disease [141, 142].

Over the next three decades, numerous articles appeared in the literature regarding intestinal neuronal dysplasia (IND). The condition seemed to be common in Europe, but was a rare entity on the North American continent. Puri and associates and Scharli were advocates of Meir-Ruge's observations regarding IND and reported series of cases with this condition and other variants of Hirschsprung's disease [122–124, 140, 141]. IND is divided into two subtypes, A and B, with the former being quite rare and the latter far more common and can be treated conservatively in most cases. Puri and colleagues noted that IND can coexist with Hirschsprung's disease and might be responsible for the persistence of motility disturbances seen in some patients following pull-through operations [122]. Controversy surrounds this condition regarding whether it is a distinct primary entity or a secondary phenomenon resulting from stasis or obstruction.

Recently, Meir-Ruge and colleagues (2004) have reported follow-up studies in patients with IND-B [106]. IND–B was identified in 6% of their patients with Hirschsprung's disease and 2.3% of other children evaluated for chronic constipation. The criteria for diagnosis were a rectal biopsy obtained 8–10 cm above the pectinate line in which 15–20% of the ganglia were giant-sized and there were more than eight nerve cells in 30 sections of the same biopsy [106]. He considered the findings consistent with delayed maturation of the enteric nervous system (ENS) and recommended conservative management up to 4 years of age. In contrast, the authors suggested that children with hypoganglionosis required surgical intervention [106]. The precise management of IND in association with Hirschsprung's disease remains unclear.

In regard to anal achalasia, in 1934, Hurst considered that this was related to parasympathetic underactivity [65]. Others suggested this was a manifestation of very low segment Hirschsprung's disease. Thomas (1967) and Holschneider et al. (1976) performed a posterior sphincterotomy and Thomas (1970) and Lynn and van Heerdon (1975) recommended a transanal posterior rectal myectomy for those with low-segment disease [64, 95, 166, 167]. In 1990, Neilson and Yazbeck described five children with "ultra-short segment Hirschsprung disease" [110]. Each of the children had loss of anorectal reflex relaxation on manometry but ganglion cells were found on rectal biopsy. They responded to posterior sphincterotomy [110]. In 1994, Krebs and Acuna noted that internal sphincter pressures initially are reduced following sphincter myotomy, but with time they return to above normal levels [82]. Currently, the diagnosis of anal achalasia requires both a rectal biopsy showing the presence of ganglion cells and absence of anorectal reflex relaxation on manometric studies [165]. Puri and Rolle suggested this condition is associated with nitrergic nerve depletion and can be treated with internal sphincter myectomy [124]. Prato and associates have reported the benefit of myectomy in anal achalasia using a posterior sagittal approach [121]. This approach is the author's personal preference as well.

As experience was obtained, it became clear that Hirschsprung's disease is more common in boys and in 80–85% of patients aganglionosis is limited to the rectum and rectosigmoid. However, in 10% of patients aganglionosis extends to more proximal areas of the colon, and in 5–8% TCA is noted with proximal extension of the aganglionic segment to various levels of the small intestine. As noted above, Bodian documented the first instance of aganglionosis of the entire bowel in 1951 [8]. Talwalker's review on the subject in 1976 identified 11 patients [160]. Sporadic reports have documented even more rare extension of aganglionosis to the stomach and esophagus [178]. In 1985, Caniano et al. described an additional patient and noted that no intestinal distension, evidence of bowel obstruction or transition zone could be detected at laparotomy. In addition, a review of similar patients in the literature indicated that 33% pass meconium at birth and 25% do not demonstrate hypertrophied nerve fibers on histologic study [18]. In 1986, Rudin et al. described three neonates with absence of the entire ENS and described 13 additional patients from the literature [136].

As noted above, Sandegard performed the first successful operative repair of TCA with colon resection and ileoanal anastomosis in 1953 [138]. The morbidity and mortality with TCA was greater than in those with the typical rectosigmoid involvement [60, 68, 153]. In an effort to improve the absorptive capacity of the colon, in 1968, Martin described a modification of the Duhamel procedure utilizing a side-to-side anastomosis to the aganglionic colon up to the level of the splenic flexure [98]. In 1981, Kimura used an aganglionic right colon patch inserted in the antimesenteric surface of the ileum to slow transit and improve absorption following ileostomy. The patch was left in place at the time of the pull-through procedure [79]. Boley used the left colon as a patch in 1984 [11]. In 1982, Martin further revised his procedure for TCA by using the entire aganglionic colon [99]. This latter procedure was associated with severe enterocolitis and has subsequently been abandoned by most pediatric surgeons [36, 37, 165, 187]. Most recent reports suggest that reasonably good results can be achieved in TCA affecting the distal ileum up to the mid-small bowel using a standard modification of the Duhamel procedure, endorectal pull-through or a Swenson operation [37, 111, 153, 159, 165, 187]. Rintala and Lindahl and Lal et al. have suggested that an ileoanal J pouch or S pouch may also be of benefit in these patients [85, 133].

The outlook for extension of aganglionosis into the more proximal small bowel remains guarded. These children essentially have short bowel syndrome and frequently require long-term support with total parenteral nutrition (TPN). Escobar et al. [37], Kimura [79], Kottmeier et al. [81] and Nishijima et al. [112] have found the aganglionic patch procedure beneficial in this subset of patients; however, iron deficiency anemia is a late complication. In 1987, Ziegler described the concept of myotomy/myectomy of aganglionic bowel for patients with near total aganglionosis (NTAG) with less than 40 cm of normally innervated small bowel [191]. The concept of myotomy in Hirschsprung's disease was first described by Martin-Burden in 1927 [33] using the procedure in the rectosigmoid, and by Kasai et al. in 1971 [77] who performed myotomy of the intact aganglionic rectal segment following proximal colon resection. In 1993, Ziegler et al. reported the outcomes of 16 myotomy/myectomies for NTAG that had been performed at multiple centers [192]. At the time, 10 of 16 patients were still alive; however, only two were enterally independent. They suggested that myectomized aganglionic bowel has the capacity to adapt and absorb nutrients, and that the procedure may be viewed as a bridge to intestinal transplantation [192]. In 2000, Saxton et al. described their experience with seven patients with NTAG of the bowel. Only two of the seven survived despite the use of myectomy and aganglionic patch procedures. These adjunctive procedures were associated with a high complication rate [139].

In the 1990s intestinal transplantation became an option in the management of patients with NTAG of the small intestine. Instances complicated by TPN-induced liver failure are candidates for combined liver and bowel transplantation. In 1995, Tzakis et al. from Dr. Starzl's group in Pittsburgh, described a 16-month-old girl with extensive aganglionosis who had a successful combined liver/bowel transplantation and a Soave endorectal pull-through using donor descending colon [172]. In 1998,

Reyes et al. found that 4 of 55 children undergoing small bowel transplantation had Hirschsprung's disease [131]. In 1999, Goulet et al. described preliminary experience with small-bowel transplantation at the Enfants Malades Hospital in Paris. Four of 20 patients had Hirschsprung's disease with aganglionosis extending to the proximal jejunum [47]. In 2003, Revillon et al. from the same institution, reported an improved quality of life in three children with extensive aganglionosis who underwent successful combined liver/bowel transplantation and a subsequent pull-through procedure (two had a Duhamel procedure; one a Swenson procedure) [130]. Also in 2003, Sharif et al. from Birmingham, UK, reported a successful outcome in four of five infants with extensive aganglionosis (between 10–50 cm of normal jejunum remaining) and TPN-related liver failure following combined liver/bowel transplantation in four and an isolated small-bowel graft in one [145]. The authors stressed preservation of the aganglionic bowel and avoidance of extensive enterectomy to preserve the size of the abdomen for subsequent graft insertion. At present this group is recommending transplantation in patients with NTAG and severe TPN-related liver disease [145]. The long-term outcomes of children with Hirschsprung's disease and NTAG who undergo organ transplantation will have to be further assessed over time.

One of the major complications observed in children with Hirschsprung's disease, both prior to and after a pull-through operation, is enterocolitis. This was probably the cause of the demise of both of the infants described by Hirschsprung in his original report in 1886, and continued to be a problematic cause of morbidity and mortality over the next century. Swenson was the first to key in on the significance of this complication in babies with Hirschsprung's disease [157]. Enterocolitis is likely the result of functional obstruction and stasis [17, 163, 165]. The reported incidence of enterocolitis varies considerably, but is in the range 14–40% depending on the diagnostic criteria used [52, 163]. Enterocolitis is associated with explosive diarrhea (70%), vomiting (50%), fever (34%) and lethargy (27%) [163]. The diarrhea is often associated with abdominal distension suggesting an obstructive cause. Acute inflammatory infiltrates have been noted in the anal crypts and colon mucosa that may lead to crypt abscesses and mucosal ulceration. The exact etiology is still unknown, but impaired mucosal defense mechanisms have been implicated with deficiency in secretory IgA, absence of mucin precursors and muc-2 gene [4,163, 188]. Although enterocolitis has been observed after all of the procedures used to treat Hirschsprung's disease, the incidence is higher after a Soave pull-through (presumably because of a tight anastomosis or snug aganglionic muscular cuff), in patients with TCA (especially after a long Martin modification of the Duhamel procedure), and in infants with Down syndrome probably related to immunologic factors. These observations led to further operative modifications such as division of the posterior muscular cuff in the Soave procedure and abandoning the long Martin modification of the Duhamel procedure.

Aside from the availability of intestinal transplantation as a treatment option, the 1990s and the first few years of the 21st century has been the era of continued technical modifications with a trend toward one-stage procedures earlier in life using advances in minimally invasive technology, employing the transanal approach and managing treatment failures. In addition, this has been a time characterized by significant advances in understanding the ENS in general and the genetic basis of Hirschsprung's disease in particular due to a veritable explosion of new information especially following the elucidation of the human genome.

In 1981, So and colleagues were the first to report a one-stage pull-through procedure in neonates with Hirschsprung's disease without a preliminary colostomy [148]. In 1982, Carcassone and associates from Marseilles similarly described a favorable experience with a one-stage procedure in the first 3 months of life [19]. These reports refuted Swenson's contention that a definitive procedure in early infancy is associated with an increased morbidity and mortality. The one-stage approach became increasingly popular in the 1990s [51, 88, 164]. Georgeson et al. described a laparoscopically assisted Soave endorectal pull-through procedure avoiding an open laparotomy [42]. He adapted this to a primary procedure in 1999 [43]. Successful application of the laparoscopic technique has also been reported by pediatric surgeons performing the Swenson procedure [22, 61, 83] and modified Duhamel operation [25, 46, 147, 173]. In 1993, Rinatala and Lindahl of Helsinki described a predominantly transanal pull-through operation but performed a laparotomy to mobilize the proximal colon [132]. In 1998, de la Torre-Mondregon and Ortega-Salgado of Mexico were the first to perform a one-stage totally transanal pull-through procedure [26]. Results with the transanal endorectal pull-through were favorable when compared to the open procedure [27]. Since then, the transanal operation has been used extensively in the neonatal period by Langer et al. [86], Albanese et al. [3] and Teitelbaum et al. [164]. Three multicenter studies in Europe [62], North America [89] and Egypt [34] have supported the use of this approach.

The Swenson, modified Duhamel and Soave endorectal pull-through procedures all give satisfactory results and each has its advocates and detractors [30, 36, 89, 116, 129, 149, 154, 158, 159, 165, 175]. Each of the procedures has required modification since their inception in attempts to deal with subsequent postoperative complications [10, 54, 79, 100, 101, 157, 165, 166, 176, 179, 191]. Although most patients do well over time, aside from the previously mentioned instances of enterocolitis and IND, there are a subset of patients who have other recurring problems [36,

165, 174]. These include instances of "acquired" aganglionosis following a pull-through performed with normally innervated proximal bowel. These problems are likely related to ischemia of the pull-through segment and respond to a second pull-through procedure [21, 28, 182]. Similarly, occasional poor outcomes related to persistent postoperative stricture or severe obstipation also require a re-do pull-through procedure [83, 87, 174, 181, 185]. Persistent stooling problems have been treated with partial internal sphincterotomy, rectal myotomy/myectomy, botulinum toxin injections and topical nitric oxide [36, 107, 108, 157, 186].

While the exact etiology of Hirschsprung's disease is still unknown, the last two decades have provided new insights into the complexities of this condition and its variants. Hirschsprung's disease has been observed to co-exist with anorectal malformations, ileal atresia, colon atresia, achalasia of the esophagus and the Currarino syndrome [5, 41, 66, 74, 78, 146, 180]. A better understanding of the ENS and the molecular genetic basis of this disorder has provided a wealth of new information. Since the early studies of Okamoto and Ueda [115] on the embryogenesis and migration of the intraneural ganglia of the gut in 1967, many investigators have focused on uncovering the mysteries surrounding the ENS through genomic analysis of ENS and neural crest development, and migration and colonization of enteric neurons. The association of Hirschsprung's disease with other neurocristopathies is linked to various genetic disturbances. These include instances of Ondine's curse (Congenital central hypoventilation syndrome; PHOX-2B), Waardenburg-Shah syndrome (SOX-10), Mowat-Wilson syndrome (ZFHX1B), Goldberg-Shprintzen syndrome, Smith-Lemli-Opitz syndrome, MEN-2A and B, neuroblastoma, and ganglioneuromatosis of the bowel [97, 109, 120, 161, 165, 190].

While early studies by Passarge [118] and Engum and Grosfeld [35] identified familial instances of Hirschsprung's disease, it was the elucidation of the human genome that opened the door to the genetic basis of the disease. Collaboration between basic scientists, medical geneticists and pediatric surgeons led the way to these discoveries. In 1992 Martucciello et al. of Genoa reported the association of TCA with interstitial deletion of the long arm of chromosome 10 [102]. This was confirmed in 1993 by Angrist et al. [96] and Yin et al. [189] who described the close linkage of the RET protooncogene in autosomal dominant Hirschsprung's disease and by Pasini et al. in 1995 [117]. Mutations were identified in 50% of the patients from families with Hirschsprung's disease. Romeo et al. in 1994 identified point mutations affecting the tyrosine kinase domain of the RET protooncogene [135]. That same year Edery et al. [31] reported that loss of function of the RET protooncogene led to Hirschsprung's disease, whereas gain of RET function led to MEN-2B. Additional studies have uncovered genetic linkages involved in the development of the ENS. Most belong to the RET and endothelin signaling pathways. In 1995 Gershon demonstrated that endothelin and the endothelin-B receptor are necessary for the development of the ENS in the colon [44]. In 1997, Kusafuka et al. identified mutations in endothelin-B and endothelin-B receptor in isolated cases of Hirschsprung's disease [84]. Iwashita et al. noted that the glial cell line-derived neurotropic factor receptor (GDNF) RET is necessary for neural crest stem cell migration in the gut [72]. Gene expression profiling, reverse genetics and analysis of stem cell function have implicated neural crest stem cell function as the likely cause of Hirschsprung's disease [72]. These studies suggest that Hirschsprung's disease is a genetically complex and heterogeneous inborn error of neural crest cell development that may involve a number of mutations affecting different genes and signaling pathways and other biologic and molecular factors yet to be determined.

Since the clinical presentations by Harald Hirschsprung in Berlin in 1886, the condition that bears his name has had a rich history. The seminal events that influenced progress in the understanding and management of this complex congenital disorder have been briefly covered in this historical review. More than 100 years ago, the condition was considered incurable and uniformly fatal over time [20, 33]. Mortality rates continued to be high in the 1940s (70%) and remained high even in the 1970s (25%). By the 1990s more than 90% of patients survived [129]. At the time of writing (2005) the survival in most advanced medical environments is greater than 95% [165]. While mortality has improved, there remains much to be learned. Why some patients with Hirschsprung's disease do poorly following operative repair remains an enigma. Similarly, the proper management of many patients with variants of Hirschsprung's disease needs to be more clearly elucidated. Continued study of the ENS and the molecular genetics of these conditions may shed further light on these issues and provide a better understanding of the choice of management in the future for affected children.

Most of the early major contributors to the care of infants and children with Hirschsprung's disease are recognized herein posthumously with the exception of Dr. Orvar Swenson who is currently 98 years of age. He and his wife Melva reside in Charleston, South Carolina. Dr. Swenson remains alert and well and continues to publish his views regarding Hirschsprung's disease with the same fervor and passion that led to the performance of the first successful operation for this condition 59 years ago [154, 158, 159]. Similarly, Dr. Lester Martin is 82 years of age, in good health, living with his wife Joan in Washington Courthouse, Ohio, 43 years following his important modifications of Duhamel's retrorectal pull-through procedure [100, 101]. Space limitations prevent individual mention of many other deserving physicians who have made significant contributions to the care of children with Hirschsprung's disease.

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Development of the Enteric Nervous System

P. Puri and U. Rolle

2

2.1 Introduction

The enteric nervous system (ENS) is the largest and the most complex division of the peripheral nervous system [1]. The ENS contains more neurons than the spinal cord and is capable of mediating reflex activity in the absence of central nervous system. About 80–100 million enteric neurons can be classified into functional distinct subpopulations, including intrinsic primary neurons, interneurons, motor neurons, secretomotor and vasomotor neurons [2]. The ENS plays a crucial role in normal gastrointestinal motility. Therefore insights into the development of the gastrointestinal tract and the ENS are relevant for the understanding of the pathophysiology and treatment of infants and children with motility disorders.

2.2 Embryonic Origin of ENS

There are two major steps in the development of the gastrointestinal tract: (1) formation of the gut tube, and (2) formation of individual organs, each with their specialized cell types (Table 2.1) [3].

Gastrulation is an early step in the development of all multicellular organisms. During gastrulation the axes of the embryo are determined and the development of the gastrointestinal tract starts. Gastrulation gives rise to three germ layers, endoderm, mesoderm, and ectoderm [3]. The mammalian gastrointestinal system originates from all three embryonic germ layers. The epithelial lining of the gastrointestinal tube and the parenchymal cells of the liver and pancreas are formed by the endoderm. The mesoderm provides mesenchymal elements including smooth muscle and stromal cells. The neurons of the ENS which regulates gastrointestinal motility are derived from ectoderm.

The ectoderm divides into three types of cells; outer ectoderm, neural tube, and neural crest (NC). The NC arises from the dorsal region of the neural tube. Melanocytes, the adrenal medulla, the dentine of teeth, the sympathetic and parasympathetic arms of the peripheral nervous system, and the neurons of the ENS are derived form the NC. These tissues and cell types originate from

Table 2.1 Developmental milestones of human gastrointestinal tract

Developmental stage	Gestation week
Gastrulation	3
Gut tube largely closed	4
Liver and pancreas buds	4
Growth of intestines into cord	7
Intestinal villus formation	8
Retraction of intestines into abdominal cavity	10
Organ formation complete	12
Parietal cells detectable, pancreatic islets appear, bile secretion, intestinal enzymes detectable	12
Swallowing detectable	16 and 17
Mature motility	36

different regions of the NC, which means that the cells need to migrate to the site of the mature organs. The gene mutations that result in disrupted NC cell migration to one region also cause altered migration of other NC-derived tissues [4].

2.3 Origin and Development of Neural Crest-Derived Cells

The NC is located along the entire length of the body axis. Two groups of undifferentiated cells, derived from NCs, colonize the gut wall and migrate in craniocaudal and caudocranial directions.

The embryonic NC arises in the neural tube, originating with the central nervous system, but NC cells detach from this tissue via reduction of cell–cell and cell–matrix adhesion. The epitheliomesenchymal transformation allows NC cells to migrate along pathways of defined routes to various tissues, where they stop moving and differentiate into various cell types. Pathway selection is most likely achieved by balanced combinations of molecules that promote and reduce adhesions [5, 6]. NC cells give rise to neuronal, endocrine and paraendocrine, craniofacial, conotruncal heart, and pigmentary tissues. Neurocristopathies encompass tumors, malformations, and single or multiple abnormalities of tissues, mentioned above in various combinations [7].

In the human fetus, NC-derived cells first appear in the developing esophagus at the 5th week of gestation, and then migrate down to the anal canal in a craniocaudal direction during the 5th and 12th week of gestation. The NC cells first form the myenteric plexus just outside the circular muscle layer. The mesenchymally derived longitudinal muscle layer then forms, sandwiching the myenteric plexus after it has been formed in the 12th week of gestation. In addition, after the craniocaudal migration has ended, the submucous plexus is formed by the neuroblasts, which migrate from the myenteric plexus across the circular muscle layer and into the submucosa; this progresses in a craniocaudal direction during the 12th to 16th week of gestation [5]. The absence of ganglion cells in Hirschsprung's disease has been attributed to a failure of migration of NC cells. The earlier the arrest of migration, the longer the aganglionic segment is.

It is generally accepted that the enteric ganglion cells are derived primarily from the NC cells [8–11]. Studies in the avian system provide strong evidence for the contribution of the sacral NC to the hindgut ENS [12–14]. Whether the sacral NC contributes to the ENS in the mammalian hindgut is less clear. Failure of the vagal derived NC cells to colonize the hindgut results in failure of hindgut ENS development, suggesting that interaction between sacral and vagal enteric NC cells may be necessary for sacral NC cell contribution to the ENS [15].

Yntma and Hammond first performed NC ablations in chick embryos and identified the vagal NC (somites 1 to 7) as the source of the ENS stem cells [11]. Le Douarin and Teillet showed an additional source of NC stem cells originating from the lumbosacral region to colonize the gut [12]. Later the lumbosacral derived crest cells were found principally in the myenteric plexus, with very few in the submucous plexus. The number of these cells declines rostrally. Cells derived from the lumbosacral NC were never observed in any gut region above the umbilicus [14].

The colonization of the gut by sacral NC-derived cells and the contribution of the cells to the development of the ENS is controversial [16]. The dual origin of enteric neurons has been negated by studies on chick embryo as well as human embryo. Allen and Newgreen [17] isolated bowel segments from fowl embryos at various stages of development, and grew these segments in the chorioallantoic membrane and found that enteric neurons appeared in a craniocaudal sequence, showing a vagal source. Meijers et al. [18] transected the chicken bowel in ovo at an early stage, before the passage of NC cells had occurred, preventing craniocaudal migration of vagal NC cells. They found that the hindgut remained aganglionic, showing that there was no colonization by sacral NC cells.

Some studies have shown that sacral NC-derived cells migrate from the neural plate early in development and extraenteric pelvic ganglia. Later these cells are able to colonize the gut and contribute to the ENS, coincident with the migration of vagal NC-derived cells [14, 19–21]. In contrast, other studies suggest that sacral NC-derived cells invade the hindgut mesenchyme several days before the colonization of the hindgut by vagal NC cells and contribute to the development of ENS [13, 22–24].

In contrast the mouse ENS is derived embryologically from cells of the vagal, truncal, and sacral regions of the NC. The vagal NC originates in somites 1 to 5 in the mouse, the truncal NC from somites 6 and 7, and the sacral NC posterior to somite 28. Cells from each of these regions of the NC migrate into the developing gut by defined pathways. Cells of the vagal and truncal NC enter the foregut, migrating in a proximal to distal direction. Truncal NC cells populate only the foregut, whereas those of the vagal NC migrate more distally to colonize the rest of the gastrointestinal tract. Cells arising from the sacral crest seem first to colonize pelvic autonomic ganglia, from which they then migrate into the distal gut, colonizing it from distal to proximal [19].

The current concept is that the development of the ENS in humans is derived primarily from cells of the vagal segment of the NC [2, 12]. Fujimoto et al. [25] studied NC cell migration in the developing gut in the human embryo using antineurofilament protein triplet antibody and found that enteric ganglia originated from a single vagal NC source. The vast majority of studies have revealed that vagal NC cells provide the main source of enteric neurons and sacral NC additionally innervates the distal bowel [12–14, 26–28].

The final requirement for development and maturation of the ENS is the formation of ganglia. Several days after NC cells have colonized the gut these cells are evenly distributed, with no indication of cell clustering, except the cecum. As the gut later increases in length and diameter, the cells start forming ganglionic groups [29]. A previous study has shown that cells forming a ganglion do not arise from a single precursor cell [30]. A recent study used human fetal intestine to investigate nitrergic neurons in the developing myenteric plexus. The distribution of nitrergic neurons was found to change markedly between 14 and 22 weeks of gestation. Nitrergic neurons were randomly distributed at week 14 and were later aggregated in the plexus and within individual ganglia at week 19 [31]. It is currently not known what factors induce cells to cluster into ganglia.

2.4 Functional Development of the ENS

The complexity of mature ENS is exemplified by many different functional types of neurons containing various neurotransmitters occurring in various combinations. Types of neurotransmitters vary according to the time of their appearance [29, 32]. The development of the human enteric nervous system is characterized by the early appearance (between 9 and 12 weeks' gestation) of adrenergic and cholinergic nerves. Strong evidence has emerged that the enteric nervous system is not only composed of adrenergic and cholinergic nerves but also nonadrenergic, noncholinergic (NANC) autonomic nerves, which contain different peptides. These peptides act as neurotransmitters, or neuromodulators, or both. These nerves have been termed *peptidergic nerves*. The development of peptidergic innervation occurs much later.

In recent years, pharmacologic and physiologic studies have provided evidence that nitric oxide (NO) is the most important mediator in nonadrenergic, noncholinergic relaxation of the gastrointestinal tract. By 12 weeks' gestation, nitrergic neurons appear in the myenteric ganglia, at all levels of the gut, and begin plexus formation. Nitrergic innervation in the submucous plexus becomes evident after 14 weeks. As gestational age increases, nitrergic innervation becomes richer and more organized. Increasing numbers of nitrergic nerve fibers are seen in the circular muscle; some of these fibers project from the myenteric plexus. Thus, the onset and pace of development of nitrergic innervation are similar to adrenergic and cholinergic innervation and occur before peptidergic innervation [33].

Serotonin (5-HT) together with glucagon, insulin, peptide XY, gastrin, and somatostatin are the earliest neurohumoral substances to be expressed at about 8 weeks of gestation. By 24 weeks of gestation, most of the known gastrointestinal neurohumoral substances can be identified.

Further contacts between enteric nerves and effectors are developed at 26 weeks and the first signs of motility can be detected at 25 weeks of gestation [3].

2.5 Development of Intestinal Motility

The innervation of the gastrointestinal tract in utero is accompanied by functional activity of increasing complexity. The first studies to measure intestinal transit in humans used amniography; aboral transport of contrast agent did not occur in the intestinal tract of fetuses younger than 30 weeks of gestation [34]. With increasing gestational age, increasing aboral transit and rate of propagation develops. Subsequent studies of gastrointestinal motility in premature infants have been performed using intraluminal catheters [35]. The data from these studies reveal no regular periodicity or rhythmicity at 25 weeks of gestation. Further development occurs during the next 15 weeks, so that by term, mature motor patterns of the gastrointestinal tract are well established. Responses to feeding vary considerably among preterm infants; in general, intestinal motility studies can predict feeding intolerance [36].

Enteric nerve cells continue to differentiate throughout the first couple of years of life, which means that the infant's nervous system is plastic and developing [37]. There is clear evidence that the development of the ENS continues after birth. In rats, NO synthase-expressing neurons are already present at birth but increase in number and location during the first 3 weeks of postnatal life [32]. Normal ganglion cell distribution is present at 24 weeks of gestation in humans. These ganglia continue to mature on into childhood. Previous studies on human bowel specimens have revealed that the density of NADPH-diaphorase-positive ganglion cells decreases in the submucous plexus of the human distal colon and the myenteric plexus of human small bowel, colon and rectum [38, 39].

2.6 Genes Involved in ENS Development

Normal development of ENS is related to migration, proliferation, differentiation and survival of NC-derived cells [40]. Several genes and signaling molecules have been identified that control morphogenesis and differentiation of the ENS. These genes, when mutated or deleted, interfere with ENS development (Table 2.2) [7, 42–44].

2.6.1 RET/GDNF/GFRα1 Signaling System

This signaling pathway is of importance for subpopulations of both peripheral and central neurons, having been shown by in vitro and in vivo assays to promote survival of neurons, mitosis of neuronal progenitor cells, and dif-

Table 2.2 Genes involved in the morphogenesis and differentiation of the ENS

Genes	Chromosomal assignment	Function
RET	10q11.2	Tyrosine kinase receptor
GDNF	5p12–13.1	Glial cell-derived neurotropic factor
NTN	19q13.3	Neurturin, RET ligand
GFRα	10q26	GDNF family receptor alpha 1
EDNRB	13q22	Endothelin-B receptor
EDN-3	20q13.2–13.3	Endothelin-B
ECE-1	1p36.1	Endothelin-converting enzyme
SOX 10	22q13.1	Sry/HMG box transcription factor
PHOX2B	4p12	Paired-like homeobox 2b
PAX3	2q35	Paired box gene 3
SIP1	2q22	Siah-interacting protein

ferentiation of neurons and neurite extension [41, 45, 46]. The RET receptor is the signaling component of receptor complexes with four ligands, glial derived neurotropic factor (GDNF), neurturin (NTN), artemin (ATM), and persephin (PSP) [45, 47]. The complete receptor complex includes the RET receptor tyrosine kinase and a glycosylphosphatidylinositol-anchored binding component (GFRα1, GFRα2, GFRα3, and GFRα4) [47–49]. In vivo the absence of GDNF/GFRα1-mediated signaling leads to the failure of ENS development, whereas the absence of NTN/GFRα2-mediated signaling leads to more subtle abnormalities in ENS development [47]. The importance of RET in mammalian organogenesis has been further illustrated by the generation of RET knockout mice [50]. These mice exhibit total intestinal aganglionosis and renal agenesis. The RET protooncogene has been demonstrated to be a major gene causing Hirschsprung's disease [51–55]. Mutations of RET account for 50% of familial and 15% to 20% of sporadic cases of Hirschsprung's disease [55, 56].

The development of the ENS is dependent upon the actions of GDNF, which stimulates the proliferation and survival of NC-derived precursor cells in the embryonic gut [57–60]. It has been reported that GDNF is the ligand of RET [61]. Mice carrying the homozygous null mutation in GDNF have been generated, and these mice demonstrate the lack of kidneys and ENS, confirming the crucial role of GDNF in the development of the ENS [62, 63]. Although a causative role for GDNF mutations in some patients with Hirschsprung's disease has been suggested, the occurrence of such cases is uncommon, and it is more likely that the GDNF mutations are involved in modulation of the Hirschsprung's disease phenotype via its interaction with other susceptibility loci such as RET [7, 64].

2.6.2 Endothelin Signaling Pathway

The endothelins (EDN1, EDN2, and EDN3) are intercellular local messengers that act via the cell surface receptors, EDNRA and EDNRB [45]. EDN is initially produced as an inactive preproendothelin that undergoes two proteolytic steps to produce an active peptide. The first cleavage produces inactive big endothelins, and these are finally cleaved by a specific protease, endothelin-converting enzyme (ECE) to produce biologically active EDN [7, 16, 45].

EDN3 and EDNRB have a role in the migration and development of the ENS [65–67]. In mice in which the EDN3 or EDNRB gene is disrupted, intestinal aganglionosis has been demonstrated experimentally. Several reports suggest that the downregulation of EDN3 expression may play a role in the pathogenesis of Hirschsprung's disease in the sporadic cases [68–74].

ECE1 knockout mice show craniofacial and cardiac abnormalities in addition to colonic aganglionosis [75].

2.6.3 SOX10

The SOX10 (sex determining region Y-box) gene is expressed in neuronal crest derivates that contribute to the formation of the peripheral nervous system during embryogenesis [76, 77]. The involvement of SOX10 in the development of enteric neurons was demonstrated in the Dom (dominant megacolon) mouse model of Hirschsprung's disease which exhibits distal intestinal aganglionosis [76]. Mutations in SOX10 have been identified as a cause of the dominant megacolon mouse and Waardenburg-Shah syndrome in humans, both of which include defects in the ENS and pigmentation abnormalities [78, 79].

2.6.4 PHOX2B

The PHOX2B gene is a homeodomain-containing transcription factor that is involved in neurogenesis and regulates RET expression in mice, in which disruption of the PHOX2B gene results in a Hirschsprung's disease-like phenotype [80, 81]. Enteric PHOX2B expression begins in vagal and truncal NC-derived cells as they invade the foregut mesenchyme and is contained in the adult submucosal and myenteric plexus [81].

2.6.5 HOX11L1

HOX11L1 is a homeobox gene involved in peripheral nervous system development and is reported to play a role in the proliferation or differentiation of NC cell lines. Two different HOX11L1 knockout mouse models have been generated [82, 83]. In both cases, homozygous

mutant mice were viable but developed megacolon at the age of 3 to 5 weeks. Histologic and immunohistochemical analysis showed hyperplasia of myenteric ganglia, a phenotype similar to that observed in human intestinal neuronal dysplasia.

2.7 Other Factors Implicated in the Control of ENS Development

Kit, another receptor with tyrosine kinase activity, is involved in the development of the interstitial cells of Cajal (ICCs) [84]. These are nonneuronal cells that serve as pacemaker cells and are responsible fro the spontaneous, rhythmic, electrical excitatory activity of gastrointestinal smooth muscle. Recent studies have found that the c-kit receptor is essential for the development of the ICCs. Mesenchymal ICC precursors that carry the c-kit receptor require the kit ligand (KL), which can be provided by neuronal cells or smooth muscle cells. According to the influence of the KL from either neuronal or smooth muscle cells, the ICCs develop as either myenteric ICCs or muscular ICCs [85]. These cells are also important in modulating communications between nerve and muscle. Mice with mutations in the KIT gene lack ICCs and have changes in skin pigment and abnormal intestinal motility [86]. No such mutations have been reported in humans so far, but several studies have shown disturbed expression of ICCs in patients with motility disorders [87–91]

Further studies have indicated the importance of the gut microenvironment during development of ENS. Mice lacking EDN-3 show increased expression of laminin, one of extracellular matrix (ECM) proteins, which leads to the conclusion that EDN-3 also affects the environment through which the NC cells migrate [92]. Altered ECM proteins such as tenascin, fibronectin and nidogen have been shown in patients with Hirschsprung's disease which suggests the importance of ECM molecules during development of ENS [93, 94].

2.8 Conclusions

During the past decade there has been an explosion of information about genes that control the development of NC. Molecular-genetic analysis has identified several genes that have a role in the development of Hirschsprung's disease. The major susceptibility gene is RET, which is also involved in multiple endocrine neoplasia type 2. Recently, genetic studies have provided strong evidence in animal models that intestinal neuronal dysplasia (IND) is a real entity. HOX11L1 knockout mice and endothelin B receptor-deficient rats demonstrated abnormalities of the ENS resembling IND type B in humans. These findings support the concept that IND may be linked to a genetic defect [95]. The development of the ENS requires the complex interaction of genes encoding transcription factors, signaling molecules, and their receptors. Normal ENS development is based on survival of NC-derived cells and their coordinated proliferation, movement and differentiation into neurons and glia. These processes are influenced by the microenvironment of the developing gut. Alterations in gene function, defects in NC cells or changes in the gut microenvironment may result in abnormal development of the ENS.

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Functional Anatomy of the Enteric Nervous System

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3

3.1 Introduction

Congenital birth defects, of which Hirschsprung's disease is an example, are among the most difficult of illnesses to study in the human patients who suffer from them. By the time the condition is identified in an affected individual, the process that brought it about is over and done with. It is thus impossible to study the ontogeny of birth defects, such as Hirschsprung's disease, in a fetus while the problems develop. An investigator seeking to uncover the pathogenesis of such a condition must search, like a detective, for clues left behind by the perpetrator who has fled the scene of a crime. Even the identification of genes that may have mutated, important an achievement as that is, does not, by itself, explain why the defect develops. Human life, moreover, is so precious that human subjects are terrible laboratory animals. As a result,

more can often be learned about the origins of human illness by studying animal models, than by investigating the patients themselves. Invasive research, which is only possible on animals, can be used to develop a conceptual framework to devise hypotheses that can subsequently be tested for applicability to human patients. Experiments, based on these hypotheses, can be targeted to what can be confirmed or denied by diagnostic tests or by analyzing the restricted materials available from human subjects. Human biology is thus made approachable by knowledge of animal biology.

The importance of animal models in learning why developmental defects occur and what can be done to prevent them cannot be emphasized too strongly. Recent animal research has greatly advanced our understanding of the factors that govern the development of the enteric nervous system (ENS). Clearly, comprehension of the pathogenesis of the neuromuscular defects of the bowel, including Hirschsprung's disease, requires a detailed understanding of the processes that govern normal enteric neuronal and glial ontogeny. This research has already provided enough insight to systematize current thinking about the origin of Hirschsprung's disease. This review is concentrated on the important progress made in the developmental biology of the ENS (provided mainly by research on animals) that now provides a logical basis for explaining the origin of the human disease.

Hirschsprung's disease is a well-defined clinical entity. It is a congenital absence of neurons in the terminal portion of the gut. The length of the aganglionic region varies and short and long segment varieties have been distinguished, although these entities represent the extremes of a continuum. In fact, classical Hirschsprung's disease, in which a segment of the bowel is totally aganglionic, is itself only one (accounting for about 25%) of a series of conditions that encompass a variety of allied disorders that include hypoganglionosis, neuronal intestinal dysplasias (hyperganglionosis), immaturity of ganglion cells, and dysganglionoses that have yet to be thoroughly classified. Most often Hirschsprung's disease is limited to the colon, although rarely, greater lengths of bowel may be involved. The gut is hypoganglionic rostral to the aganglionic segment and, in some patients, the junction between the abnormal hypoganglionic tissue and the normal bowel may not be obvious. The aganglionic segment is invariably narrowed in comparison to the bowel rostral to it, which often becomes massively dilated, so that another name for Hirschsprung's disease is congenital megacolon. The aganglionic portion of the gut evidently functions as an obstruction causing the ganglionated orad bowel to dilate.

Although various investigators have proposed a number of hypotheses to explain why the aganglionic tissue should be a functional obstruction, including denervation hypersensitivity of the smooth muscle and a selective deficiency of fibers able to relax the bowel [1, 2], a more general explanation is that the ENS is essential for normal propulsive intestinal motility [3, 4]. Given the absence of the ENS from the aganglionic zone, a failure of propulsive reflexes and thus a functional obstruction are to be expected. Aside from propulsion, moreover, the net effect on intestinal muscle of the ENS is relaxant [5, 6]; therefore, contraction and narrowing would be the predicted behavior of gut that lacked ganglia.

In thinking about the physiology of the colon in a patient with Hirschsprung's disease, it is important to emphasize the difference between aganglionosis and denervation. Although the terminal bowel is aganglionic in Hirschsprung's disease, it is not denervated [1, 2, 7–9]. Actually, many investigators have reported that the aganglionic gut may be hyperinnervated, especially by catecholaminergic and cholinergic nerve fibers [2, 10]. What is missing in the diseased bowel are the cell bodies of intrinsic enteric neurons, which are essential for the mediation of reflexes, not nerve fibers. Certain types of intrinsic axon are also selectively lost, including those which contain serotonin (5-HT) [11] or nitric oxide synthase (NOS) [12, 13]; however, the apparent selectivity of these deficiencies may be attributable to the absence of intrinsic neurons from the aganglionic region. Given the lack of intrinsic neurons, one might expect that the transmitter of virtually any type of intrinsic neuron would be diminished. The confirmation that what is expected actually occurs is thus of limited value in understanding the pathogenesis of the disease (although a loss of relaxant fibers (such as those which contain NOS) is often invoked to explain the narrowing of the aganglionic segment as a contracted region. To understand why a loss of nerve cell bodies, despite an abundance of axons should be so devastating, it is important to consider the nature of the ENS.

3.2 The Normal Enteric Nervous System

The mature ENS is absolutely unique and different from any other region of the peripheral nervous system (PNS). First, the ENS is independent and can function in the absence of input from the brain or spinal cord [3, 4]. Second, in contrast to the remainder of the PNS, the ENS can mediate reflexes, even when it is isolated from the central nervous system (CNS). This ability of the ENS is often overlooked, even though it has long been known to be true. As the 19th Century turned to the 20th, Bayliss and Starling reported that enteric reflexes could be mediated by "the local nervous mechanism" of the gut [14, 15]. These investigators described what they called the "law of the intestine" (now known as the peristaltic reflex) in extrinsically denervated loops of dog intestine. This is a reflex, evoked by increased intraluminal pressure, that consists of a wave of oral excitation and anal relaxation that descends in the bowel and is propulsive.

Essentially the same reflex can also be elicited in vitro in preparations of guinea pig intestine [16]. The fact that reflex activity can be manifested by segments of gut in vitro, which have clearly lost all connection to dorsal root or cranial nerve ganglia, the brain and the spinal cord, indicates that every neural element of the peristaltic reflex arc (sensory receptors, primary interneurons, motor neurons, and effectors) must be intrinsic components of the wall of the gut.

These observations were taken into account by Langley in his seminal work on the autonomic nervous system [17]. Together with Langley's idea that most enteric neurons receive no direct input from the CNS, the independence of the ENS caused Langley to classify the ENS as a third component of the autonomic nervous system. The sympathetic division was defined as that with a thoracic and lumbar outflow of preganglionic axons from the CNS, while the parasympathetic was the division with a cranial and sacral outflow. The ENS, which mainly lacks either outflow had to be classified as a separate division, since it met the criteria of neither of the other two. Anatomical observations have more recently confirmed the distinct nature of the enteric innervation. The internal ultrastructure of the ENS is more similar to that of the CNS than to any other region of the PNS [3, 18–21]. The ENS lacks internal collagen and its neurons receive support from enteric glia, which resemble astrocytes, and not from Schwann cells. Phenotypic diversity of peripheral neurons peaks in the ENS, and every class of neurotransmitter known to be present in the CNS is also represented in the ENS [3, 4]. Intrinsic neuronal reflexes evoke secretion as well as motility [22]; furthermore, most enteric neurons not only lack connection to the CNS, but some actually project centripetally, beyond the confines of the gut, to innervate extra-enteric targets. These outside-the-bowel projections of enteric neurons make it possible for the ENS to affect directly the function of prevertebral sympathetic ganglia [23–25], the gallbladder [26], and the endocrine and exocrine pancreas [27, 28].

3.3 Organization of Enteric Neurons

The ENS of most adult mammals is comprised of two major interconnected ganglionated plexuses, the submucosal and the myenteric [3, 4]. The submucosal plexus is the smaller of the two. In larger animals, including humans, the submucosal plexus can be divided into separate plexuses of Schabadasch (external) and Meissner (internal) [29]; however, these plexuses interconnect extensively and clear functional distinctions are not yet known. The submucosal plexus is thus usually treated a single entity [4], although this practice will probably have to be changed in the future as new information accumulates that suggests a significant segregation of function to the subplexuses of Schabadasch and Meissner [30]. Submucosal plexus neurons project to one another, to the mucosa, and to the myenteric plexus. The neurons that project to the mucosa include intrinsic sensory [31–33] and secretomotor neurons [22, 34, 35]. Some submucosal neurons are bipolar or pseudounipolar in shape and also project to the myenteric plexus; these have been postulated to be sensory in function [31]. A newly discovered subset of submucosal neurons, which evoke vasomotor responses when activated by mucosal stimuli, project both to the mucosa and to blood vessels [36]. These cells may actually function as a unicellular reflex arc, which if true would be a structure that, in vertebrates, is unique to the bowel.

Both the submucosal and the myenteric plexuses contain many interneurons involved in interganglionic projections and the formation of complex microcircuits that are just beginning to be mapped. Motor neurons that excite or relax the muscularis externa are located exclusively in the myenteric plexus [3, 4]. The myenteric plexus of rodents, but not that of humans [37], probably also contains intrinsic sensory neurons that project to the mucosa as well. The extreme complexity of the ENS and the behaviors of the gut that it regulates have only recently been appreciated. Certainly, the ENS is not, as used to be thought, a system of "relay ganglia" interposed between the brain and effector in the bowel. Because the ENS is so different from the other components of the PNS, it stands to reason that the factors and/or processes that dictate the development of the ENS are likely to be different from those of other peripheral ganglia.

The search for the developmental basis of Hirschsprung's disease is likely to be a long one, not simply because of the complexity of the system, but also because it is unlikely that the multitude of neuronal developmental dysganglionoses, of which classical Hirschsprung's disease is but one, are a single disease entity.

3.4 The ENS is Derived from the Neural Crest

The first clear demonstration that the ENS is derived from the neural crest was made by Yntema and Hammond who noted that enteric ganglia fail to appear when the "anterior" neural crest is deleted in chick embryos [38, 39]. Their work was confirmed, and levels of the crest that contribute to the ENS were more precisely identified by Le Douarin and her colleagues [40, 41]. These investigators took advantage of the distinctive nucleolar-associated heterochromatin of quail cells, which allows these cells to be readily identified following their transplantation into embryos of other species. Le Douarin and her co-workers replaced segments of the chick neural crest with those of quail (or the reverse) and traced the migration of crest-derived cells in the resulting interspecies chimeras by identifying cells of the donor (chick or quail, depending on the particular experiment). These

studies suggested that the ENS is derived from both the vagal (somites 1–7) and the sacral (caudal to somite 28) crest. The vagal crest colonizes the entire bowel, while the sacral crest colonizes only the postumbilical gut.

The conclusion that there are two sites of origin of enteric neuronal precursors was soon challenged, because other investigators could recognize only a single proximodistal progression of cells thought to be "neuroblasts" in the avian gut [42]. This progression was believed to imply that neuronal precursors in the bowel only descend, as would be expected of vagal progenitors. No ascent, of the kind predicted for precursors from the sacral crest, could be found. These observations led to the suggestion that the data derived from experiments with interspecies chimeras could have been obtained if crest-derived cells were to be more invasive in a foreign embryo than they are when they migrate in embryos of their own species. If so, then quail cells might reach ectopic destinations in a chick embryo and chick cells might behave in a similarly abnormal manner in a quail embryo. There are, however, reasons why only a single proximodistal progression of cells that can be recognized as belonging to a neuronal lineage can be detected, even though multiple levels of the crest contribute precursors to the bowel. Neuronal progenitors have been shown to colonize various levels of the gut before they actually give rise to progeny that express recognizable neural properties [43]; thus, neurons develop in vitro in segments of gut that appear to be aneuronal at the time of explantation, thereby demonstrating that otherwise unrecognizable neural precursor cells were present in the explants. The delay, however short it might be, between the arrival of progenitors and their differentiation into neurons provides an opportunity for crest-derived precursors to interact with, and be influenced by, the enteric microenvironment. In fact, the enteric microenvironment has been demonstrated to play a critical role in the development of enteric neurons and glia [44–46]. The observed proximodistal progression of perceived "neuroblasts" (which is not found in all species), therefore, may be due to a proximodistal gradient in the maturation of the enteric microenvironment, rather than to the timing of the descent of the neuronal precursors.

More recent studies, in which endogenous crest cells have been traced by labeling them with a vital dye or a replication-deficient retrovirus, have confirmed that both the avian and murine gut are each colonized by cells from both vagal and sacral levels of the neural crest [47, 48]. The human bowel, like that of mice, appears to be colonized by sacral as well as vagal crest cells [49, 50]. In the mouse, studies with labeled crest-derived cells have also revealed that a third site, truncal crest, contributes to the rostral-most foregut (esophagus and adjacent stomach) [51]. Retroviral tracing in avian embryos has suggested that the entire vagal crest does not contribute to the formation of the ENS; instead, the bulk of the enteric neuronal progenitors evidently originate from only the portion of the vagal crest lying between somites 3 and 6 [52]. The specificity of vagal and sacral regions as sources of enteric neuronal progenitors is well illustrated by back-transplantation experiments. Back-transplantation consists of grafting a developing organ or piece of tissue from an older to a younger host embryo. It is a technique that provides insight into whether cells in the older tissue retain and can manifest, in a suitably permissive environment, properties associated with earlier stages of development. Crest-derived cells that have colonized the bowel will leave segments of gut that are back-grafted into a younger embryo and remigrate in their new host [53]. These cells will only reach the bowel of their host if the graft is situated so as to replace the host's vagal or sacral crest [54].

A subset of the vagal crest-derived cells that colonize the gut can be visually identified in transgenic mice directed to express *lacZ* by the promoter for dopamine β-hydroxylase (DBH) [55]. The *DBH-lacZ* transgene is permanently expressed in these mice by neurons that are not catecholaminergic in the adult gut. The colonization of the bowel by the transgenically labeled cells has been studied in detail in both normal mice and in murine models of Hirschsprung's disease [56, 57]; however, it is important to note that the *DBH-lacZ* transgene probably demonstrates only a subset of vagal crest-derived cells and does not reveal those of sacral origin. Some enteric neurons develop from precursors that are transiently catecholaminergic (TC) [58–61]. DBH is one of the enzymes that participate in the formation of norepinephrine (NE) and thus its presence is a component of the catecholaminergic phenotype. Even in normal mice, and especially in rats, the genes encoding DBH are not completely repressed in the noncatecholaminergic neurons that develop from TC cell progenitors. Neurons derived from TC cells continue to express DBH, although they inactivate other elements of the catecholaminergic phenotype [59]. It is likely that the cells that are marked by the expression of the *DBH-lacZ* transgene are members of this lineage, that is they are cells that originate from catecholaminergic progenitors. Unfortunately, not every enteric neuron originates from a TC cell precursor. In fact, the subset of neurons that arises from progenitors that never exhibit catecholaminergic properties is larger than that which is TC cell-derived [61]. As a result, many enteric neuronal precursors are not subject to surveillance by the *DBH-lacZ* transgene tracing technique.

However cells are traced, it is now apparent that in both fetal mice and in avian embryos, the ENS arises from multiple regions of the neural crest, not just one. Although the number of sources of enteric neurons in the neural crest is limited, it is necessary to take account of this multiplicity in attempting to explain the abnormal colonization of the gut that arises in Hirschsprung's disease and other dysganglionoses.

3.5 The Crest-Derived Cells that Colonize the Gut are Originally Pluripotent and Migrate to the Bowel Along Defined Pathways in the Embryo

The restriction of the levels of the premigratory crest that contribute precursors to the ENS raises the possibility that the crest cells in these regions might be predetermined to migrate to the bowel and give rise to enteric neurons and/or glia. Such a predestination, however, is not supported by experimental evidence, which indicates instead that premigratory crest cells are pluripotent. For example, when levels of the crest are interchanged so as to replace a region that normally colonizes the gut with one that does not, the heterotopic crest cells still migrate to the bowel and there give rise to neurons the phenotypes of which are ENS-appropriate, not level of origin-appropriate [62, 63]. An analogous process, moreover, is seen when the interchange of crest cells is reversed. Vagal and sacral crest cells give rise to non-enteric neurons in ectopic locations, such as sympathetic ganglia, when they are grafted so as to replace crest cells at other axial levels. Clones derived from single crest cells, furthermore, give rise, both in vitro [64–68] and in vivo [69–71], to progeny that may express many different phenotypes. A single cell that gives rise to a clone containing many phenotypes has to be pluripotent. The crest-derived cells that colonize the gut, moreover, remain multipotent with respect to their ability to give rise to neurons and glia, even after they have completed their migration to the bowel. This potency is well demonstrated by back-transplantation experiments. When segments of gut are back-transplanted into a neural crest migration pathway at a truncal level, which normally colonizes sympathetic ganglia and the adrenal gland, donor crest-derived cells leave the graft, but they do not migrate to the host's gut. Instead, they migrate to the host's sympathetic ganglia, adrenal gland and peripheral nerves; moreover, instead of giving rise to enteric neurons and glia, the donor crest cells, despite their previous migration to and residence in the bowel, now form catecholaminergic neurons in the ganglia, chromaffin cells in the adrenals, and Schwann cells in the nerves [53].

Analogous results have been obtained from in vitro studies of cells developing from cloned crest-derived cells of enteric origin. The progeny found in these clones express a variety of different phenotypes, including some that are not present in the normal ENS [72]. Despite their multipotent nature, however, the developmental potential of enteric crest-derived cells in vivo [53] and in clonal culture is not as great as that of their progenitors in the premigratory crest [72, 73]. The pluripotency of the crest-derived cells that colonize the gut, revealed by studies of clones and the behavior of cells emigrating from back-transplants [54], indicates that the bowel does not become colonized by precursors from restricted regions of the neural crest because only these regions contain crest cells endowed with homing information that programs them to migrate to the gut. Instead, these regions are the only levels of the crest from which there are defined migratory pathways that lead to the bowel. The pathway from the vagal crest conveys the largest cohort of crest-derived émigrés to the gut and in avian embryos leads crest-derived cells to the entire bowel between the proventriculus and the cloaca. In mammals the equivalent region would extend from the corpus of the stomach to the rectum. The cohort that follows the sacral pathway is much smaller and leads crest-derived émigrés only into the postumbilical bowel. The cohort following the truncal pathway is still smaller and leads crest-derived cells only to the presumptive esophagus and the most rostral portion of the stomach.

The possibility that crest-derived cells of different origins are not identical exists and has some experimental support. It is also conceivable that the crest-derived émigrés from different levels interact with one another during the formation of the ENS. The molecular nature of the migratory pathways and the nature of the mechanisms that guide progenitors to their correct destinations within the gut itself have yet to be identified. Chemoattractant or repellent molecules for growing axons have been identified in the vertebrate CNS [74]. These molecules include netrins [74–77] and semaphorins [78–80]. The directional growth of migrating crest-derived cells is a property also shown by path-finding axonal growth cones [81, 82]. Both netrins 1 and 2 ($2 > 1$) are expressed in the developing bowel [75] and mice with a targeted mutation in netrin-1 die at birth with a bloated bowel and no milk in their stomach (Tessier-Lavigne, personal communication). It is thus conceivable, although there is as yet absolutely no direct supporting evidence, that netrins play a role in the guidance of crest-derived progenitors and/or axons to their proper destinations in the gut. The roles, if any, of the netrins or semaphorins in the formation of the ENS are thus intriguing possibilities that remain to be investigated.

3.6 Enteric Neurons are Derived from More Than One Progenitor Lineage

The developmental potential of the originally pluripotent population of premigratory crest cells becomes progressively restricted as development proceeds. This restriction is accompanied by the sorting of crest-derived progeny into recognizable lineages [83–85]. A lineage restriction has occurred in the crest-derived population that colonizes the bowel [61]. At least two lineages of enteric neuronal progenitor have been distinguished. Recognition of these lineages is significant, because the fate of the neuronal precursors in the bowel depends, not just on the enteric microenvironment, but also on the lineages of the crest-derived cells. Lineages, as much as environmental factors, determine patterns of phenotypic expression. In

order for any progenitor to respond to a microenvironmental signal (whether that signal is a growth factor or a molecule of the extracellular matrix) the responding cell first has to have expressed receptors capable of being stimulated by the microenvironmental signal. The expression of these receptors is lineage-dependent. Lineage thus establishes which developmental options are open to precursor cells and which are not, and which growth factors can affect the cells and which cannot. The development of the ENS can thus be understood as a symphony in which lineage-determined properties provide the themes and environmentally provided factors provide the counterpoint.

Perhaps the earliest indication of the multiplicity of the lineages of crest-derived precursors contributing to the formation of the ENS was the discovery in the developing mammalian bowel [86, 87] and vagal crest migration pathway [58, 60] of TC cells. These remarkable cells mimic all of the known properties of sympathetic neurons except one. TC cells express tyrosine hydroxylase (TH) and DBH, and take up and store NE [88–90]. The one property of sympathetic neurons that TC cells do not mimic is that sympathetic neurons, like every other neuron, are postmitotic cells, while TC cells proliferate [58, 59, 91, 92]. TC cells, therefore, cannot by definition be neurons, which are postmitotic. Still, TC cells do express neural markers, including neurofilament proteins and peripherin [58, 59]; moreover, TC cells give rise to neurons in vitro [60]. TC cells thus are crest-derived neural precursors [59, 60]. In fact, the persistence of DBH after the cessation of transcription of TH made it possible to demonstrate that TC cells are the ancestors of at least some mature enteric neurons [59]. The persistence of DBH in a subset of enteric neurons probably explains the ability, discussed above, of a transgene driven by the DBH promoter to label these cells and their precursors [55].

TC cells have more in common with sympathoadrenal progenitor cells than just their catecholaminergic characteristics. Both TC cells and sympathoadrenal progenitors express the same cell surface differentiation antigens and each changes these antigens at the same time of development. The first common antigens to be expressed are "SA" proteins, recognized by a series of monoclonal antibodies [93, 94]. The SA antigens disappear at the time another transiently expressed antigen, recognized by "B2" antibodies, appears. The sharing of characteristics by enteric and sympathetic neuronal precursors led to the suggestion that there is a common sympathoadrenal-enteric precursor lineage from which both the sympathetic nervous system and ENS are derived [93]. Studies of catecholamine expression in clonal cultures of chicken enteric crest-derived cells led investigators to conclude that there is a also a common sympathoadrenal-enteric precursor lineage in avians [72].

Two recent lines of evidence have shown that the hypothesis that the ENS arises from a single sympathoadrenal-enteric progenitor lineage is only partially correct [61]. Dissociated cells of the fetal rat gut were repeatedly exposed to B2 antibodies in vitro in the presence of complement. This treatment causes all of the crest-derived cells that express surface antigens in common with sympathoadrenal progenitors to lyse, thereby eliminating the putative common sympathoadrenal-enteric progenitor. If such a precursor were to be the sole source of enteric neurons, its destruction by complement-mediated lysis would be expected to prevent the in vitro development of neurons in the treated cultures of cells from the dissociated fetal bowel. In fact, complement-mediated lysis reduces the number of neurons differentiating in the cultures and eliminates all that express TH, DBH, or B2; nevertheless neurons that express none of these antigens continue to arise in the cultures. These findings suggest that at least two precursor lineages contribute to the development of the ENS. Only one of these lineages can be ablated by destroying cells that express sympathoadrenal markers.

The second line of evidence showing that multiple precursor lineages contribute to the development of the ENS has come from studies of mice with a homozygous targeted mutation in a gene encoding a transcription factor, *mash-1*. This gene is a mammalian analog of *achaete-scute* of *Drosophila* [95]. *Mash-1* is expressed by both sympathetic and enteric neural precursors [96, 97] and thus its expression is one more shared property that implies a sympathoadrenal-enteric commonality. The sympathetic nervous system fails to develop in *mash-1*–/– mice [98], indicating that sympathetic neurons are *mash-1*-dependent. The common precursor idea suggests that enteric neurons should also be *mash-1*-dependent. The ENS, however, is not absent in *mash-1*–/– animals. Instead, enteric neurons are lacking only in the esophagus. In the remainder of the bowel, neurons develop, but there is a delay of about two days in the timing of their appearance. In the absence of additional evidence, it was initially impossible to know whether this delay was due to the elimination of a *mash-1*-dependent set of early-developing neurons, or to the slower development of all neurons. Subsequent studies, however, revealed that the delay was caused by the interference in *mash-1*–/– mice with the development of a *mash-1*-dependent set of early-developing neurons.

The birth dates of enteric neurons vary in relationship to their phenotype [99]. Enteric serotonergic neurons are among the first to be born, some becoming postmitotic (at E8.5), even before they colonize the gut. Others, such as neurons containing calcitonin gene-related peptide (CGRP) originate quite late and continue to be born postnatally. The first CGRP neuron (E16) is not born until about two days after the last serotonergic neuron has become postmitotic. Mash-1 and TH immunoreactivities are colocalized, indicating that *mash-1* is expressed in TC cells [61]; moreover, TC cells do not develop in *mash-1*–/– mice. The ENS of *mash-1*–/– animals, furthermore, contains no serotonergic neurons,

although it does contain neurons that express CGRP. These findings suggest that TC cells are the postulated *mash-1*-dependent common sympathoadrenal-enteric progenitor; moreover, serotonergic neurons would appear to be an example of an enteric neuron derived from this lineage. CGRP-containing neurons, which arise later, and are *mash-1*-independent could not be derived from such a common lineage. These suggestions have been confirmed by examining which types of neuron do or do not develop in cultures of dissociated rat intestine that have been subjected to complement-mediated lysis with antibodies to common sympathoadrenal-enteric antigens (B2). Serotonergic neuronal development is prevented, but neurons that contain CGRP continue to appear.

These findings confirm that the ENS is derived from at least two progenitor lineages. One of these is related to sympathoadrenal precursors. This lineage expresses and depends on *mash-1*, arises early in ontogeny, is transiently catecholaminergic, and gives rise to limited subsets of enteric neurons, including all of those that populate the esophagus and all of the remainder that express a serotonergic phenotype. The second lineage, which may not be homogeneous, is unrelated to sympathoadrenal cells, develops late, does not express or depend on *mash-1*, is not catecholaminergic, and gives rise to gastric and intestinal neurons, some of which contain CGRP.

3.7 Dependence of Enteric Neuronal Subsets on Different Microenvironmental Signals (Growth/Differentiation Factors) Defines Sublineages of Precursor Cells: RET and Glial Cell Line-Derived Neurotrophic Factor

The *c-ret* protooncogene is a gene upon which most enteric neurons are critically dependent for survival [51, 100, 101]. This gene encodes a receptor tyrosine kinase, for which glial cell line-derived growth factor (GDNF) has recently been identified as the functional ligand [102–104]. GDNF was first identified as a factor, produced by a glial cell line (B49), that promotes the survival of midbrain dopaminergic neurons [105]. GDNF was later observed to enhance the survival of spinal motor neurons [106]. GDNF is a distant relative of transforming growth factor-β (TGF-β). It is a homodimer, consisting of two peptide chains of 134 amino acids linked by a disulfide bridge. A larger precursor of 211 amino acids is synthesized first. This big molecule is proteolytically cleaved intracellularly to produce mature GDNF, which is secreted. During development, GDNF is not restricted to the brain, but rather is very highly expressed in the gut and other peripheral organs [106, 107]. In keeping with its peripheral distribution, GDNF is not just a survival factor for central CNS neurons [103], but also enhances the in vitro survival of peripheral sensory and sympathetic neurons, and also promotes their extension of neurites [106].

The observation that GDNF affects sympathetic neurons suggests that it should also affect at least some neurons of the ENS. In fact, both enteric and sympathetic neurons express *c-ret*, at least transiently [100, 108]. When *c-ret* is knocked out in transgenic mice, the ENS totally fails to develop in the entire bowel, with the exception of the rostral foregut [51, 101]. Since Ret is the functional receptor for GDNF, the fact that a similar lesion has recently been found to occur in the bowel of knockout mice lacking GDNF [109–111] is not surprising. Neither is the observation surprising that, in contrast to the trophic effects GDNF exerts on autonomic neuroblasts from control mice, GDNF fails to exert trophic effects on analogous cells from *c-ret*–/– animals [104]. Activation of the Ret receptor by GDNF is thus a critical event in the formation of the ENS. Actually, GDNF does not appear to bind directly to the Ret receptor itself. Instead, GDNF binds to a glycosylphosphatidylinositol-linked cell surface protein called GDNFR-α, which then complexes with Ret to trigger the autophosphorylation and other actions of Ret [102, 112].

Despite the fact that most of the bowel is aganglionic in *c-ret*–/– mice [101], there are neurons in the portions of the gut that develop from the rostral foregut of these animals [51]. Although the superior cervical ganglion is missing in *c-ret*–/– mice, most other sympathetic ganglia do develop. The crest-derived cells that colonize the rostral foregut and the superior cervical ganglion have been traced by injecting a fluorescent dye (DiI) that intercalates into the lipid of the plasma membrane. The DiI-labeled cells that colonize the presumptive esophagus and rostral stomach originate from the same pool of truncal crest cells that gives rise to the sympathetic chain ganglia below the superior cervical ganglion. In contrast, the post-otic vagal crest cells that colonize the entire bowel distal to the rostral foregut also contribute the crest-derived cells that form the superior cervical ganglion. There thus appears to be not one but two common sympathoadrenal-enteric lineages. One of these is *c-ret*- and GDNF-dependent, while the other is *c-ret*- and GDNF-independent. The bulk of the ENS is constructed of cells in the *c-ret*/GDNF-dependent sympathoadrenal-enteric lineage, which evidently also gives rise to the superior cervical ganglion. The *c-ret*/GDNF-independent lineage forms the ENS of the rostral foregut and the entire sympathetic chain, except for the superior cervical ganglion.

The *mash-1*-dependent and *c-ret*-dependent lineages seem superficially to be opposite sides of a single coin [51]. For example, the ENS of the esophagus, which is totally *mash-1*-dependent, happens to be the region of the gut that is *c-ret*-independent. In contrast, the ENS of the bowel below the proximal stomach is totally *c-ret*-dependent, yet it contains neurons in *mash-1* knockout mice. Still, as noted above, there is no region of the ENS that is completely *mash-1*-independent. Although there are neurons in the intestines of *mash-1* knockout mice, TC cells and all the neurons derived from TC cells are

missing. Still to be explained as well is why the presumably *c-ret*-independent crest-derived cells of the rostral foregut do not migrate distally in the bowel of *c-ret*–/– mice (or mice lacking GDNF). Possibly, the evident inability of the *c-ret*-independent cells of the rostral foregut to expand their territory in *c-ret*–/– mice is due to an inhibition of their migration, or possibly proliferation. Alternatively, all enteric neurons may be GDNF/Ret-dependent but able to survive in the rostral foregut, despite the absence of GDNF or Ret, because a compensatory factor (currently unknown) is expressed only in this region of the bowel.

3.8 The Development of the ENS is Probably Influenced by a Neurotrophin

For a long time neurotrophins were thought to play little or no role in the development of the ENS. Unlike developing sensory and sympathetic ganglia, explanted enteric neurons can be cultured without nerve growth factor (NGF) or even in the presence of neutralizing antibodies to NGF [113, 114]. Neuritic outgrowth from organotypic cultures of gut, moreover, is not stimulated by NGF. Autoantibodies to NGF produce severe sensory and sympathetic defects in the progeny of immunized animals [115, 116]; nevertheless, the same autoantibodies to NGF do not induce ENS lesions [117]. These observations, however, suggest only that the development of the ENS is independent of NGF, not that the ENS does not require the action of any neurotrophin. NGF was the first neurotrophin to be discovered and the studies outlined above were carried out before the existence of other neurotrophins became known. NGF, of course, together with brain-derived neurotrophic factor (BDNF), neurotrophin-3 (NT-3), NT-4/5 [118–120], and NT-6 [121] are members of a family of small, very basic proteins. Each of these neurotrophins is able to interact independently with a common receptor, p75NTR, and with a specific Trk receptor tyrosine kinase, TrkA for NGF, TrkB for BDNF and NT-4/5, and TrkC for NT-3. At higher concentrations, the neurotrophins become somewhat promiscuous and activate Trks other than their primary receptor. NT-3, for example, activates TrkA and TrkB, but it binds to those receptors with an affinity that is lower than its affinity for its natural ligand, TrkC, or that of NGF or BDNF for TrkA or TrkB, respectively.

A variety of roles have putatively been assigned to the common neurotrophin receptor, p75NTR. These include an enhancement of the affinity of Trks for their neurotrophins, increasing the rate at which NGF binds to TrkA, and improving the specificity of Trk receptors by decreasing their receptivity to activation by the wrong neurotrophin. p75NTR has also been postulated to exert effects on apoptosis, retrograde transport of neurotrophins, sphingomyelin hydrolysis, and cell migration [119, 122]. Despite all these proposed functions, it has not been totally clear until recently that p75NTR actually does play a physiologically significant role in development [122]. It has been difficult to separate a function for p75NTR from that of a Trk receptor, which can function in the absence of p75NTR. Schwann cells, however, express p75NTR, but they do not express a Trk receptor; nevertheless, despite the absence of a Trk, their expression of p75NTR enables them to respond to neurotrophin stimulation. p75NTR is thus a receptor in its own right. Its transduction mechanism involves the activation of the transcription factor, nuclear factor kappa-B (NF-κB) [123]. This activation does not occur in Schwann cells from transgenic knockout mice lacking p75NTR. Multiple transcripts may be produced from each of the *trk* genes [118–120, 124], which can be translated into proteins with variations in their kinase domains. There are also truncated variants of TrkB and TrkC lacking intracellular kinase domains and variants that contain inserts that inactivate their kinase activity.

The discovery of multiple neurotrophins and Trks has caused the idea that one or more neurotrophins play roles in enteric neuronal development to be reconsidered. The first observation to suggest that one or more neurotrophins probably are important in the formation of the ENS was the discovery that the common neurotrophin receptor, p75NTR, is expressed by the crest-derived cells that colonize the fetal mouse and rat gut [58, 59]. The cells that express p75NTR give rise to neurons and glia in vitro [60]. Antibodies to p75NTR specifically immunoselect crest-derived cells from the fetal bowel [125, 126]; moreover, almost no cells able to give rise to neurons or glia remain in dissociated cell populations after p75NTR-expressing cells have been removed by immunoselection. These observations suggest (but do not prove) that all, and not just some, of the crest-derived cells that colonize the gut express p75NTR. No marker has yet been found that reveals a greater number of enteric crest-derived neural precursors than p75NTR. Although p75NTR may not be required for stimulation of cells by a neurotrophin, which can activate a specific Trk, p75NTR is commonly expressed by cells that are neurotrophin-responsive. The fact that enteric neuronal precursors express p75NTR, however, is not the only reason to believe that a neurotrophin plays an important role in the development of enteric neurons and/or glia.

The concept, that at least one lineage of enteric neurons arises from a common sympathoadrenal-enteric progenitor suggests that at least the enteric neurons of this lineage should share the neurotrophin-dependence of their sympathoadrenal equivalents. Sympathetic neural precursors are not at first NGF-dependent [127–130]. Instead, they are supported by NT-3 before they respond to, and become dependent on, NGF [127, 128]. This change in neurotrophin responsivity and dependence is matched in sympathetic neural precursors by a change from TrkC

to TrkA expression [128, 129, 131]. This switch in receptor expression may occur spontaneously [131], or it may require the exposure of cells to NT-3 [127]. NT-3 thus promotes the development of sympathoadrenal precursors [127, 129, 132]; moreover, both the knockout of NT-3 in transgenic mice [133, 134] and the administration of neutralizing antibodies to NT-3 impair the normal development of sympathetic neurons [135]. Excessive apoptosis of sympathoadrenal neuroblasts occurs when NT-3 is absent during development [136].

If the enteric neurons that arise from a common sympathoadrenal-enteric progenitor were to diverge from the common lineage before TrkA and NGF-dependence are acquired, then the evident NGF-independence of virtually all enteric neurons could be explained. In this model, the acquisition of NGF-dependence would be considered, for sympathetic neurons the time when their progenitors diverge from the common lineage. Acquisition of NGF-dependence would also be an event that does not occur in the enteric microenvironment, where the successors of TC cells lose their catecholaminergic properties and acquire other, gut-specific, phenotypes. Since NT-3 plays such an important role in the early development of sympathoadrenal cells, NT-3 might be expected to play a similar role in the development of those enteric neurons that are derived from the common sympathoadrenal-enteric lineage. NT-3 would be predicted to affect the enteric neuronal progenitors during the predivergent phase, when they share properties with sympathetic neural precursors. Clearly, the logic of this argument suggests that NT-3 would support the development of the subset of enteric neurons that is derived from the *mash-1*-dependent TC cells (the common sympathoadrenal-enteric progenitor). What the argument does not suggest is that NT-3 or any other neurotrophin is likely to exert a global effect similar to that of GDNF. GDNF stimulation of the Ret receptor appears to be critical at a very early stage of development, so that the loss of precursor cells that are GDNF/Ret-dependent results in the total failure of both neurons and glia to arise in the affected region of the bowel.

The idea that NT-3 is the critical neurotrophin in enteric neuronal development is supported by the observations that TrkC is expressed by enteric neurons, where both full-length and truncated forms of the receptor can be detected in newborn mice [124] and fetal rats [126, 137]. Transcripts encoding TrkC have been shown by in situ hybridization to be located in the developing and mature ENS [137, 138]. mRNA encoding the full-length TrkC (containing a kinase domain) is enriched in purified populations of crest-derived neural and glial precursor cells immunoselected from the fetal rat bowel [126]. NT-3 binding to both full-length and truncated forms of TrkC has been detected in the E13.5 chick gut [139], although affinity labeling has not revealed the presence of significant amounts of NT-3 binding to TrkC in the bowel of newborn mice [139]. NT-3, as well as TrkC, is expressed in the developing gut [140]. The expression of *lacZ* driven by the NT-3 promoter in transgenic mice has enabled cells that express NT-3 to be located and identified in the fetal bowel [140]. The cells that express NT-3 are located in the outer gut mesenchyme of fetal mice. The outer gut mesenchyme is the layer of the bowel within which myenteric ganglia arise, suggesting that NT-3 is secreted in situ, where it can reach and affect TrkC expressed by developing enteric neuronal precursors and/or neurons. NT-3 expression has not been detected in the submucosa. The development of submucosal neurons follows that of myenteric neurons [141, 142] and all submucosal neurons are born late [99]. The neurons of the submucosal plexus, therefore, probably are not derived from the *mash-1*-dependent TC cell lineage, which gives rise only to neurons, such as serotonergic cells (of which there are none in the submucosal plexus) that are born early [61]. These considerations are consistent with the idea that a subset of enteric neurons, most likely the *mash-1*-dependent TC cell lineage, are affected by NT-3.

3.9 NT-3 Promotes the Development of Enteric Neurons

A major breakthrough, which has enabled the effects of growth factors on the development of enteric neurons or glia to be studied in vitro, has been the development of a means of isolating crest-derived cells from within the wall of the fetal bowel. If crest-derived cells are not so isolated, then the direct actions of growth factors on crest-derived neural and/or glial precursors cannot be distinguished from indirect effects of these molecules on other cells of the enteric mesenchyme. The isolation of enteric crest-derived cells takes advantage of the phenomenon that these cells express cell-surface differentiation antigens or markers that are not expressed by non-neuronal cells of the gut wall. Antibodies to these cell surface antigens are utilized for immunoselection of the crest-derived cells. The SA and B2 antigens, which are discussed above in the context of the identification of cells in a putative sympathoadrenal-enteric lineage, are examples of differentiation antigens that could potentially be used for immunoselection, although they have not yet been so employed. Immunoselection is not dissimilar in concept to the use of the B2 antigen for the immunoelimination of crest-derived cells by complement-mediated lysis [61].

The first differentiation antigen used for the immunoselection of crest-derived cells from the fetal gut of chicks and rats was a protein recognized by HNK-1 monoclonal antibodies [125, 126]. Since then, p75NTR [143] and Ret [73] have each been employed with good effect. In general, the fetal gut is dissociated and the separated cells are incubated with primary antibodies, which selectively decorate the surfaces of the crest-derived cells.

The antibody-labeled cells can then be immunoselected with secondary antibodies coupled to magnetic beads, and eventually isolated with a magnet [125, 126]. Alternatively, the primary antibody-labeled cells can be identified with fluorescent secondary antibodies and isolated with a cell sorter [73] or by manual selection [72]. The non-immunoselected cells proliferate much more than do the immunoselected crest-derived cells. The crest-derived precursors that colonize the gut are still dividing when they arrive in the bowel [58, 92, 99]; however, crest-derived cells withdraw from the cell cycle when they give rise to neurons. In contrast, the non-neuronal cells of the residual population do not give rise to cells that become postmitotic and thus continue to divide in vitro.

The ability of isolated populations of crest-derived cells, immunoselected from the fetal rat gut, to differentiate into neurons and glia is promoted by NT-3 [126, 144]. In contrast to the immunoselected cells, NT-3 has no effect on crest-depleted populations of cells that remain after the crest-derived cells have been removed by immunoselection. In these experiments, it is necessary to identify cells as neurons or glia by demonstrating chemical markers, because the morphological appearance of the cells in culture can be misleading. Neurons can be identified by their expression of the immunoreactivity of nerve-specific markers such as neurofilament proteins, peripherin, neuron specific enolase, or PGP9.5 (a neuronal form of ubiquitin). Glia can be identified by their expression of the immunoreactivity of markers such as S100 and glial fibrillary acidic protein (GFAP).

The ability of NT-3 to promote neuronal and glial development is concentration-dependent and is maximal at 40 ng/ml. In addition to promoting the development of enteric neurons and glia, NT-3 enhances neurite outgrowth, but it is not mitogenic. Similarly, NT-3 does not induce dorsal root ganglion cell precursors to proliferate; on the contrary, when administered early in ontogeny, NT-3 causes sensory neurons to differentiate prematurely, thereby reducing their ultimate numbers [145]. NT-3 thus exerts an effect on the postmigratory crest-derived cells that colonize the bowel and dorsal root ganglia that is different from its action on premigratory crest cells, which are stimulated to proliferate by NT-3 [146, 147]. The action of NT-3 on immunoselected cells, in common with the effects of most growth factors, is associated with the transient induction of the *c-fos* protooncogene in responding cells [126]. Other neurotrophins, such as NGF, BDNF, and NT4/5 affect neither the in vitro development of neurons and glia in populations of immunoselected cells, nor the in vitro proliferation or differentiation of the non-immunoselected cells. NT-3 thus specifically promotes the in vitro differentiation of crest-derived cells as enteric neurons and glia and is probably the only neurotrophin that can do so. The in vivo importance of NT-3 in the development of the ENS remains to be established.

Although a physiological role for NT-3 in the normal development of the ENS has not yet been identified, NT-3 has been shown to be able to affect the development of enteric ganglia in vivo. The DBH promoter has been used to direct the overexpression of NT-3 in the developing ENS [148]. When this is done, the myenteric plexus of the small and large intestines of the DBH/NT-3 transgenic animals becomes hyperganglionic. There are significant increases in the number of neurons/ganglion, the number of neurons per unit length of gut, the packing density of neurons within ganglia, the proportion area of ganglia, and the size (maximal diameter and volume) of individual neurons. In contrast, none of these parameters are changed in the submucosal plexus and there is no change in the numbers of CGRP-containing neurons (the majority of which are submucosal). CGRP-containing neurons are the latest-born of enteric neurons and are derived from cells in the *mash-1*-independent lineage [61, 99]. In fact, the entire set of submucosal neurons tends to be born late. These findings suggest that the late-developing *mash-1*-independent lineage of enteric neurons is probably not affected by the DBH/NT-3 transgene. Both the myenteric hyperganglionosis and the increase in neuronal size induced by the overexpression of NT-3 in transgenic mice are thus probably due to a response of the *mash-1*-dependent precursor lineage. This conclusion still needs to be confirmed.

Surprisingly, analysis of mice with knockout of genes encoding neurotrophins or neurotrophin receptors has not revealed that any of these growth factors are required for enteric neuronal development. For example, no defects have been found in the ENS of mice lacking TrkC [149], NT-3 [133, 134, 140], or p75NTR [150]. It remains possible, of course, that the ENS of some, or even all, of these mice is abnormal, despite the presence of a detectable ENS. The ENS is a very complex nervous system that cannot be adequately evaluated just by noting its presence or absence in an all-or-none fashion. At most, disruption of *trkC* or NT-3 would be predicted to affect only the subset of crest-derived cells that are derived from *mash-1*-dependent TC cells [51, 61]. The presence of other receptors or growth factors might also compensate for the loss of NT-3 or its receptors. For instance, the knockout of *trk*C does not reduce the number of neurons in sympathetic ganglia; moreover, the knockout fails to prevent the expression of sympathoadrenal markers such as TrkA and TH [151]. If NT-3 plays a physiologically important role in sympathoadrenal development, therefore, it would have to be able to stimulate receptors other than TrkC to do so.

To detect an anatomical defect in the ENS, which may be quite limited in scope, a more detailed examination than the simple visualization of stained ganglia is likely to be required. Similarly, a physiological deficit may only become evident if the gut is perturbed, or if one analyzes motility and secretion in a sophisticated manner. Neither

a detailed anatomical investigation, nor a physiological analysis of motility or secretion have been carried out in mice with knockouts of genes encoding TrkC, NT-3, or p75NTR. The survival of newborn mice that lack NT-3 and the presence of enteric ganglia in these animals [134], however, does show that a functional, if not necessarily normal, ENS can arise in the absence of NT-3. The relatively poor weight gain and survival of mice that do not express NT-3 are compatible with the possibility that the ENS that develops in the absence of NT-3 is not entirely normal.

3.10 The Development of the ENS is Probably Influenced by a Cytokine

Ciliary neurotrophic factor (CNTF) was identified as a factor in the eye that promotes the survival of chick ciliary ganglion neurons [152]. CNTF has now been purified, cloned, and found to affect many different neurons, both developing and mature [153]. CNTF does not resemble any of the neurotrophins and is a member of the cytokine family, which includes distantly related molecules, such as leukemia inhibitory factor (LIF), interleukin-6 (IL-6), interleukin-11 (IL-11), oncostatin M (OSM), and cardiotrophin-1 [153–155]. CNTF primarily acts on neurons, while the actions of the other cytokines (with the possible exception of cardiotrophin-1) are exerted on many other types of cell. The active CNTF receptor (CNTFR) is an assembly of three molecular components, only one of which, CNTFRα, actually binds CNTF [156]. There are two β receptor subunits, gp130 and LIFRβ. These are signal-transducing molecules and also serve as components of receptors for cytokine relatives of CNTF, such as LIF and IL-6 [155, 157–160]. The three molecular components are not initially associated with one another on cell surfaces, but are recruited to form a complex when stimulated by CNTF. CNTF binds first to CNTFRα and the β components then join to form the tripartite complex [160]. The signal transduction process thus begins with formation of the CNTFRα/LIFRβ/gp130 complex, involves the dimerization of LIFRβ with gp130, and proceeds by activating Jak tyrosine kinases, which are constitutively associated with the cytosolic tails of each of the β components [161, 162]. CNTF neither binds to, nor activates the β components in the absence of CNTFRα. CNTFRα, moreover, is restricted to the nervous system, which thus explains the neural specificity of the actions of CNTF.

Other cytokines have different specificity determinants, which are expressed extraneuronally. Levels of CNTF in embryonic and fetal animals are very low [153], although expression of mRNA encoding CNTF can be detected in the developing bowel by using reverse transcriptase and the polymerase chain reaction [163]. In contrast to CNTF, CNTFRα is expressed by many cells of the developing nervous system, including the ENS [164]. The natural or targeted knockout of genes encoding CNTF does not cause notable developmental defects in mice [165] or humans (about 2.5% of the Japanese population) [166]. CNTF, furthermore, unlike the majority of secreted proteins, lacks a signal sequence and thus is probably cytosolic. In the absence of cell death, it is thus not clear how such a protein could be secreted. It has therefore been proposed that CNTF is an emergency factor, which is released only in response to injury.

Conceivably, CNTF itself may not play a very significant role in development. In contrast to the relatively normal development of CNTF knockout mice, there are profound motor and other defects at birth in animals with targeted deletions of CNTFRα [154]. Mice lacking CNTFRα fail to feed and die with a massive dilation of the bowel during the perinatal period. Neurons immunoreactive for substance P (SP) and NOS are markedly reduced in the enteric plexuses of these animals (Kirchgessner A et al., unpublished data). Almost no SP- or NOS-immunoreactive axons are found in the circular muscle of CNTFRα knockout mice. Motor neurons that excite smooth muscle contain SP [167] and motor neurons that relax smooth muscle contain NOS [168–172]. An identical defect is seen in the gut of mice in which the expression of *LIFRβ* has been knocked out. These observations suggest that the tripartite CNTFR, and particularly its α component, plays a vital role in the development of enteric motor neurons. Although enteric ganglia are present in mice that lack CNTFRα or LIFRβ, the ENS cannot function in the absence of motor neurons. Since a similar effect is not seen in mice lacking CNTF, there may be another endogenous ligand in the fetal gut that can bind to CNTFRα. If so, this yet-to-be-identified ligand is essential for the development of enteric motor neurons. The known relatives of CNTF, LIF, OSM, IL-6, IL-11, and cardiotrophin-1, do not require CNTFRα and thus are unlikely to be the unknown CNTFRα ligand [154].

LIF [173], CNTF [174], and cardiotrophin-1 [155] promote the development of sympathetic neurons and cause these cells, which are normally catecholaminergic, to become cholinergic. By analogy, the effects of these agents on sympathetic neurons suggests that CNTF (and its putative physiological ligand) might act on enteric neurons that arise from the common sympathoadrenal-enteric progenitor and thus be within the *mash-1*-dependent TC cell lineage. mRNA encoding CNTFRα is expressed and developmentally regulated in the fetal bowel [163]; furthermore, mRNA encoding CNTFRα is specifically expressed by crest-derived cells immunoselected with antibodies to p75NTR (Chalazonitis A et al, unpublished data). Addition of CNTF to cultures of crest-derived cells immunoselected from the fetal rat gut with antibodies to p75NTR mimics the action of NT-3 and promotes the development of neurons. In fact, the in vitro effects of NT-3 and CNTF are synergistic [143]. This

synergism is consistent with the possibility that the cells that respond to CNTF and NT-3 arise within the same progenitor lineage.

3.11 An Aganglionosis Similar to That in Hirschsprung's Disease Occurs in *ls/ls* and *sl/sl* Mice

The terminal colon of lethal spotted (*ls/ls*) and piebald-lethal mutant mice (*sl/sl*) becomes aganglionic [175]. These murine aganglionoses, inherited as autosomal recessives, provide the best known animal models of Hirschsprung's disease [176]. Additional models have now been discovered. These include megacolon inherited as a recessive trait in species other than mice, such the spotting lethal rat (*sl/sl*) [12, 177–181] and the homozygous spotted rabbit (*en/en*) [182]. There is also *Dominant megacolon* (*Dom*), a mouse in which the development of aganglionosis is inherited as a dominant characteristic [183, 184]. What all of these models have in common, whether they are inherited as a recessive or a dominant trait, is that the terminal region of the gut is aganglionic, megacolon develops, and the animals exhibit a spotted coat. The megacolon can be attributed to a loss of the reflexes normally mediated by the ENS. The presence of nerve fibers thus does not compensate for the aganglionosis which contain the neural circuitry responsible for enteric reflexes. The spotted coat color reflects an abnormality of melanocytes, which like enteric neurons are derivatives of the neural crest. The defects in the animal models, however, like that which occurs in patients with Hirschsprung's disease, does not extend to all derivatives of the neural crest, or even to all crest-derived neurons. The constant association of enteric neuronal and melanocytic deficiencies thus suggests that there is a common factor or requirement for normal differentiation that the ENS of the terminal gut shares with melanocytes.

3.12 Genetic Abnormalities in Genes Encoding Endothelin-3 or its Receptor, Endothelin-B, are Associated with Spotted Coats and Aganglionosis

The genes that are abnormal in lethal spotted (*ls/ls*) and piebald lethal (*sl/sl*) mice, as well as the spotting lethal rat, have recently been identified. The loci that are involved in these models are also abnormal in a subset of patients with Hirschsprung's disease. Aganglionosis in *ls/ls* mice is associated with a mutation in the gene encoding the peptide hormone, endothelin-3 (EDN3) [185], while the somewhat more severe aganglionosis that occurs in *sl/sl* mice [186], spotting lethal rats [178, 180, 181], and some patients with Hirschsprung's disease [187] is linked to abnormalities of genes encoding the endothelin-B receptor (EDNRB). This is the receptor normally activated by EDN3. The discovery that EDN3 and the EDNRB are important in the development of the ENS (at least in the colon) was made as a result of analyses of the effects of knockouts of the genes that encode these molecules in mice.

Endothelins 1–3 represent a family of peptides, each with a chain length of 21 amino acids that activate one or both of two serpentine (G-protein coupled) receptors, endothelin-A (EDNRA) and/or EDNRB [188, 189]. Each of the endothelins has an equivalent potency for stimulating the EDNRB, but that for activating the EDNRA is EDN1 > EDN2 >> EDN3 [190]. EDN1 was discovered as a product of vascular endothelial cells that is a strong vasoconstrictor [191]. Since their initial discovery, however, the endothelins and the EDNRs have been found to be widely distributed [188]. The endothelins are initially synthesized with a signal sequence (a preproendothelin) that is responsible for translocation of the proteins across the membranes of the rough endoplasmic reticulum into the cisternal space. This translocation enables the proteins to be packaged for secretion. The signal sequence is removed cotranslationally to yield an inactive precursor, called a big endothelin. Big endothelins, in turn, are again cleaved by a specific membrane-bound metalloprotease, the endothelin-converting enzyme-1 (ECE-1), to produce the smaller active peptides [192].

Craniofacial defects arise in transgenic knockout mice that fail to produce EDN1 due to the abnormal development of first branchial arch derivatives [193]. Missense mutations in *ednrb* occur in *sl/sl* mice [186]. Similar mutations can be found in *EDNRB*, which is the analogous human locus, in patients with Hirschsprung's disease [187]. When *ednrb* is knocked out by homologous recombination, an aganglionosis of the colon develops that is identical to that seen in *sl/sl* mice [186]. More recently, lethal spotting in rats has also been demonstrated to arise as a result of an interstitial deletion in an exon of the *ednrb* gene that prevents expression of the rat EDNRB [178, 180]. The *edn3* gene is mutated in *ls/ls* mice so that an arginine is replaced by with a tryptophan residue in the C-terminus of big EDN3 [185]. This defect prevents the conversion of big EDN3 to the active EDN3 by ECE-1. In an analogous fashion, the knockout of *edn3* also causes the terminal colon to become aganglionic. It is thus clear that both the receptor, EDNRB, and the ligand, EDN3, play critical roles in the development of the ENS. The nature of these roles, however, remains to be identified.

The genetic loss of EDN3 stimulation could, in theory, lead to aganglionosis by affecting the crest-derived precursors of enteric neurons themselves. Alternatively, the effect of EDN3 could be mediated indirectly, through an action on another cell type that interacts with crest-derived cells in a manner that is essential for neuronal

and/or glial development. Why the absence of EDN3 interferes with the development of neurons only in the colon is also an issue that must be resolved. The inability of EDN1 or EDN2 to compensate for the loss of active EDN3 in *ls/ls* or *edn3* knockout mice [185] is also hard to understand, in view of the fact that all endothelins are equally good as ligands for the EDNRB [190]. The effects of EDN3, therefore, must be quite local and the circulating concentrations of EDN1 and EDN2 must be too low to be effective at those EDNRBs that are critical for development of the ENS of the terminal bowel.

3.13 An Action of EDN3 on Crest-Derived Precursors Does Not, by Itself, Account for the Pathogenesis of Aganglionosis

Several hypotheses have been advanced to explain the critical role played by EDN3 on the development of enteric neurons. One idea is that EDN3 is an autocrine growth factor [185]. This proposal considers (1) that EDN3 is essential for the development of migrating crest-derived cells as enteric neurons or melanocytes, and (2) that the crest-derived cells themselves are both the source and target of EDN3. The nice feature of this hypothesis is that it explains why the coats of all of the animal models of Hirschsprung's disease are spotted or white. The lack of EDN3 deprives both the precursors of melanocytes and enteric neurons of a necessary growth factor. The hypothesis postulates that the migrating crest-derived cells that colonize the bowel synthesize big EDN3, convert it to active EDN3, and express EDNRBs. A problem for this autocrine hypothesis is that it fails to explain why the development of enteric neurons in mice lacking EDN3 [185] and in both piebald mice [186, 194] and spotting lethal rats [178, 180, 181] that lack EDNRBs only becomes abnormal in the colon. If no factors other than the crest-derived cells themselves were to be involved, then there is no obvious reason why enteric neuronal development should be independent of EDN3 in the esophagus, stomach, and small intestine, but EDN3-dependent in the terminal colon. An idea that has been advanced to account for this problem is to assume that the ability of the vagal population of crest-derived cells to migrate as far as the terminal colon requires that the starting population be large. This hypothesis postulates that EDN3 is a mitogen that is required to provoke vagal crest cells to multiply sufficiently to generate a population that is large enough to colonize the entire bowel.

EDN3, in fact, has been demonstrated to be a mitogen for cells cultured from the premigratory neural crest [195]. Addition of EDN3 causes these cells to proliferate massively; however, following their multiplication, the cultured crest cells go on to develop primarily as melanocytes. The neural crest cells, therefore, do not respond to EDN3 exactly as predicted by the hypothesis that EDN3 is required to generate adequate numbers of neural precursors to colonize the entire gut. EDN3 promotes the formation of melanocytes, not neurons, suggesting that, at least in culture, the precursors that proliferate in response to EDN3 are not neurogenic but melanogenic. The data are even consistent with the possibility that EDN3 shifts the originally pluripotent neural crest population toward the melanocytic lineage. Unless EDN3, therefore, were to exert a different effect in vivo, this outcome would not enhance the formation of neurons in the colon. The proliferative action of EDN3 in vitro thus is consistent with the idea that its mitogenic properties are needed to enlarge the number of melanogenic precursors enough to colonize the skin, but the data do not, by themselves, support the concept that the mitogenic properties of EDN3 are needed for the formation of the ENS. To apply the hypothesis to the ENS, it is necessary to assume that the effects of EDN3 on crest-derived cells that have colonized the gut are different from those which EDN3 exerts on cells isolated from the neural crest itself. There are no longer any cells with a melanogenic potential in the crest-derived cell population that colonizes the bowel; moreover, the cohort of crest-derived cells that colonizes the gut is still proliferating [58, 59, 92]. Conceivably, after differentiation along a melanocytic lineage is no longer an option, the proliferation of crest-derived cells in response to EDN3 in the enteric microenvironment increases the number of neurons in the bowel.

Recent studies, however, in which EDN3 has been applied to crest-derived cells immunoselected from the developing murine bowel with antibodies to p75NTR, have provided surprising results (Wu J et al., unpublished data), which indicate that EDN3 does not increase the number of neurons generated in vitro. In these cultures, the development of neurons is not inhibited by an EDNRB antagonist, suggesting that there is no autocrine promotion of neuronal differentiation; furthermore, addition of EDN3 to the medium, not only fails to increase the number of neurons developing in the cultures, but both EDN3 and other EDNRB agonists actually inhibit neuronal differentiation. It is possible to envision a means by which the ability of EDN3 to inhibit the differentiation of neurons could have the seemingly paradoxical consequence of promoting the colonization of the bowel. Crest-derived precursors are migratory; neurons are not. Therefore, by preventing the differentiation of crest-derived cells as neurons, EDN3 might sustain them in a migratory state. The premature differentiation of crest-derived émigrés, by ending migration prior to its completion, would prevent the colonization of the terminal portion of the gut. Whatever the physiological action of EDN3 on crest-derived cells turns out to be, however, it is highly doubtful that these effects are sufficient by themselves to account for the development of aganglionosis.

3.14 The Pathogenesis of Aganglionosis Is Not Explained by an Abnormality Limited to Crest-Derived Neural Precursors

The enteric microenvironment may become inhospitable for colonization by crest-derived cells if EDN3 is deficient or if the EDNRB is lacking [56, 57, 194, 196]. Such an effect could be the result of an action of EDN3 on EDNRBs expressed by non-neuronal cells of the bowel wall. Alternatively, the crest-derived cells themselves may respond to EDN3 by secreting a factor that stimulates their non-neuronal neighbors to make the enteric microenvironment tractable for invasion by crest-derived émigrés. As noted above, the advancing front of crest-derived cells in the developing gut cannot be recognized by the expression of neural or glial markers, but can be detected indirectly by explanting and culturing the bowel [43, 197, 198]. Neurons develop in cultures of the normal murine terminal colon explanted after stage 33, but not before [199]. In contrast, neurons never arise in the terminal 2 mm of an EDN3-deficient (*ls/ls*) gut, no matter what the stage of the fetus at the time of explantation [197, 198]. These observations establish that the final segment of the *ls/ls* bowel is the presumptive aganglionic region and they suggest that viable crest-derived cells do not enter this zone.

In coculture experiments, crest-derived cells from a variety of sources, including the ganglionated proximal gut of *ls/ls* mice, have been shown to enter explants of the terminal bowel from control mice and give rise to neurons; however, no source of crest-derived cells migrates into an *ls/ls* terminal colon [199]. In contrast to the normal colon, moreover, the *ls/ls* colon also fails to promote the acquisition of gut-appropriate phenotypes when it is cocultured with sources of crest cells [45]. These observations are inconsistent with the hypothesis that a deficiency of EDN3 causes aganglionosis because crest-derived cells lack a critical autocrine factor. The crest-derived cells of *ls/ls* mice are genetically unable to produce EDN3, but they can colonize a normal colon; furthermore, wild-type crest-derived cells have no reason not to be able to produce EDN3, but they cannot colonize the colon of an EDN3-deficient *ls/ls* mouse. These observations suggest that EDN3 is not only an autocrine factor produced by crest-derived cells. Instead, there may be other sources of EDN3 and other EDNRB-expressing targets in the colon. The EDNRB may thus be expressed both by neurogenic cells and by non-neuronal cells and each may contribute to making it possible for crest-derived cells to complete their colonization of the bowel.

The possibility that EDN3 acts on non-neuronal cells of the gut wall has received strong experimental support. Aganglionosis does not occur in *ls/ls* × C3H aggregation chimeric mice, as long as >5% of enteric cells are of C3H origin; moreover, *ls/ls* neurons, identified with an endogenous marker (β-glucuronidase activity), are found even in the most distal enteric ganglia [196]. Similarly, ganglia containing mutant neurons (marked by the expression of a transgene, *lacZ* driven by the DBH promoter) develop in the terminal colon of aggregation chimeras constructed between wild-type and either *ls/ls* [56, 57] or *sl/sl* embryos [194]. It might be argued that the autocrine secretion of EDN3 by normal crest-derived cells could rescue their *ls/ls* neighbors in chimeric embryos. Although the *ls/ls* cells lack EDN3, they express the EDNRB, and thus should be responsive to EDN3 supplied by nearby cells. On the other hand, this explanation does not account for the ability of *sl/sl* crest-derived cells, which lack the EDNRB [186], to colonize the bowel. Such cells should be unable to respond to EDN3 from any source. Clearly, therefore, the simple autocrine model, in which crest-derived cells stimulate themselves by secreting EDN3, is inadequate by itself to explain the development of aganglionosis. It has therefore been postulated that intercellular signals "downstream" from the EDNRB mediate colonization of the terminal gut by crest-derived cells [194].

An alternative hypothesis is that there is an additional, non-neuronal cell in the wall of the colon that expresses the EDNRB and must be stimulated by EDN3 in order to open the colon to colonization by crest-derived neuronal precursors. This latter idea is supported by the observation that the migration of vagal crest-derived cells, visualized by their expression of the *DBH/lacZ* transgene, is entirely normal in *ls/ls* mice until the cells reach the colon; however, the migration of vagal crest-derived cells becomes abnormal within the colon, which is not fully colonized [55, 57]. These observations imply that the ability of crest-derived cells to migrate within the colon is influenced by the enteric microenvironment, which is abnormal in EDN3-deficient *ls/ls* mice. This suggestion has been confirmed by back-transplantation experiments [196]. When segments of wild-type or *ls/ls* colon are placed in a neural crest migration pathway of a quail embryo, the avian crest-derived cells enter wild-type, but not *ls/ls* grafts. There is no reason to suppose that the quail crest-derived cells in these experiments fail to express either EDN3 or the EDNRB. Their inability to enter the *ls/ls* colon, therefore, cannot be explained by the autocrine hypothesis; furthermore, the back-transplantation experiment demonstrates that the absence of active EDN3 in the aganglionic *ls/ls* colon has produced an environment that crest-derived cells do not enter.

In sum, the accumulated evidence suggests that crest-derived cells are capable of colonizing the gut and forming enteric neurons whether or not they produce or respond to EDNRB, but that the enteric microenvironment becomes abnormal in the absence of EDN3/EDNRB stimulation, so that the colon becomes resistant to colo-

nization by crest-derived cells, whether or not these cells produce or respond to EDN3. In fact, extracellular matrix abnormalities have been described, both in the colon of *ls/ls* mice and in human patients with Hirschsprung's disease.

3.15 The Extracellular Matrix is Abnormal in the Presumptive Aganglionic Bowel of *ls/ls* Mice

A variety of defects involving components of the extracellular matrix have been found in *ls/ls* mice [200–202] and in human patients with Hirschsprung's disease [203, 204]. A common feature that unites these abnormalities is that they all involve an over-abundance and/or maldistribution of constituents of basal laminae. Molecules that have been noted to be over-abundant include laminin, collagen type IV, nidogen non-sulfated glycosaminoglycans, and proteoglycans. In the developing colon of fetal *ls/ls* mice, the abnormal molecules are diffusely distributed throughout the mesenchyme of the colon and the surrounding pelvis and are not, for the most part, aggregated in formed basal laminae [200–202]. The mucosal basal lamina of the terminal and distal colon, however, is also thickened relative to that of a wild-type fetus of the same age. The location of the accumulated molecules of the extracellular matrix is in the paths both of vagal crest-derived cells migrating down the bowel [205] and of sacral crest-derived cells approaching the gut [206]. Double-label electron microscopic immunocytochemistry, moreover, has revealed that crest-derived cells, identified by their expression of HNK-1 immunoreactivity, migrate through the enteric mesenchyme of the developing bowel in contact with what appears to be diffuse tufts of electron-opaque material that is laminin-immunoreactive [207].

The over-abundance of laminin and type IV collagen can be detected in the colon of *ls/ls* mice at an earlier age [200] than that when crest-derived cells colonize the terminal colon in wild-type mice [199]. This timing and the fact that the extracellular matrix molecules accumulate in the path of incoming crest-derived cells are consistent with the possibility that the abnormal extracellular matrix in *ls/ls* mice (and by analogy in patients with Hirschsprung's disease) contributes to the pathogenesis of aganglionosis. This suggestion, however, presumes that the accumulation of laminin and other constituents of the extracellular matrix is a primary event rather than a secondary response to the absence of neurons and/or their precursors.

Recent studies with *ls/ls* mice have indicated that, at least in that model, the accumulation of molecules of the extracellular matrix in the fetal bowel is probably due to an increase in their biosynthesis [202]. mRNAs encoding the β1 and γ1 subunits of laminin, as well as the α1 and α2 chains of collagens type IV, were found by quantitative Northern analysis to be increased in the colons of *ls/ls* mice. Transcripts encoding laminin α1 were also found to be increased; however, the abundance of mRNA encoding the α1 chain was so much less than that of the β1 and γ1 subunits that the α1 protein had to be evaluated quantitatively with reverse transcription and the competitive polymerase chain reaction (RT-cPCR). The abundance of mRNA encoding laminin α1 was developmentally regulated and declined as a function of age after E11; nevertheless, at all ages the abundance of mRNA encoding laminin α1 was higher in the *ls/ls* colon than in an age-matched wild-type colon or in the small intestine of the same *ls/ls* animals. The location of the cells responsible for the bulk of the biosynthesis of laminin α 1 and β1 and the α2 chain of collagen type IV was found by in situ hybridization (with 35S-labeled antisense riboprobes) to change as a function of developmental age. In the fetal colon, transcripts of mRNA encoding these molecules are first concentrated in the endodermal epithelium; however, by day E15, the transcripts are more abundant in mesenchymal cells of the outer gut wall than in the epithelium. More mRNA was found in the colonic mesenchyme of the *ls/ls* colon than in the wild-type colon at an equivalent age.

To determine whether the increase in mRNA encoding subunits of laminin is a primary or secondary event, the expression of laminin-1 in E15 and newborn *c-ret* knockout mice were compared with that in age-matched *ls/ls* and wild-type animals. The assumption behind this comparison was that the aganglionosis that occurs in both *ls/ls* and *c-ret* knockout mice does so for different genetic reasons. In *c-ret* knockout mice, the entire bowel distal to the rostral foregut becomes aganglionic because early crest-derived precursors lack functional Ret receptors and thus cannot respond to GDNF [101–104, 109]. In the *ls/ls* mice the animals lack EDN3 and the aganglionic region is restricted to the colon [185]. If the increase in transcripts of laminin and the associated accumulation of laminin and other molecules of the extracellular matrix in the colon of *ls/ls* mice were to be a secondary response to the absence of neural precursors, then one would expect to see the same increase in the aganglionic bowel of *c-ret* knockout mice. In contrast, the increase in mRNA encoding laminin subunits should not occur in the aganglionic bowel of *c-ret* knockout mice if the change is *ls/ls*-specific and related to an effect of the absence of EDN3 on the colonic mesenchyme. No difference from controls either at E15 or in newborn mice was detected by RT-cPCR in the abundance of mRNA encoding laminin α1 in the *c-ret* knockout colon [202]; furthermore, the over-abundance of immunocytochemically visualizable laminin characteristic of the *ls/ls* colon was seen in *c-ret* knockout mice.

The results of these experiments suggest that the increase in abundance of mRNA encoding components of the extracellular matrix occurs in *ls/ls* mice as a primary effect of the genetic defect in EDN3, and is not a consequence of the aganglionosis. The observations also suggest that at least one isoform of laminin that is present in excess in the *ls/ls* mouse is laminin-1 (α1-β1-γ1). It should be noted that the accumulation of laminin-1 and other molecules of the extracellular matrix is not limited to the colon, although it occurs there. The excess of these molecules is also found in the pelvic mesenchyme that surrounds the terminal bowel. As a result, the abnormal extracellular matrix is located in the paths both of the vagal crest-derived cells that descend within the gut and of the sacral crest-derived cells that approach the bowel within the pelvis. The location, as well as the *ls/ls* specificity of the abnormal matrix, therefore, are compatible with the possibility that it contributes to the pathogenesis of aganglionosis. Whether the extracellular matrix defects are actually contributory to the condition, however, remains the be confirmed.

Although molecules of the extracellular matrix have been demonstrated to inhibit the migration of crest cells in a number of locations, including the dorsolateral path between the ectoderm and the somites [208, 209], the posterior sclerotome [210–212], and the perinotochordal mesenchyme [213], in none of these regions have the inhibitory effects been linked to accumulations of components of basal laminae [208, 211, 212, 214]. In fact, the extracellular matrix in these regions behaves rather differently from that of either the aganglionic *ls/ls* [142] or Hirschsprung's bowel [9, 215]. The aganglionic bowel in each of these conditions is heavily innervated both by axons of neurons from the more rostral hypoganglionic gut and from extrinsic ganglia [142]. The defect in the colon of *ls/ls* mice and patients with Hirschsprung's disease thus impedes its colonization by crest-derived cells, but it does not antagonize the ingrowth of axons.

In contrast, the other regions that normally exclude crest-derived cells also inhibit the outgrowth of axons [214]. It also seems paradoxical that laminin-1 should be one of the molecules that is overly abundant in a zone where crest-derived cells fail to migrate. Laminin is a favorable substrate for the adherence of crest-derived cells [81, 216]; moreover, laminin-1 also stimulates the migration of cells away from the neural crest itself [82, 217]. Antibodies to integrins that block attachment of crest-derived cells to laminin [218, 219], as well as antibodies that bind to a laminin-proteoglycan complex [220] inhibit cranial crest cell migration in vivo. The abundance of laminin in the aganglionic *ls/ls* colon, therefore might be expected to promote rather than inhibit the colonization of this region of the bowel by cells from the neural crest. On the other hand, the abundance of laminin-1 in the aganglionic colon of *ls/ls* mice and human patients with Hirschsprung's disease could explain why this region of the gut is so well innervated by extrinsic axons; laminin promotes neurite extension and axonal growth [221–226].

3.16 Laminin-1 Promotes the Development of Neurons from Enteric Cells of Neural Crest Origin

Molecules of the extracellular matrix have been demonstrated to be biologically active and able to alter the fate of stem cells from the neural crest in vitro [227]. Extracellular matrix molecules, therefore, can provide more than just an adhesive substrate for crest-derived cells; they are also able to provide signaling information and are, at least potentially capable of influencing the differentiation of crest-derived cells. Specifically, with respect to crest-derived cells that colonize the bowel, a substrate that includes laminin-1 has been found to increase the in vitro development of neurons relative to that which occurs on substrates of tissue culture plastic or type I collagen [125, 228, 229]. Neurons in these studies were defined as cells that express markers (such as peripherin, neurofilament proteins, neuron-specific enolase, or PGP9.5) that were visualized by immunocytochemistry. The ability of laminin-1 to promote the development of enteric neurons was initially observed in cultures of crest-derived cells immunoselected from the developing avian or rat gut with HNK-1 monoclonal antibodies. An even more pronounced effect of laminin-1 [228–230] is seen in cultures of cells immunoselected from the mouse gut with antibodies to a cell-surface laminin-binding protein, known as LBP110 [222, 231–233].

3.17 The Effect of Laminin-1 on Enteric Neuronal Development Depends on the Binding of its α1 Chain to LBP110

LBP110 is not an integrin, but is similar to a β-amyloid precursor protein [234]. The domain of laminin that binds to LBP110 contains an isoleucine-lysine-valine-alanine-valine (IKVAV) sequence and is located on the laminin α1 chain, near its globular C-terminal end [235–237]. Expression of LBP110 by PC12 cells is downregulated by transfection of the cells with an antisense amyloid precursor protein cDNA [234, 235]. The ability of NGF to induce neurite extension on a laminin-1 substrate is reduced in such antisense-treated PC12 cells. Kleinman and colleagues have concluded that LBP110 is a laminin-1 receptor that mediates the effects of laminin-1 on neurite outgrowth and also is responsible for controlling a variety of behaviors in non-neuronal cells [234, 235, 238–243].

The only cells in the bowel that express LBP110 are those of neural crest origin; therefore, LBP110 immunoreactivity colocalizes in the gut with crest markers [207]

and cells immunoselected from the fetal mouse gut with antibodies to LBP110 preferentially differentiate as neurons or glia [125]. The ability of laminin-1 to promote the development of crest-derived cells as neurons or glia is specifically blocked by a synthetic peptide that contains the IKVAV sequence (IKVAV peptide) [228, 230]. A variety of control peptides exert no effect on neuronal differentiation, including a nonsense peptide, a peptide with the same amino acids in a different sequence, or a peptide with a sequence found elsewhere in the laminin-1 molecule. The IKVAV peptide, moreover, does not affect the development of neurons and glia when similar populations of anti-LBP110-immunoselected crest-derived cells are cultured on poly-d-lysine or fibronectin. The IKVAV peptide, therefore, does not exert a generally inhibitory action on the development of enteric neurons, but only blocks the increment in neuronal development that is a response to laminin-1. Since the addition of an IKVAV peptide does not reduce the total number of cells in culture, the IKVAV peptide appears not to antagonize the adhesion of cells to laminin-1. Adhesion is probably integrin-dependent [218, 219] and independent of LBP110 [235].

Further evidence that the IKVAV peptide does not interfere selectively with the attachment of a small neurogenic subset of crest-derived cells (which could be too small to affect the total number of cells counted in the cultures) has come from the observation that laminin-1 is just as effective when added in soluble form to already adherent cells as it is when it is used as the substrate upon which cells are plated [228–230]. Soluble laminin is also equally efficacious when applied to cells immunoselected from the fetal mouse gut with antibodies to p75NTR as when it is applied to cells immunoselected with antibodies to LBP110. The effectiveness of soluble laminin-1 does not necessarily indicate that laminin-1, in a soluble form, is able to activate the receptors responsible for its effect on enteric neuronal development. Even when added as a soluble molecule, laminin-1 might bind to the substrate and then, after becoming bound, activate the receptors on cell surfaces that mediate its effects; nevertheless, the observation that laminin-1 retains its efficacy many hours after cells have adhered to poly-d-lysine, indicates that the ability of laminin-1 to increase the numbers of neurons developing in vitro is not due to the selective adherence of neurogenic crest-derived cells to laminin-1 at the time of plating.

As is true of the responses of cells to the addition of a growth factor, the response of immunoselected crest-derived cells to laminin-1 is associated with a rapid, but transient induction of the expression of the *c-fos* proto-oncogene. The effect of laminin-1 on *c-fos* expression is evident within one hour of adding laminin-1 and is no longer detectable by 24 hours. The *c-fos* response to laminin-1, like the promotion by laminin-1 of neuronal development, is abolished by the IKVAV peptide, but not by control peptides. The specific antagonism by the IKVAV peptide of both the laminin-1-induced development of neurons and the expression of *c-fos* suggests that both of these responses are mediated by LBP110, which is the cellular binding site for the IKVAV domain of laminin-1. Since the IKVAV peptide is an antagonist, and not an agonist, the observations also imply that activation of the putative receptor function of LBP110 requires more than simply its binding to the IKVAV domain of laminin α1. It is likely that the binding of the IKVAV domain to laminin-1 is necessary but not sufficient to stimulate the LBP110 receptor. Other sequences of laminin-1 and/or the whole laminin-1 molecule must be required for agonist activity. However, although the IKVAV peptide does not stimulate LBP110, its presence in excess in the medium indicates that it probably occupies IKVAV binding sites on LBP110 and competitively antagonizes the binding of laminin-1.

These ideas have recently been supported by additional experiments that have shown that an antipeptide neutralizing antibody directed against the IKVAV domain of the α1 chain of laminin-1 mimics the effect of the IKVAV peptide and blocks the promotion of the development of enteric neurons in vitro by laminin-1 (Chalazonitis A et al., unpublished data). In contrast, precipitating antibodies to the β1 chain of laminin-1, applied in the same manner, fail to interfere with the in vitro differentiation of enteric neurons. Neither the antibodies to the α1 chain, nor those to the β1 chain, cause cells to detach from a laminin-1-containing substrate. As might be expected from its effect on PC12 cells, laminin-1 promotes the extension of neurites, as well as the development of neurons. This action is also specifically antagonized by an IKVAV peptide and by antibodies to the IKVAV domain of laminin α1.

3.18 The Effects of Laminin-1 on Crest-Derived Cells Immunoselected from the Fetal Bowel Are Different from those of Laminin-1 on Cells Isolated from the Crest Itself

In contrast to its action on crest-derived cells immunoselected from the fetal gut, laminin-1 does not induce neural crest stem cells to differentiate as neurons [227, 244]. The ability of crest-derived neuronal precursors to respond to laminin-1 must thus be a characteristic the cells acquire, either while migrating to the bowel, or after they enter it. The difference in responsivity to laminin-1 between neural crest stem cells and their crest-derived successors, could be accounted for by the timing of LBP110 expression. Although premigratory and early-migrating crest cells express integrins, and thus are able to bind to laminin [81, 216, 217, 219, 220], which is abundant in the embryonic mesenchyme and basal laminae [206, 207, 245, 246], premigratory and early-migrating crest cells

do not express LBP110 [207]. LBP110 is expressed only in target organs; moreover, the crest-derived émigrés that colonize the bowel express LBP110 for the first time within the gut itself. If the induction of neuronal development by laminin-1 depends on the interaction of LBP110 with the IKVAV domain of the α1 chain of laminin-1, as suggested by the in vitro studies outlined above, then enteric neuronal precursors could adhere to laminin-1 while migrating to the bowel without being induced to prematurely differentiate into neurons. The premature differentiation of crest-derived cells into neurons prior to their arrival in the gut would prevent them from colonizing the bowel. Neurons are not notably migratory; thus, for ganglia to develop within a given region of the bowel, that region must first be colonized by crest-derived neural precursors. Crest-derived cells, within the gut, acquire LBP110 asynchronously. Some of the vagal crest-derived émigrés express LBP110 as soon they enter the proximal bowel. Others, however, acquire LBP110 later and by the time they express LBP110 they have moved distally [207]. This asynchronous delay in the timing of LBP110 expression may enable the late-responding crest-derived cells to make their way distally into the caudal bowel before they differentiate and cease migrating.

3.19 Premature Neuronal Differentiation May Result When Inadequately Resistant Progenitors Encounter an Excessively Permissive Extracellular Matrix

The expression of LBP110 and the evidently related ability of laminin-1 to promote enteric neuronal development from crest-derived precursors may explain the seemingly paradoxical association of an excess of laminin-1 with aganglionosis in the terminal colon of EDN3-deficient *ls/ls* mice and human patients with Hirschsprung's disease. As has been noted previously, it is likely that the deficiency of EDN3 removes an inhibitory influence on neuronal differentiation. By simultaneously leading to an excess laminin-1 in the colonic mesenchyme, the lack of EDN3 also causes crest-derived cells to become exposed to an over-abundance of a signal that promotes neuronal development. On the one hand, a brake to neuronal differentiation is absent, while on the other, a drive to differentiate is enhanced. The consequence of the combined effect may be the premature differentiation of crest-derived émigrés as neurons. Premature differentiation in turn causes the cells to cease migrating before colonization of the gut is complete. The genetic deficiency of EDN3 may thus exert both direct and indirect effects, which combine synergistically to prevent the formation of ganglia in the terminal bowel. These ideas predict that vagal crest-derived cells of *ls/ls* mice (or the subset of patients with Hirschsprung's disease with defects in EDN3 or the EDNRB) would encounter an abnormally strong inducement to differentiate (the over-abundance of laminin-1) when they enter the proximal colon. Consistent with this prediction, is the observation that the progression of crest-derived cells, visualized in *ls/ls* mice by their expression of the *DBH-lacZ* transgene, is comparable to that in wild-type animals until the cells cross the ileocecal threshold, but becomes abnormal immediately thereafter [57].

Since laminin-1 is present in excess in the pelvic mesenchyme that surrounds the bowel, the hypothesis also predicts that sacral crest-derived precursors will not even enter the gut [200]. This prediction too has been confirmed, in that unique ectopic ganglia are present outside the terminal bowel in *ls/ls* mice [142, 197]. It is likely that these extra-enteric ganglia are formed by migrating sacral crest-derived cells that prematurely differentiate and stop before entering the gut. In the hypoganglionic region of the *ls/ls* colon, the aberrant ganglia actually pierce the longitudinal muscle and fuse with ganglia of the myenteric plexus. This peculiar configuration of ganglia, partly in and partly out of the gut, provides strong support for the idea that sacral crest-derived cells cease migrating short of their destination in the *ls/ls* bowel. This concept, that the aganglionosis of EDN3 deficiency (or absence of EDNRB) has a dual origin in an abnormal extracellular matrix driving an inadequately resistant crest-derived progenitor, would account for the observations that the failure of neurogenesis in the terminal bowel in these conditions is not neural crest-autonomous.

3.20 Both Crest-Derived and Non-Neuronal Cells of the Colon Probably Respond to EDN3

There is evidence that EDN3 affects both crest-derived and non-crest-derived cells in the colon. Clearly, the excess of laminin-1, which occurs independently of crest-derived cells in the *ls/ls* bowel, is most easily explained by the postulate that EDN3 normally acts on one or more of the cells of the fetal enteric mesenchyme to downregulate their secretion of laminin-1. This postulate assumes that the EDNRB must be expressed, not only by crest-derived cells, but also by other cells of the fetal mesenchyme. Smooth muscle precursors and cells that form interstitial cells of Cajal (ICCs) are each candidates to be cells that express EDNRBs. In the mature gut, EDNRBs have been demonstrated to be expressed by the smooth muscle cells of the muscularis externa of both the large intestine [247] and small intestine [248]; moreover, intestinal smooth muscle responds directly to EDN3. When, during development, smooth muscle cells acquire EDNRBs is unknown. Transcripts of mRNA encoding EDN3 and those encoding the EDNRB are each found in the totally aganglionic bowel of *c-ret* knockout mice (Chen J et al., unpublished data), confirming (albeit indirectly) that enteric neuronal and glial precursors are not the only cells in the bowel wall that synthesize these molecules.

Direct evidence that non-neuronal cells contain mRNA encoding the EDNRB has been provided by in situ hybridization carried out in mice in which the crest-derived cells are marked by their expression of the *DBH-lacZ* transgene (Kapur R and Yanagisawa M, reported at the 1996 Meeting of the American Motility Society). Both the *lacZ*-expressing crest-derived cells in primordial myenteric ganglia and non-*lacZ*-expressing cells that surround the ganglia were found to express the EDNRB. The location of the *lacZ*-negative cells that contain mRNA encoding the EDNRB is compatible with the idea that these cells are ICCs. That possibility must still be confirmed; however, ICCs have been found to be abnormal in patients with Hirschsprung's disease [249, 250].

3.21 Interstitial Cells of Cajal are Present, but Abnormal, in the Aganglionic Bowel of Hirschsprung's Disease

The nature of the ICC has long been the subject of debate [251, 252]. An old idea that ICCs might be fibroblasts [253] has now been discarded [253]. A more recent suggestion is that ICCs are modified or primitive smooth muscle cells [254, 255]. Whether or not they are related to smooth muscle, ICCs can be identified as a distinct cell type by their expression of, and dependence on, the *c-kit* protooncogene [256–259]. *c-kit* encodes a receptor tyrosine kinase (Kit) and is allelic with *White Spotting* (*W*) [260]. Kit ligand (KL; also known as *Steel* factor or stem cell factor) is allelic with *Steel* (*Sl*). Activation of Kit by KL is probably critical for the development and/or maintenance of ICCs, because *W* [256, 257] and *Sl* [259] mutations interfere with the appearance of ICCs, the injection of neutralizing antibodies to Kit causes ICCs to disappear [258, 261], and the development of Kit-expressing ICCs in vitro is dependent on KL in the culture medium [262]. ICCs appear to be the pacemakers for myogenic intestinal slow waves because these waves are impaired when the network of ICCs is lost or fails to develop [256–259, 261]. Once the ICC network is disrupted and slow waves are lost, intestinal motility becomes abnormal and the bowel dilates in a manner that is not dissimilar to that seen in aganglionosis.

During fetal development and, in some regions (the longitudinal muscle) extending into postnatal life, ICCs express markers in common with smooth muscle cells [251]. These markers include the intermediate filament protein, desmin, and smooth muscle isoforms of actin and myosin. ICCs never express Ret, which can serve as a marker for crest-derived cells in the wall of the gut [100, 101, 263]. These observations suggest that ICCs are not crest-derived cells, but that instead, they share a common precursor with smooth muscle. A similar conclusion has been reached from studies of stably marked crest-derived cells in avian interspecies chimeras [264]. Interestingly, Kit-immunoreactive ICCs assume a variety of shapes in different locations in the intestinal wall and may be divided by the timing of their divergence from the common smooth muscle/ICC precursor into subtypes of ICC [251]. It has been proposed that those ICCs that surround myenteric ganglia and those that are found within the deep muscle plexus, circular, and longitudinal muscle layers constitute functionally distinct cell classes.

Since ICCs are not crest-derived cells, it follows that their abnormality in the affected region of the bowel of patients with Hirschsprung's disease [249, 250] demonstrates that the genetic lesion in these patients affects more cells than just neurons and their precursors. ICCs, however, are reduced in number and disrupted in pattern, but they are not totally absent from the aganglionic region of the colon in Hirschsprung's disease. ICCs are also found in the terminal colon of *ls/ls* mice and in the aganglionic bowel of *c-ret* knockout mice, although again, their numbers are reduced in comparison to those of wild-type mice, and the distribution pattern of ICCs is abnormal [262]. These observations indicate that ICCs can develop in the absence of EDN3 and even in the absence of neurons. Conceivably, the abnormal numbers and distribution of ICCs in the aganglionic bowel of patients with Hirschsprung's disease and *ls/ls* mice are secondary effects, resulting from the aganglionosis. Supporting this possibility, in situ hybridization has indicated that enteric neurons do contain mRNA encoding KL [251]. Enteric neurons thus are likely to be a source of KL; moreover, the physiologically active form of KL is not the secreted protein, but a membrane-bound ligand [265, 266]. To be stimulated by neuronal KL, therefore, neurons probably must come into contact with target cells so that the Kit receptors of the targets can be activated by the KL bound to neuronal surfaces. The requirement that cell-to-cell contact must occur for the KL/Kit interaction to take place could explain the close spatial relationship of a subset of ICCs to myenteric ganglia.

The aganglionosis of Hirschsprung's disease and that of *ls/ls* mice, therefore, might each be expected to be associated with ICC abnormalities; the KL-dependent ICCs would be deprived of neuronal KL in the aganglionic bowel in these conditions. Neurons, however, are probably not the only source of KL in the bowel. First, if they were, then ICCs would be expected to be totally absent from the aganglionic zone of the Hirschsprung's and *ls/ls* colon, but they are not. Second, ICCs develop in the *c-ret* knockout gut, which contains no neurons at all; moreover, mRNA encoding KL (as well as that encoding Kit) can be detected in this tissue. It is possible that ICCs do not require EDN3 for their development, but they might still express the EDNRB and be EDN3-responsive. The abnormalities noted in the numbers and distribution of ICCs in the aganglionic regions of the Hirschsprung's and *ls/ls* colons are consistent with this idea. Certainly, the location of the non-neuronal cells of the colon of

DBH-lacZ mice found by in situ hybridization to contain mRNA encoding the EDNRB conforms to the known location of Kit-immunoreactive ICCs in the bowel. One might speculate that EDN3 speeds the development of ICCs or smooth muscle. In its absence, the respective precursors might remain secretory for a longer period of time than normal and secrete more laminin-1. As the cells mature as smooth muscle and/or ICCs, laminin-1 secretion diminishes. This hypothesis is consistent with the observed developmental regulation of laminin-1 and the slower than normal rate of decline found in its expression in the *ls/ls* colon [202].

3.22 Hirschsprung's Disease is Associated with Many Different Genetic Abnormalities: Conclusion From Animal Models

Congenital neuromuscular disorders of the gut are commonly encountered during the neonatal period. These conditions include, in addition to Hirschsprung's disease (long and short segment varieties), the allied disorders, hypoganglionosis, neuronal intestinal dysplasias (hyperganglionosis), ganglion cell immaturity, and dysganglionoses. There are also additional defects such as hypertrophic pyloric stenosis, volvulus, and intussusception, that may also involve abnormalities of the development of the ENS. Hirschsprung's disease is quite common and occurs in up to 1 in 5,000 live births [267]. In some patients, Hirschsprung's disease has been shown to be associated with loss-of-function mutations in the *RET* protooncogene [267–271]. Only a small minority of patients with Hirschsprung's disease can be accounted for by *RET* mutations [267–269]. Both long and short segment Hirschsprung's disease can occur in patients with identical *Ret* abnormalities and patients may also exhibit other problems, including multiple endocrine neoplasia type A (more commonly associated with gain-of-function mutations in *RET*), maternal deafness, talipes, and malrotation of the gut. Identical mutations in *RET* may thus give rise to distinctly different phenotypes in affected individuals. Unfortunately, there is no obvious relationship between the *RET* genotype and the Hirschsprung's phenotype; moreover, the frequency of *RET* mutations in Hirschsprung's disease is so low that other genetic and/or environmental conditions must be invoked to explain susceptibility to Hirschsprung's disease in the majority of patients.

Another important genetic defect that has been associated with Hirschsprung's disease involves mutations in *EDNRB* [187]. Again, many patients with Hirschsprung's disease do not exhibit mutations of *EDNRB* or *RET* and there are individuals who carry these mutations (and also those of *RET*) who do not express the Hirschsprung's disease phenotype [187]. As might be expected, not only are some cases of Hirschsprung's disease linked to mutations in *EDNRB*, but mutations of genes encoding the ligand, EDN3, are also associated with Hirschsprung's disease. In the case of the EDN3 mutations, the phenotype is reminiscent of that which is seen in *ls/ls* mice. Hirschsprung's disease occurs together with pigmentary abnormalities and is combined with a Waardenburg type 2 phenotype (Shah-Waardenburg syndrome) [272, 273]. Hirschsprung's disease is thus a multigene abnormality and a wide variety of mutations (many of which are still to be identified) predispose toward it [187, 267]. The environmental background within which these mutations operate probably also influences the phenotypic outcome.

Not all of the genetic abnormalities that have been correlated with Hirschsprung's disease are exactly comparable to the analogous mutations in animal models. Knockout of *c-ret* only leads to aganglionosis in mice when the mutated gene is homozygous [101, 104]. The ENS is not abnormal in the bowel of heterozygous mice that carry only a single mutated *c-ret* allele. Even when *c-ret* +/− mice are crossed with animals carrying the *ls* gene, the double heterozygotes do not exhibit aganglionosis (Rothman T et al., unpublished observations). In contrast, the *RET* mutations detected in patients with Hirschsprung's disease have only been heterozygous. This discrepancy in the effects of mutations between humans and mice is difficult to explain and may be due to the effects of additional genes or environmental factors. Despite these discrepancies, animal models provide the best hope of achieving an understanding of the pathogenesis of Hirschsprung's disease. The EDN3/EDNRB-deficient mouse and rat models appear to be especially useful. The resemblance of these models to Hirschsprung's disease are striking both from a genetic and an anatomical point of view. Molecular abnormalities that have been found in the extracellular matrix of the murine models [202] also occur in patients with Hirschsprung's disease [203, 204]. The thickening of the muscularis mucosa and the overabundance of laminin and type IV collagen that characterize the aganglionic gut of *ls/ls* mice characterize human megacolon as well. In addition, it has been reported that laminin and type IV collagen normally accumulate before neurogenesis begins at the sites where ganglia will form [274]. Observations made on *ls/ls* [54, 56, 57, 196, 197, 199, 200], *sl/sl* [194], and *Dom* [184] mice have been able to demonstrate that the pathogenesis of the aganglionosis that occurs in these animals is not neural crest autonomous, but involves an intrinsic abnormality of the colon.

3.23 Summary

The ENS is a complex and independent nervous system that is formed by precursors that migrate to the bowel from vagal, truncal, and sacral regions of the neural crest.

The crest-derived enteric neuronal progenitors are initially multipotent; however, their developmental potential decreases as a function of time and place during ontogeny. The crest-derived émigrés that arrive in the bowel have lost the potential to give rise to some derivatives, such as ectomesenchyme and melanocytes, but the émigrés retain a high degree of pluripotency and their ultimate fate is influenced by the enteric microenvironment. The effects of the microenvironment are played out on cells that vary in their receptivity according to the lineages and sublineages into which they have been sorted. Two such lineages have been defined. One is born early, is transiently catecholaminergic, is dependent on expression of the *mash-1* gene, and gives rise to serotonergic neurons. The other, from which CGRP-containing neurons are derived, is born late, is never catecholaminergic, and is *mash-1*-independent. A variety of signals have been identified that influence the differentiation and/or survival of enteric neurons. An early-acting factor is GDNF, which activates the Ret receptor. A later-acting factor is the neurotrophin, NT-3. This factor acts synergistically with a still-to-be-identified member of the cytokine family that activates the CNTFRα.

Natural or targeted mutations in genes that encode factors required by crest-derived precursors early in development affect cells that are still relatively multipotent; therefore, the resulting defects tend to be large, such as those associated with the knockout of GDNF or Ret. Later-acting factors give rise to many fewer global abnormalities, although even a small loss of a critical neuron may be lethal. Knockout of the CNTFRα, which results in an apparent loss of motor fibers to smooth muscle, is an example. A still more localized abnormality occurs in mice lacking EDN3 or the EDNRB. The terminal colon becomes aganglionic. This defect may result from an effect of the mutation both on the crest-derived precursors of enteric neurons and on the non-neuronal cells of the bowel wall that produce the matrix through which crest-derived cells must migrate to colonize the gut. There is an excess of laminin-1 in the colon that may combine with the loss of the effect of EDN3 on crest-derived cells to cause premature differentiation of precursors as neurons. Since neurons do not migrate, the consequence of premature differentiation is an early cessation of migration leading to a distal aganglionosis.

Hirschsprung's disease of humans has been associated with a number of mutations, including *RET*, *EDNRB*, and *EDN3*. Hirschsprung's disease, however, is a multigene abnormality that cannot be completely accounted for by known mutations. Each of the factors that are critical for the formation of the normal ENS are potential targets of mutations that might cause Hirschsprung's disease or other birth defects in humans. Future research should begin to reveal genes that, in their abnormality, cause hypoganglionosis, neuronal intestinal dysplasias, and intestinal dysganglionoses, as well more contributors to Hirschsprung's disease. Hopefully, progress made in understanding the pathogenesis of Hirschsprung's disease and allied disorders will provide better means of treating these conditions and, better yet, preventing them.

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Animal Models of Aganglionosis

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4

4.1 Introduction

Humans are not the only mammals to suffer from aganglionosis. Aganglionosis has also been described in mice, rats, horses, cats and dogs. Rodent animal models have contributed significantly to our understanding of Hirschsprung's disease (HSCR). Over the last decade, the understanding of the genetics and cell biology of the development of the enteric nervous system (ENS) has made great progress. Rodent animal models have shown many points of correlation with humans in regard to ENS development, both normal and abnormal. Nevertheless, the link between the genotype and the phenotype is often indirect, and so many questions have yet to be answered. This chapter deals with the characteristics of aganglionosis in rodents with emphasis on how knowledge of the animal models has contributed to our understanding of the genetics and pathogenesis of HSCR and allied disorders.

4.2 History

4.2.1 Rodents

The first description of aganglionosis in mice was by Derrick and St George-Grambauer in 1957 [1]. They found approximately 3.2 per 1000 of their colony developed aganglionosis. The average length of aganglionosis was 15–20 mm with colonic distension extending into the first few millimeters of aganglionic colon. There was no association with a white or patched color coat. The next description was by Bielschowsky and Schofield in 1962 [2]. In this colony 10% of the offspring were affected and there was an association with a white colored coat. These mice also had a high incidence of mammary cancer and pituitary adenomas. Outbreeding experiments suggested an autosomal recessive trait with modification of the trait by other genetic factors.

In 1966, Lane described two strains of mice which developed aganglionosis as an autosomal recessive condition [3]. The lethal spotting (ls) mice have approximately 2 mm of aganglionosis with a patched coat, and later studies linked the defect to chromosome 2. Piebald lethal (s^1) mice had approximately 10 mm of aganglionosis and linkage studies suggested that the defect was on chromosome 14. In 1979, Ikadai et al. [4] described aganglionosis in spotting lethal (sl) rats. The animals had two lengths of aganglionosis: total colonic aganglionosis (TCA) and mid colon. These animals again showed autosomal recessive inheritance and had a white colored coat. A fourth rodent model is the Dominant megacolon (Dom) mouse

in which the aganglionic colon has a long hypoganglionic transition zone (Table 4.1) [5].

4.2.2 Other Mammals

There have been isolated reports of aganglionosis in a range of animals including cats [6, 7], horses [8–11] and pigs [12, 13].

4.3 Histologic Anatomy

Are rodents good histologic models of HSCR? The first histologic studies were performed by Lane [3]. These were restricted to showing there was aganglionosis in the terminal bowel, and in documenting the length of aganglionosis.

Bolande and Towler [14] and Bolande [15] investigated the lethal spotting mouse using histochemical and ultrastructural studies. Histology showed hypoganglionosis in the distal bowel but there was no dense ingrowth of nerve fibers. Boley [16] suggested that these findings of no hypertrophied nerve trunks indicated that these mice were not a good model of human disease. In the distal narrowed segment there was a reduction in adrenergic and cholinergic fibers. In the dilated part of the bowel there was an increase in adrenergic fibers. The ultrastructural studies showed that just above the transition zone, there were secondary degenerative changes in the ganglion cells, which increased with age, resulting in what appeared to be secondary cell death and abiotrophy. Webster [17, 18] performed detailed studies in lethal spotting and piebald lethal mice using cholinesterase stains and fluorescence to delineate the adrenergic nerves. In postnatal mice of both strains he demonstrated normal innervation in the proximal bowel followed by a transition zone and then an aganglionic zone with increased nerve trunks and a decrease in the innervation of the circular muscle fibers. In the most distal aganglionic colon, just above the anal sphincters, there appeared to be a variable, but denser, innervation to the circular muscle involving cholinergic nerves. Bu'Lock et al. [19] found a selective depletion of substance P in the transitional zone in piebald lethal mice. However, the change was from 10% in normal mice to 5% in mutant mice, and the study failed to confirm a previous report from the same laboratory of a decrease in substance P in the mutant ileum, indicating that the variability between animals and sensitivity of the techniques can make conclusions difficult.

In the spotting lethal (sl) rat model Ikadai et al. [4, 20] and Horie et al. [21] studied the length of aganglionosis and found that there were two subgroups, one in which there was TCA and a second, less numerous group, in which ganglion cells extended to the proximal half of the colon. The visible cone was often distal to the commencement of aganglionosis. In a histologic study of sl rats using whole-mounts and AchE, tyrosine hydroxylase and substance P, Nagahama et al. [22] showed aganglionosis of the colon along with increased nerve trunks. These changes are similar to those seen in humans with TCA. However, even in the proximal ganglionated duodenum there were changes in the two dimensional structure of the enteric plexus, with the lattice pattern being irregular. This raises the question as to whether the proximal gut is entirely normal, and if there is histologic abnormality in some of the subtle architecture, does this mean diminished function? The bowel also has many functions, so it may be possible for example that water absorption is affected while propulsive activity is normal.

An ultrastructural study in the sl rat model confirmed that almost no nerve terminals were present in the circular muscle layer of any regions of the constricted intestine, but some terminals were observed in the longitudinal muscle layer of that segment. The authors concluded that the denervated circular muscle layer is related to the production of a constricted segment, irrespective of the presence or absence of nerve terminals in the longitudinal muscle layer [23].

Table 4.1 Naturally occurring rodent animal models of HSCR

Name	Length of aganglionosis	Pigment	Inheritance	Locus	Genetic defect	Percent in human HSCR
Lethal spotting mice (ls)	2mm	Patched	Recessive	Chr.2	Point mutation of EDN3	5
Piebald lethal mice (sl)	10mm	White	Recessive	Chr.14	Absent EDNRB	<10
Spotting lethal rat (sl)	TCA	White	Recessive	Chr.6	301 bp deletion in EDNRB	<10
Dominant megacolon (Dom)	Variable	White	Dominant	Chr.15	Point mutation of SOX10	<1

Studies in our laboratory using AchE whole-mounts have demonstrated that the three rodent animal models have a histologic picture identical to that of humans. There was distal aganglionosis with increased nerve trunks, followed by a transition zone which was often asymmetric and variable in length, and more proximally the plexus was near normal in two-dimensional architecture. In spotting lethal rats, particularly those with TCA, the proximal small bowel had an abnormal architecture, but inconsistently so. We could not demonstrate as clearly as Nagahama et al. [22] that the duodenal architecture was abnormal. In our experiments the normal two-dimensional architecture of the enteric plexus was itself variable with some areas looking open and other areas with a tight regular lattice-like structure.

In the lethal spotting mice we were able to identify increased nerve trunks, in contrast to Bolande [15] who could not detect them. The animals we investigated had a longer length of aganglionosis (about 4 mm) and it may be that the genetic background of the animals used by Bolande was such that they had a very short length of aganglionosis, and were more a hypoganglionic model.

The published literature suggests that the length of aganglionosis in each animal model is relatively consistent within each strain [3]. However, in reality there is a considerable variability and while in the majority of cases the length of aganglionosis in piebald lethal is greater than in lethal spotting mice, there is overlap. We have noticed that if heterozygote animals with the least pigment are chosen as mating pairs then the length of aganglionosis tends to be longer. Alternatively, if the mutant animals are back-crossed with C57 or Castaneus stock, the length of aganglionosis reduces. Some F2 offspring from a lethal spotting mouse crossed with Castaneus apparently had anatomically normal distal colon although the color of the coat was that of a mutant animal. The most dramatic alteration in length of aganglionosis occurs when spotting lethal rats are crossed with DA rats: the predominant ileal aganglionosis changes to distal colonic aganglionosis. The change is a dramatic step-like decrease in the length of aganglionosis, suggesting that an extra quantum of enteric neuronal precursors has been created or there are regional differentiations in the bowel such that a region is either filled or remains aganglionic. It is of interest that in humans there also appear to be two common points of cessation of enteric innervation, either the sigmoid colon (in 80% of patients) or about 10 cm proximal to the ileocecal valve (in 10% of patients).

The sl rat has also been instructive in further elucidating the distal aganglionic bowel, namely that the gut is not completely aganglionic. There are groups of ganglion cells (seen best with NPDH-diaphorase staining) associated with the hypertrophied nerve fibers. We postulate that these can only have arisen from the sacral neural crest (there is a gap of many centimeters before ganglion cells are seen in the small bowel). Furthermore, we postulate that the presence of occasional clusters of ganglion cells in the "aganglionic" distal bowel in HSCR patients, if sampled on rectal biopsy, may cause a temporary diagnostic error. This may be the basis of the rarely described patients with "acquired aganglionosis", where the patient clinically has HSCR but the first biopsy suggests the presence of ganglion cells.

Morphologic studies in mice have shown that the density and distribution of the interstitial cells of Cajal (ICC) in the aganglionic region of the colons are similar to those of ICC at the same level of the colon in age-matched wild-type controls [24, 25]. Therefore, it appears that the enteric neurons are not necessary for the development of ICC. Data from humans are inconsistent which could be attributed to the regional differences in the density of ICC in the colon [26–28]. More recently Taniguchi et al. [29] have shown that the aganglionic intestine of ls/ls mice induces secondary disturbances during the normal development of ICC, in the form of fewer cytoplasmic processes and lack of attachment to the intermuscular nerves.

4.4 Physiology

The principle work, which has been on the piebald lethal mouse, is that of Wood et al. [30–32]. In a series of experiments the colon was inspected under video cameras and at the level of aganglionosis there was a functional obstruction. Pellets would move down the bowel and as soon as they reached the aganglionic zone they would stop and at that stage reverse peristalsis would commence [31].

Electrophysiologic studies in piebald lethal mice have shown that there are abnormal discharges of myogenic action potentials in the aganglionic bowel associated with tonic constriction and a reduction in the luminal diameter [30]. Furthermore, the proliferated extrinsic cholinergic nerve fibers appear to be not related to the narrowing of the aganglionic colon [33].

Electrophysiologic experiments in our laboratory have demonstrated that the aganglionic bowel in mouse models has no inhibitory neuromuscular junction potentials (IJP) and only occasional excitatory junction potentials which on repeated stimulation fatigue quickly. The aganglionic circular muscle, lacking inhibition, writhe in an uncontrolled manner and tend to contract [34]. The visual and tension studies confirm the impression that the aganglionic smooth muscle lacks the stabilizing influence of the nonadrenergic noncholinergic (NANC) inhibitory nerves. The smooth muscle appears hyperexcitable and in constant motion. This would confirm the hypothesis proposed by Alvarez [35] that the simplest explanation for the finding in HSCR is that nerves to smooth muscle normally function to keep the muscle from contracting into a knot. This is also the conclusion of Richardson

[36] who performed a pharmacologic study on the lethal spotting mouse.

The simple lack of nerve fibers is sufficient explanation for the functional obstruction seen in the rodent models and in patients with HSCR. There is no need to invoke selective and subtle disorders of various components of the autonomic nervous system to account for the clinical variability.

Thorough studies on the electrophysiology of the mutant rat colon have shown an absence of IJPs except at the sphincter where there is an evoked inhibitory response (in addition to an excitatory response) [37–40]. Another study in piebald lethal mice has shown an increase in basal contractile activity and a reduction in responsiveness to vasoactive intestinal peptide [41]. This supports a generalized reduction in the function of the inhibitory innervation of the aganglionic colon.

In 1990, Bult et al. [42] provided evidence that nitric oxide (NO) is released on stimulation of the inhibitory NANC nerves of canine ileocolonic junction. Since then, substantial evidence has accumulated indicating that NO is the primary nitrergic inhibitory neurotransmitter in the gut of various species [43–46]. More recently de Lorijn et al. [47] have shown that the inhibitory innervation of the murine internal anal sphincter and the rectoanal inhibitory reflex are mediated by NO, and the rectoanal inhibitory reflex requires an intact network of ICC in the internal anal sphincter. Thus both loss of nitrergic innervation and deficiency of ICC lead to impaired anal relaxation and may play an important role in rectal evacuation disorders.

4.5 Embryologic Studies on Rodent Models of Aganglionosis

Webster [17, 18] reported studies on both the ls and s^{l} mice in which he used a nonspecific esterase stain to follow migratory patterns of enteric neuronal precursors in embryos of mutant mice. In both cases there was slowing of migration such that the migrating vagal neural crest cells (VNCC) did not keep pace with the rapidly elongating gut, although the cells still showed signs of distal migration for several days after the usual time of cessation of migration of VNCC in the normal embryos. Webster interpreted these results as suggesting a defect in the vagal neural crest. Using the nonspecific esterase strain in the mutant and control animals there was no evidence of a sacral neural crest input. However, Rothman and Gershon found different results studying the same ls mouse strain [48, 49]. They found that the gut microenvironment in the embryo is unreceptive and cannot support enteric neuronal precursors. The principal experiments in reaching this conclusion were cocultures where isolated segments of aganglionic colon from mutant mice were placed next to sources of neural crest cells (either vagal crest or proximal gut). Aganglionic gut from the mutants was never normally innervated whereas distal gut from the normal embryo was receptive to ingrowth of enteric neuronal precursors. Rather, enteric neuronal precursors tended to avoid aganglionic gut tissue [50]. Other studies looking at the extracellular matrix have shown increases in laminin, collagen type IV and chondroitin sulfate in the distal aganglionic gut [51, 52]. These authors concluded that it is an excess of these extracellular matrix molecules, in particular laminin, which results in a hostile local microenvironment and this causes aganglionosis. They proposed that there is no defect in the émigrés from the neural crest.

Commencing in 1984 our laboratory studied the ls, s^{l} mouse and sl rat animal models, using histology, tissue culture and the kidney capsule techniques. We used three techniques in an attempt to overcome possible artifacts with any one technique, and three animal models to detect any differences between each of the animal strains. The results in all animals, and with each of the three techniques, agreed with those of Webster. In particular, there was slowing of migration in what was the one predominant source of enteric neurons, namely vagal neural crest cells. The slowing in the migration of these cells occurred well before the eventual aganglionic zone was reached and migration spluttered on for several days after it would normally have ceased. In the ls and s^{l} mice the slowing commenced in the terminal small bowel whereas in the sl rats the slowing was apparent even in the proximal small bowel. The appearances were most consistent with the interpretation of a lesser population of enteric precursors in the mutants and insufficient numbers to fully colonize the embryonic gut [53].

We made one new finding which shed some light on the contentious debate as to the existence and extent of a contribution of the second sacral neural crest to the vagal neural crest cells: there were small numbers of enteric precursors in the most distal hindgut. These cells were usually in groups of two to four (maximum ten) in contrast to the vagal émigrés which proliferated into the thousands. There was no obvious difference in the numbers of these sacral neural crest cells in the mutants compared to normal embryos [34]. This result was confirmed using the three different experimental techniques. Therefore, as in most good debates, both sides are correct—there is a sacral neural crest contribution to the ENS, but this contribution is functionally insignificant. Nevertheless, failure of the vagal derived neural crest cells to colonize the hindgut is the prime cause of failure of hindgut enteric nervous development. Furthermore, the interaction between sacral and vagal enteric neural crest cells may be necessary for sacral neural crest cell contribution to the ENS [54, 55].

However, the use of immunohistochemistry, special stains and techniques such as the kidney capsule were limited in achieving a full understanding of how aganglionosis arises in the animal models. Ultimately, all the techniques are indirect, and the debate between the

various theories (see below) could not be settled. Therefore, the new techniques of molecular genetics, that is linkage studies and gene knockouts, were utilized. Initially, the first strategy was linkage studies, as there was no knowledge of which genes were likely to be involved and so knocked out. Theoretically the linkage strategy was simple but during the laborious application of these techniques the answers came from knocking out genes known to have a completely unrelated physiologic function with the unexpected finding that aganglionosis resulted.

4.6 Molecular Genetics

We will focus here on the animal work that lead to a better understanding of the molecular genetics of HSCR and allied disorders (for further details see Chapter 5).

4.6.1 Backcross and Linkage

As the rodent model animals are inbred, if polymorphisms are introduced by outbreeding (backcrossing) then a linkage strategy should allow the chromosomal area responsible for the mutation to be progressively narrowed and finally the gene isolated [56].

In several laboratories (including ours) a lethal spotting backcross strategy was used to try and localize the gene. It was already known that the chromosomal location was on mouse chromosome 2, and backcross studies narrowed the area to between GNAS and endothelin 3 (*edn3*) (Ramu E et al., unpublished work; [57]). However before further work could be done to walk into the mutation area, the answer came from knockout experiments (see below). A similar backcross strategy was used in the s^{l} mouse. Work was more successful in these experiments, in that the regions of interest were localized and cloned, but it remained difficult to identify the gene involved [58]. Similarly in the sl rat, a project was initiated in our laboratory to localize the gene responsible for aganglionosis in the rat, using the mutant animals crossed with the DA rat. The DA rat was chosen because of its heavy pigmentation which allowed the wild types and heterozygotes to be clearly distinguished from mutants in the F2 offspring litter. However, while this work was progressing the answer came from knockouts of genes found initially in humans and adult animals whose full function was being explored by the use of experiments in which the gene was removed and then what happened in the offspring was observed.

4.6.2 Knockout Models

With advances in knockout and transgenic technology, many molecules and several signaling pathways have been identified as important in the control of mammalian ENS development (Table 4.2).

4.6.2.1 *Ret/Gdnf/Gfrα1* and *Ret/Ntn/Gfrα2* Knockout Mouse

The *Ret/Gdnf/Gfrα1* signaling pathway is of importance in ENS development, having been shown to promote survival of neurons, mitosis of neuronal progenitor cells, differentiation of neurons and neurite extension [59, 60].

The first targeted gene deletion knockout model was of the *ret* gene which unexpectedly produced a phenotype similar to aganglionosis. The *ret* gene had been initially isolated in a tumor cell line [61]. Subsequent examination of these animals showed a total absence of ganglion cells throughout the gut and associated renal anomalies [62]. *Ret* is normally expressed in the embryonic gut [63]. *Ret*–/– mice exhibit a failure of neural crest colonization of the gut distal to the gastric cardia. The esophagus and gastric cardia also exhibit a reduced population of neurons and glia [64, 65].

This animal work was critically important in identifying *RET* as a firm human candidate gene in the area previously identified as deleted on chromosome 10 [66–69]. Without this vital research, *RET* would have remained only one of a dozen or so candidate genes in this deleted area of the human chromosome. It was a combination of both the human and animal works that allowed the early identification of *RET* as the first gene responsible for HSCR [70, 71].

Likewise, *gdnf*–/– and *gfrα1*–/– mice have almost identical phenotypes to *ret*–/– mice [72, 73]. To date, a few *GDNF* mutations and no *GFRα1* mutations have been identified in humans with HSCR [74–76].

Gfrα2 and *ntn* are, like *gfrα1* and *gdnf*, capable of forming a *ret*-activation complex and are thought to be required for the maintenance of a subset of enteric ganglia [77]. *Ntn*–/– and *gfrα2*–/– mice exhibit a decrease in the density of cholinergic neurons in the ENS but no renal abnormalities, and the mice survive and breed [77, 78]. It appears that *NTN* mutation alone does not result in HSCR, but could contribute to the severity of HSCR due to other mutations [79]. To our knowledge, the *GFRα2* mutation has not been identified yet in humans with HSCR.

4.6.2.2 *Ednrb/Edn3/Ece1* Knockout Mouse

Endothelins had been discovered while searching for contractile substances in the pig aorta [80]. Scientists interested in the biologic function of endothelin genes also produced a series of targeted gene deletion knockouts in an attempt to see how animals without the gene would function. The first of these was an endothelin-1 (*edn1*) deletion, due to disruption of the endothelin-convert-

Table 4.2 Knockout mouse models of Hirschsprung's disease and allied disorders [54, 59, 119]

Mice	Gene	Function	Phenotypea	Human locus	Percent in HSCR
Ret−/−	RET	Tyrosine kinase receptor	Total intestinal aganglionosis	10q11.2	70–80% long segment, 50% familial, 15–20% sporadic
Gdnf−/−	GDNF	Glial cell-derived neurotrophic factor	Total intestinal aganglionosis	5p12–13.1	<10%
G Gfrα1−/−	GFRα1	GDNF family receptor alpha 1	Total intestinal aganglionosis	10q26	–
Ntn−/−	NTN	Neurturin, RET ligand	Hypoganglionosis	19q13.3	<1%
Gfrα2−/−	GFRα2	GDNF family receptor alpha 2	Hypoganglionosis	–	–
Etr3−/−, ls	EDN3	Endothelin-3	Distal hindgut aganglionosis	20q13	<10%
EdnrB−/−,Sl	EDNRB	Endothelin-B-receptor	Distal hindgut aganglionosis	13q22	<10%
Ece 1−/−	ECE-1	Endothelin-converting enzyme	Distal hindgut aganglionosis	1p36.1	<1%
Sox 10DOM, DOM	SOX 10	Sry/HMG box transcription factor	Complete gut aganglionosis	22q13.1	<1%
Phox2b−/−	Phox2b	Paired-like homeobox 2b	Complete gut aganglionosis	4p12	<1%
Pax3−/−	PAX3	Paired box gene 3	Total intestinal aganglionosis	2q37	–
Ncx−/−	HOX11L1	Homeobox 2	INDB-like condition	2p13.1	–
Ihh−/−	IHH	Indian hedgehog	Skip intestinal aganglionosis	2q33–q35	–
Shh−/−	SHH	Sonic hedgehog	Ectopic mucosal neurons	7q36	–

[a]Of homozygous mice

ing enzyme-1 gene (*ece1*), which produced craniofacial and cardiac defects in addition to colonic aganglionosis, suggesting that the endothelins were important in neural crest development [81, 82]. In follow-on experiments, when the endothelin-3 gene (*edn3*) was made functionally null, a phenotype resulted which looked identical to the lethal spotting mouse. Similarly when the endothelin B receptor (*ednrb*) was made nonfunctional, the offspring resembled piebald lethal animals. Examination of the gut confirmed aganglionosis. Researchers then examined the lethal spotting mice and piebald lethal mice for defects in *edn3* and *ednrb*, respectively. They confirmed that *edn3* and *ednrb* have a role in the migration and development of the ENS, and defects of the endothelin signaling pathway are responsible for the phenotypes of these animal models. Namely, in the lethal spotting mouse there was a point mutation in the proendothelial-3 gene which prevented cleavage and resulted in no *edn3*; and in the piebald lethal mouse there was a complete deletion of *ednrb* (Table 4.1) [83–86].

The shorter length of aganglionosis in the lethal spotting mouse is thought to be due to the other endothelins (*edn1* and *edn2*) partially reacting with the *ednrb* and producing a milder form of aganglionosis. This would explain why in the piebald lethal animals the length of aganglionosis is on average about 10 mm, whereas in the lethal spotting mouse the length of aganglionosis is about 2 mm.

In our laboratory we have crossed both lethal spotting and piebald lethal animals and the resultant length of

aganglionosis appears to be similar or only slightly longer than that of the piebald lethal mouse. Therefore there appears to be no additive factor between the two genes and the defect produced by the piebald lethal lesion which is due to the absence of *ednrb* is the limiting factor in the length of aganglionosis. However, when the animals are backcrossed with either C57 or Castaneus animals, the innervation of the most distal colon appears to be near normal. Therefore, there are modifier genes affecting the length of aganglionosis in these mouse models.

Knowledge that *ret* and *ednrb* defects are involved in mice suggested that defects in these genes were candidates for the spotting lethal rat. First a defect in *ret* was excluded, along with *edn3*. Working initially on a cDNA from the rat *ednrb*, we noted a 250–300 bp deletion. Further experiments using both the cDNA and genomic DNA localized the defective area to the end of the first translating exon and the next 17 bp sequence of the first intron of the *ednrb*. This 301 bp deletion results in alternative splicing which results in either a stop codon or an in-frame 270 bp deletion and a protein product with an inability to insert into the cell membrane [87, 88].

A recent study has shown that abnormalities of the ENS in heterozygous *ednrb*-deficient spotting lethal rats resemble those in intestinal neuronal dysplasia B (INDB) [89].

These mouse animal experiments again led the way to the discovery of defects in the same genes in humans. Subsequently, defects in *EDNRB* [90–94] and *EDN3* [95, 96] have been found in humans.

4.6.2.3 *Sox10* Knockout Mouse

Sox10 was identified while doing a comparative study of human/mouse sequences [97]. It is a member of the sry-related family of transcription factors [98]. The naturally occurring Dom mouse model of HSCR was used to identify the role of *sox10* in ENS development [99]. It appears that early death of neural crest cells is responsible for the complete aganglionosis of the gut in *sox10Dom*/*sox10Dom* mice [100]. *Sox10Dom*/+ mice exhibit distal hindgut aganglionosis and pigmentation abnormalities [101]. A similar gene mutation has been identified in patients with Waardenburg-Shah syndrome [102].

4.6.2.4 *Phox2B* Knockout Mouse

Phox2b is a homeodomain-containing transcription factor that regulates *ret* expression and thus it is essential for ENS development [103, 104]. *Phox2b*–/– mice exhibit neural crest colonization of foregut only. Subsequently, the foregut neural crest cells undergo apoptosis. At birth, animals exhibit complete aganglionosis of the gut [104]. *PHOX2B* is proposed to be a candidate gene in patients with Haddad syndrome [104, 105].

4.6.2.5 *Pax3* Knockout Mouse

Pax3 is a member of the paired-box-containing family of transcription factors [106]. It appears that *pax3* is required for the formation of enteric ganglia and functions with *sox10* to modulate *ret* expression, and thus there are no enteric neurons caudal to the stomach in *pax3*–/– mice [107]. Patients with Waardenburg syndrome without HSCR usually have mutations in *PAX3* [108].

4.6.2.6 *Hox11L1* Knockout Mouse

Hox11L1 is a homeobox transcription factor which may play a role in neural crest cell proliferation or differentiation [109]. *Hox11L1*–/– mice develop an INDB-like condition, followed by death of some of the enteric neurons [109, 110]. The *Hox11L1*–/– mouse has been proposed as a model for INDB. Further knowledge of the regulatory genes and the transcriptional targets of *Hox11L1* may produce candidate genes for involvement in INDB, and thus a better understanding of this controversial disease entity [59, 111].

4.6.2.7 *Ihh*–/– and *Shh*–/– Knockout Mouse

Indian hedgehog (*Ihh*) and Sonic hedgehog (*Shh*) genes may influence survival and/or differentiation of neural crest cells [112]. *Ihh*–/– and *Shh*–/– mice die during early embryonic stages. Late fetal *Ihh*+/– mice exhibit a dilated region of the colon, with missing enteric neurons in some parts of the small intestine and the dilated region of the colon [113]. *Shh*+/– mice do not lack an ENS in any part of the gut, but nerve cell bodies are present within the mucosa [113]. Both *IHH* and *SHH* are possible candidate genes for ENS defects in humans [59].

4.7 Contribution of Animal Models to Theories as to the Cause of Aganglionosis

There are two broad theories as to the cause of aganglionosis (with many internal minor refinements of the theories being possible), and workers investigating the animal models have found evidence for both theories.

4.7.1 Defect of Central Vagal Neural Crest Cell Production and Migration

Webster's and our interpretation of the slowing of migration found in mutant embryos is that this suggests an early defect in the vagal neural crest in the production of sufficient neural crest cells to adequately populate the gut.

This hypothesis would fit with the ablation experiments of Yutema and Hammond (see Chapter 2).

As a refinement to this theory we hypothesize that the uneven distribution we see in humans and mutant animal experiments suggests evidence of clones or discrete quanta of precursors or mother cells, perhaps as low as four to six quanta from which arise all progeny that normally populate the gut. If one quantum is missing, distal colonic aganglionosis results; if two quanta are missing, ileal aganglionosis results; if three are missing, mid-small-gut aganglionosis results; if four quanta are missing, total intestinal aganglionosis results [34]. Certainly in the mutant rats we do not see an even or random distribution of the site of the commencement of aganglionosis; rather there are three most frequent "nodal regions" where aganglionosis commences. Our hypothesis is that the quanta are generated at a vagal neural crest level over several somites and the defect in the mutants occurs initially at a premigratory stage, with the eventual extent of bowel aganglionosis being merely a later playing out of this early vagal neural crest defect. It is as if there is a "checkerboard" of potential spaces to be filled and a number of precursor families to fill the spaces. Because the spaces are always filled from a proximal direction, no matter which family is missing, the result will be distal aganglionosis. The only variable is that if there are more families missing then the length of aganglionosis will be longer (Cass, First World Workshop in Hirschsprung's Disease, Sestri Levante, 1993, unpublished data).

4.7.2 Defect in the Local Gut Microenvironment

Gershon and coworkers extensive experimental work supports defects in the mutants being in the gut microenvironment itself and being specifically related to excess laminin causing migrating enteric neuronal precursors to mature early and hence not to continue to divide or migrate [114]. Nishijima et al. [115] found migration down the mouse embryonic gut was not an even process but rather proceeded in bursts followed by a pause. In the lethal spotting mouse mutants, migration proceeded normally but then suddenly stopped at the last of these boundaries, resulting in aganglionosis. The authors interpreted this result as indicating that the gut had subtly different microenvironments, and an intrinsic defect in the last 2 mm of the mouse colon resulted in aganglionosis. Similarly Kapur et al. [116], using transgenic mice with a cell label DβH-inlacZ and chimeric animals, showed that the enteric neurons from ls/ls could populate the distal gut. The explanation was that the enteric neuronal precursor from the normal embryo contributed a factor that overcomes the microenvironmental defect [116–118]. In our experiments, we could not demonstrate defects in the extracellular matrix in early embryos [34, 53]. Rather the increase in the extracellular matrix components appeared to be a secondary event [34, 53].

4.8 Summary

In summary, the animal models of aganglionosis have been pivotal in the discovery of the genes of HSCR. In future, animal models will continue to contribute to the understanding of how the genes interact and are modified by yet other genes. In addition, animal models of aganglionosis will continue to contribute to the anatomic, physiologic and pharmacologic understanding of aganglionosis.

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The Molecular Genetics of Hirschsprung's Disease

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5

5.1 Epidemiology and Genetics of HSCR

Hirschsprung's disease (HSCR), or aganglionic megacolon, is a classic example of a complex genetic disease, characterized by the lack of enteric ganglia in the submucosal and myenteric plexuses, along variable portions of the distal gut. Since it is caused by a premature arrest of the migration of neural crest cells along the hindgut, it is defined also as a neurocristopathy. The variable extent of aganglionosis correlates with severity of the disease, leading to a classification of HSCR into short- and long-segment phenotypes [1, 2]. S-forms include aganglionosis confined below the rectosigmoid junction (80% of patients), while L-forms (20% of patients) can extend below the splenic flexure (colonic forms, 9%), to the whole colon (total colonic aganglionosis, TCA, 5–10%), or up to the whole bowel (total intestinal). The disease is a congenital malformation occurring in 1 in 5,000 live births, with the highest incidence in Asian populations (2.8 in 10,000), intermediate in Afro-Americans (2.1 in 10,000) and Caucasians (1.5 in 10,000) and lowest in Hispanics (1 in 10,000). The male to female ratio is 4:1, and the sex imbalance is particularly evident for S-forms (ranging from 4.2 to 5.5 in S-form and from 1.2 to 1.9 in L-form aganglionosis) [1–3] (Table 5.1). A proportion of cases are familial (20%), but HSCR most commonly presents with a sporadic occurrence. Approximately 30% of patients show an association with other disorders such as chromosomal abnormalities (12%) or different neurocristopathies, and with a variety of additional isolated or syndromic anomalies [4]. In this respect, patients with Down's syndrome are at higher risk of HSCR (5%, vs 1/5,000 in the general population), suggesting that dosage-sensitive susceptibility gene(s) are located on chromosome 21. Interestingly, the sex ratio is balanced among patients with Down's syndrome [4].

While possible environmental effects on disease pathogenesis have not yet been demonstrated, the genetic component has been recognized since the 1960s on the basis of both increased recurrence risk for sibs of affected individuals as compared to the general population, the association with other genetic diseases and the existence of several animal models of colonic aganglionosis showing Mendelian inheritance.

Notwithstanding the clear hereditability, segregation analyses suggest a complex mode of inheritance. In particular, an autosomal dominant and an autosomal recessive or multifactorial models of segregation have been suggested for L- and S-forms, respectively (Table 5.1). The recurrence risk for siblings varies from 1% to 33% depending on gender and length of aganglionosis of the probands and the gender of the sibs. In particular, it is estimated to be 3% and 17% for S- and L-forms, respectively, and to be higher for female rather than male probands and for males rather than female sibs, attesting to the fact that HSCR is a sex-modified multifactorial disorder [2]. Finally, it is higher in multiplex families (Table 5.2).

Table 5.1 Genetic and epidemiological features of different HSCR phenotypes

	Short-segment	Colonic-segment	Long-segment
Affected (%)	79	11	10
Sex ratio (M:F)	4.9:1	1.6:1	1.9:1
Genetic model	Multifactorial or recessive	Dominant or additive	Dominant or additive
Heritability (%)[a]	87	100	100
Penetrance (%)[a]			
Males	17	37	66
Females	4	29	51
Sporadics (%)[a]			
Males	4	39	41
Females	0	21	13
New mutations (%)[a]	0	0	15

[a]Based on segregation models (dominant or additive for long- and colonic-segment and recessive or multifactorial for short-segment), according to Badner et al. [2]

Table 5.2 Risk of recurrence based on segregation models (dominant or additive for long- and colonic-segment and recessive or multifactorial for short-segment, according to Badner et al. [2])

Risk Recurrence	Short-segment		Colonic-segment		Long-segment	
	Male	Female	Male	Female	Male	Female
Risk to sib (%)						
male proband	5	1	10	7	11	8
female proband	6	2	13	10	23	18
Offspring (%)						
of affected male	0	0	11	9	18	13
of affected female	0	0	15	11	28	22
Risk to second-degree relatives (%)	0	0	5	4	4–9	3–7
Risk in multiplex families (%)	5–10	1–4	19	14	33	25

In summary, the most relevant factors providing evidence that underlines the complex genetics of the disease are:

1. High proportion of sporadic cases
2. Variable expressivity, depending on the length of gut involved
3. Incomplete and sex-dependent penetrance
4. Risk to siblings varying by gender, segment length and co-occurrence of nonenteric phenotypes

5.2 The *RET* Protooncogene

5.2.1 Identification of *RET* as the Major Disease Locus in Isolated HSCR

The protooncogene *RET* is the major gene responsible for HSCR, with *RET* mutations implied also in different pathologies: multiple endocrine neoplasia of type 2A (MEN2A) and 2B (MEN2B) and medullary thyroid carcinoma, both sporadic (MTC) and familial (FMTC). The starting point for the identification of *RET* mutations in HSCR was the observation in 1992 of a patient with total colonic aganglionosis carrying a de novo interstitial deletion of chromosome 10 (46, XX, del10q11.21) [5]. The presence of a gene responsible for HSCR located on chromosome 10 was confirmed by two independent linkage studies [6, 7]. Moreover, the co-occurrence of HSCR with MEN2 syndromes, which had already been mapped to 10q and found in association with *RET* gain-of-function mutations, proved *RET* as a good candidate for HSCR as well [8–10]. Finally, the description of two other interstitial deletions allowed the smallest region of overlap (sro) among the deleted chromosomes to be narrowed to an interval of less than 250 kb, where *RET* was the only

known and already cloned gene [11, 12].

The exon–intron organization of *RET* was therefore reconstructed, starting from the published cDNA sequence [13, 14] and by using a PCR-based approach [15]. This allowed DNA fragments flanking both sides of each exon to be sequenced, thus making possible the mutation screening of the whole coding region of the *RET* gene.

5.2.2 *RET* Gene Mutations

A variety of mutations of the *RET* protooncogene have been detected in HSCR patients including microdeletions, insertions, variants affecting the correct RNA splicing, nonsense mutations, and, above all, missense mutations (Fig. 5.1). To date, more than 100 different missense mutations have been described, with a recurrent mutation described in the Chinese population (R114H) [16]. De novo mutations can be found in approximately 16–65% of patients with a *RET* mutation, are associated with L-forms [17–22], and have been demonstrated in a limited number of patients to arise equally on both paternal and maternal chromosomes [21]. Mutations found in HSCR patients are scattered throughout the gene while in MEN2 syndromes and in MTC mutations occur in specific codons, among which the cysteine residues of the cys-rich extracellular domain are the most frequently affected [23–26]. Moreover, *RET* mutations in HSCR generally result in the loss-of-function of the protein due to misfolding, failure in transportation to the cell surface or suppression of its biological activity, and the identification of deletions also supports the haploinsufficiency effect in disease pathogenesis [26–29]. As well as the localization, this loss-of-function mechanism contrasts with the MEN2 pathogenesis in which gain-of-function, due to constitutive dimerization of the RET receptor or dysregulated activation of the tyrosine kinase activity, has been demonstrated [30–32]. Surprisingly, some MEN2A-typical *RET* mutations seem to result in both gain- and loss-of-function, since they have been detected in families presenting a certain degree of co-occurrence of MEN2A and HSCR [18–35].

Despite the central role played by *RET* in HSCR and the extensive mutation screenings performed by many groups in the last 10 years, the mutation rate remains quite low, and only about 50% of familial and 7–35% of sporadic cases (15–20% in most of the series) present with *RET* mutations [17–19, 22, 36]. Moreover, *RET* mutation frequency has been shown to be higher in TCA and the L-form than in the S-form, overall suggesting the effects of multiple genes which would work particularly in the least severely affected, and providing an explanation for the still very poor genotype–phenotype correlation in HSCR. It was initially supposed that such a limited mutation detection rate might have derived from pitfalls in the screening procedure. More recently it has become clear that this is not the case and in the majority of HSCR cases still await a clarification of the underlying pathogenetic mechanism. Several hypotheses have been advanced to explain these cases, such as:

1. The possible effect of neutral gene variants acting as low penetrant alleles
2. The presence of still undetected mutations in RET non-coding regions involved in either regulatory functions or transcript processing and maturation
3. The existence of another gene in the vicinity of the *RET* locus

5.3 Other Genes Involved in HSCR Pathogenesis

To date, molecular and genetic analyses have allowed eight other different HSCR susceptibility genes to be identified (*GDNF, NRTN, ECE1, EDN3, EDNRB, SOX10, ZFHX1B, PHOX2B*), and found to be related to both the *RET*-mediated signaling pathway and other cellular programs crucial for the normal development of the enteric nervous system (ENS). A role for the *KIAA1279* gene in HSCR etiology has also recently been hypothesized.

5.3.1 The *RET* Signaling Pathway

In 1996, *GDNF*, which is known to be a major survival factor for many types of neurons, was shown to be the *RET* ligand by both phenotypic similarities between *Ret*–/– and *Gdnf*–/– knockout mice [37–39], and Xenopus embryo bioassays [40]. *GDNF* is a TGF-β-related protein of 211 residues, proteolytically cleaved to a 134-residue mature protein that homodimerizes. To activate *RET*, *GDNF* needs the presence of the coreceptor *GFRA1* linked to glycosylphosphatidylinositol (GPI) [41, 42]. Four related GPI-linked coreceptors, *GFRA1–4* [43], and four related soluble growth factor ligands of *RET* have been identified, namely: *GDNF*, *NTN* [44], persephin (*PSPN*) [45] and artemin [46]. Specific combinations of these proteins are necessary for development and maintenance of both central and peripheral neurons, and all can signal through *RET*. Based on its crucial role in *RET* activation and the presence of aganglionosis in *Gdnf*–/– mice, extensive mutation screening has been performed, but only seven mutations have been described so far [47–53]. Absence of genotype–phenotype correlation and cosegregation of *GDNF* variants with *RET* mutations and with trisomy 21 have suggested a weak role for *GDNF* in HSCR pathogenesis [49]. Moreover, none of the five *GDNF* mutations tested in vitro can interfere with *RET* activation and consequently none is causative of HSCR per se [54, 55]. Among other *RET* ligands, only *NTN* has been found mutated in a patient with familial HSCR, where a *RET* mutation also cosegregates [56].

Finally, although *Gfra1* homozygous knockout mice are phenotypically very similar to *Ret*–/– and *Gdnf*–/– mice, no *GFRA1* mutations have been identified in HSCR

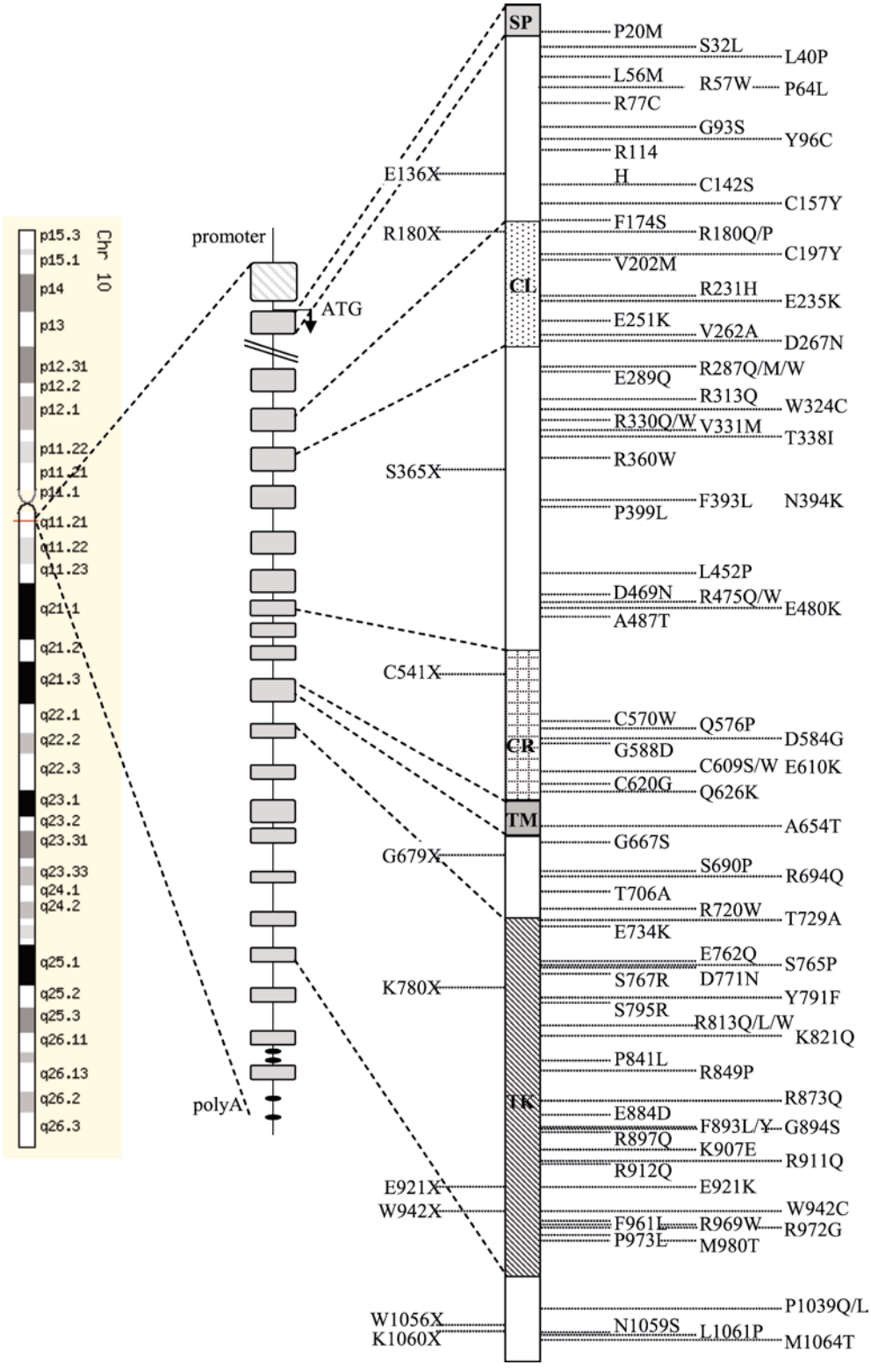

Fig. 5.1 Representation of chromosomal location, gene structure, protein domains and HSCR associated single nucleotide mutations of the *RET* protooncogene (*SP* signal peptide, *CL* cadherin-like, *CR* cysteine rich, *TM* transmembrane, *TK* tyrosine kinase)

patients in spite of extensive screenings performed to this end [57–60].

5.3.1.1 RET and GDNF Proteins in Normal and HSCR Gut

The early studies on Ret protein expression in mammalian tissues showed that this receptor tyrosine kinase (RTK) might be a receptor normally functioning in particular differentiation stages or restricted tissue lineages [61]. As expected from knockout mice, Ret mRNA is highly expressed in both the developing peripheral nervous system and the excretory system during mouse embryogenesis [62]. Successive studies, using immunohistochemical techniques on embryonic, infant and adult normal tissues from rats, showed Ret protein expression not on ly in the nervous system but also in acinal cells of the salivary glands, epithelial cells of the thymus, and follicular dendritic cells of the spleen and lymph nodes [63].

The first localization of the RET protein in the ENS was achieved in 1995 [64] through immunohistochemical studies using three different anti-RET protein antibodies, namely anti-RET R5, anti-RET C and anti-RET K [65]. In normal ganglia of control subjects, diffuse granular red-staining cells and some strongly positive ganglion cells were found, while in HSCR patients with complete deletion or stop codon mutations of the *RET* gene [64, 66] a markedly reduced staining was clearly observed (Figs. 5.2 and 5.3). This finding may support the hypothesis of loss of function due to reduced amounts of RET protein.

A possible deficit in the expression of GDNF protein in the ENS of HSCR patients with no *GDNF* mutations was searched for: the immunohistochemistry assay was performed in 30 HSCR patients and 10 control subjects with GDNF D-20, an affinity-purified rabbit polyclonal antibody raised against a peptide corresponding to amino acids 186–205 mapping within the carboxyl terminal domain of human *GDNF* [51]. GDNF immunoreactivity was localized in the ganglia of the myenteric and submucous plexuses. In normal colon and in the ganglionic segment of HSCR, a strong granular red staining was obtained in the satellite elements and on the cellular membranes of the ganglion cells. In ganglionic intestine, GDNF-positive nerve fibers were not observed. The small ganglia of the hypoganglionic segment showed a reduced GDNF immunoreactivity when compared with the proximal normoganglionic segment. The muscular interstitium showed trunks of nerve fibers and persistence of some small cellular elements of glial origin that showed GFAP and S-100 protein immunoreactivity. GDNF immunoreactivity was absent in the aganglionic segment of HSCR. A deficit in the expression of *GDNF* in the distal aganglionic segment could be a cofactor in HSCR pathogenesis. The absence of GDNF in the distal hindgut could result in a missed or reduced autophosphorylation (activation) of the RET receptor in the absence of *RET* protooncogene mutations, causing enteric neuroblast migration arrest and HSCR.

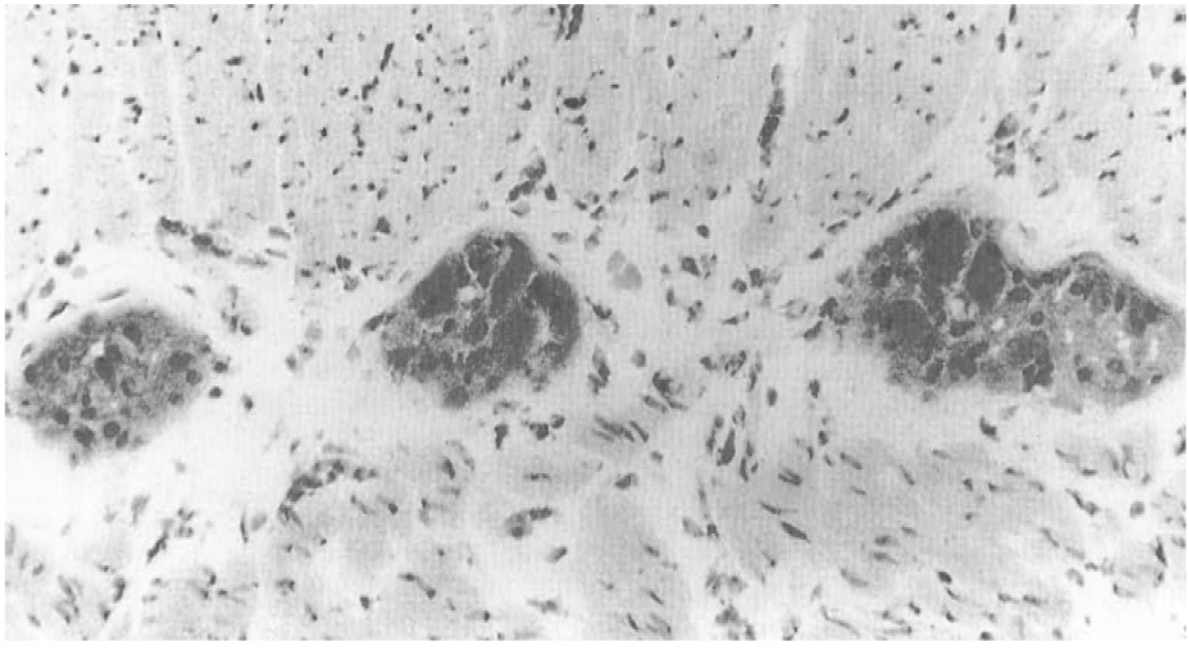

Fig. 5.2 Normoganglionic myenteric plexus. The ganglia of Auerbach plexus show a diffuse granular staining with anti-Ret K polyclonal antibody. Some strongly stained ganglion cells are present

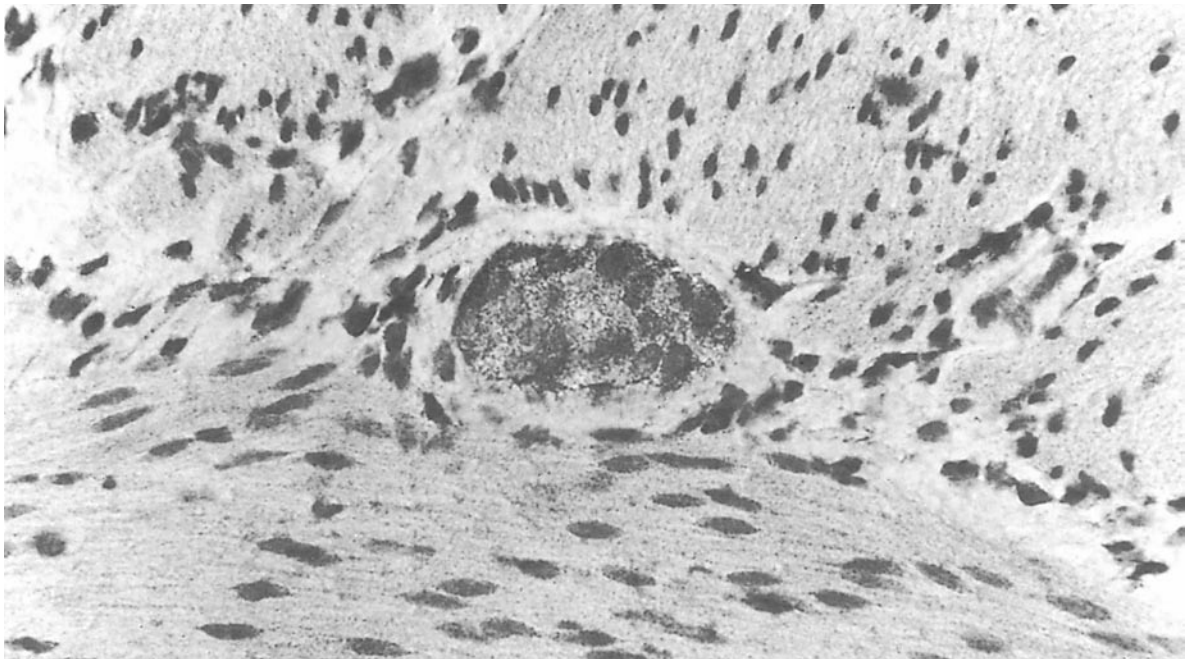

Fig. 5.3 Ultralong Hirschsprung's disease with complete deletion of the RET protooncogene. In the hypoganglionic segment, a ganglion shows a reduced content of Ret protein

5.3.2 The Endothelin Signaling Pathway

The critical role of the endothelin pathway in HSCR was demonstrated with the finding that *piebald-lethal* (s^l), a murine model of aganglionosis, is allelic to the endothelin receptor b (*Ednrb)* knockout mouse and harbors an *Ednrb* mutation (Table 5.3) [67]. Subsequently, an *EDNRB* missense mutation (W276C) was identified in a large inbred Old Order Mennonite community with multiple patients with HSCR [68–70].

However, the W276C mutation was neither necessary (since the presence of affected wild-type homozygotes in the pedigree) nor sufficient (nonaffected mutant homozygotes were found) to cause HSCR, and penetrance showed sex-dependence (more in males than in females) [68]. *Piebald-lethal* can be considered a mouse model for Shah-Waardenburg type 4 (WS4) in humans and, in agreement with this notion, some of the affected Mennonite individuals have pigmentary anomalies and sensorineural deafness in addition to HSCR [69]. This prompted a study of the *EDNRB* gene in WS4, and homozygous mutations in some families were found [71]. At the same time, an *Edn3* mutation was identified in the *lethal spotting* (ls) mouse, another natural murine model of human WS4 [72], and *EDN3* homozygous mutations were accordingly identified in patients (Table 5.3) [73, 74].

Both *EDNRB* and *EDN3* were screened in large a series of isolated HSCR patients. While *EDN3* mutations were seldom found [75], *EDNRB* mutations could be identified in approximately 5% of the patients [20, 76–78]. It is worth mentioning that these studies were able to demonstrate that penetrance of *EDN3* and *EDNRB* heterozygous mutations is incomplete in HSCR patients as de novo mutations are not observed and that S-HSCR is largely predominant. The observation of interstitial 13q22 deletions, encompassing the *EDNRB* gene, in HSCR patients makes haploinsufficiency the most likely mechanism for *EDNRB*-mediated HSCR development (Table 5.3).

Although *EDNRB* binds all three known endothelins (*EDN1*, *2*, *3*), the similarity of the phenotypes associated with both *Ednrb* knockout mice and *Edn3* knockout mice [67, 72] suggests that *EDN3* is the major ligand of *EDNRB*. Pre-proendothelins are proteolytically cleaved by two related membrane-bound metalloproteases giving rise to the mature 21-residue endothelins. Ece1 processes Edn1 and Edn3 and *Ece1* knockout mice show colonic aganglionosis in addition to craniofacial defects and cardiac abnormalities [79]. Accordingly, a heterozygous *ECE1* mutation has been identified in a patient with HSCR and associated craniofacial and cardiac defects (R742C) [80].

5.3.3 *SOX10*

Dominant megacolon (*Dom*) is a mouse model of human WS4, the homozygous *Dom* mutation being embryonically lethal [81]. The *Dom* gene is *Sox10*, a member of the

Table 5.3 Genes involved in Hirschsprung's disease (*CCHS* congenital central hypoventilation syndrome; *GOSHS* Goldberg-Shprintzen syndrome; *MEN2* multiple endocrine neoplasia type 2; *PCWH* peripheral demyelinating neuropathy, central dysmyelinating leukodystrophy, Waardenburg syndrome and Hirschsprung's disease; *WS4* Waardenburg syndrome type 4)

Gene	Map locus	Related syndromes	HSCR reported mutations	Animal model
RET	10q11	MEN2	Heterozygotes	Knockout
GDNF	5p13	–	Heterozygotes	Knockout, s[l]
EDNRB	13q22	WS4	Hetero/homozygotes	Knockout, s
EDN3	20q13	WS4	Hetero/homozygotes	Knockout
SOX10	22q13	WS4/PCWH	Heterozygotes	Dom
NTN	19p13	–	Heterozygotes	Knockout
ECE1	1p36	–	Heterozygotes	Knockout
SIP1	2q21–23	Mowat-Wilson	Heterozygotes	Knockout
PHOX2B	4p12	CCHS	Heterozygous deletion[a]	Knockout
KIAA1279	10q21.3–22.1	GOSHS	Homozygotes	–

[a] One patient reported [100]

SRY (sex-determining factor)-like, high-mobility group (HMG) DNA-binding proteins [82]. Both inherited and de novo heterozygous *SOX10* mutations have been identified in familial and isolated HSCR patients with WS4 [83–85] and, more recently, in a severe phenotype designated PCWH (*P*eripheral demyelinating neuropathy, *C*entral dysmyelinating leukodystrophy, *W*aardenburg syndrome and *H*irschsprung's disease) [86]. These two phenotypes, PCWH and WS4, are caused by two distinct molecular mechanisms. While all mutations have enhanced DNA-binding affinity, and potent dominant-negative activity, only the WS4 mutation activates the nonsense-mediated decay (NMD) while PCWH escapes it as the stop codon lies in the last exon [86].

5.3.4 *ZFHX1B*

The SMAD-interacting protein1 gene (SIP1), better named as *ZFHX1B*, located in 2q22, encodes a transcriptional corepressor of Smad target genes. In 2001, the gene was found to be mutated in Mowat-Wilson syndrome (MWS) by the cloning of two de novo translocation break points [87, 88]. MWS is a multiple congenital anomaly syndrome characterized by dysmorphic features, severe intellectual disability and microcephaly, and is commonly associated with congenital anomalies, including HSCR, heart defects, hypospadias, genitourinary anomalies, postnatal microcephaly, agenesis of the corpus callosum, severe mental retardation, short stature and facial dysmorphic features. The facial gestalt is so distinctive that the diagnosis can be suspected in patients with no congenital malformation [89, 90]. HSCR, which at first was considered a mandatory feature to suspect the diagnosis of MWS, is now described in about 60% of the patients only (see reference [91] for a review). The MWS phenotype is the result of de novo heterozygous deletions or truncating mutations of the *ZFHX1B* gene, suggesting that haploinsufficiency for *ZFHX1B* is sufficient to cause the disease phenotype. The study of the expression pattern of the gene in early embryonic and fetal stages in humans argues for a pleiotropic role of the gene [92]. *Zfhx1b* knockout mice do not develop postotic vagal neural crest cells, the precursors of the ENS that are affected in patients with HSCR, and they display a delamination arrest of cranial neural crest cells, which form the skeletomuscular elements of the vertebrate head [93]. This suggests that *Zfhx1b* is essential for the development of vagal neural crest precursors and the migratory behavior of the cranial neural crest in the mouse.

5.3.5 *PHOX2B*

The paired-like homeobox gene *PHOX2B*, located in 4p12, encodes a transcription factor (homeodomain protein) that has been regarded as a candidate gene in the

association of HSCR and congenital central hypoventilation syndrome (CCHS; Haddad syndrome). Indeed, the neuronal circuits of the autonomic nervous system that control vegetative functions have been shown to depend on the expression of the *Phox2b* homeodomain transcription factor as the neurons either fail to form or degenerate in mouse mutants null for *Phox2b* (paired-like homeobox 2B) [94, 95]. Heterozygous *PHOX2B* mutations, clustered in the C terminus of the gene, have recently been detected in 50–98% of patients affected by CCHS [96, 97], a very rare neonatal disorder characterized by an abnormal ventilatory response to hypoxia and hypercapnia owing to failure of autonomic respiratory control [98]. Subsequently, *PHOX2B* was reported to be the first susceptibility gene in TSNS (tumors of the sympathetic nervous system). Therefore, HSCR, CCHS and TSNS can be found in various combinations and can be ascribed to various *PHOX2B* gene mutations with some genotype/phenotype correlation. Although a deletion encompassing the *PHOX2B* gene has been described in a patient with HSCR, mental retardation and failure to thrive, there is only weak evidence to support a role of *PHOX2B* in the development of isolated HSCR [99, 100].

5.3.6 *KIAA1279*

The *KIAA1279* gene has been found to carry homozygous nonsense mutations in two different families diagnosed with Goldberg-Shprintzen syndrome (GOSHS) [101], a disorder characterized by microcephaly, mental retardation, facial dysmorphisms, and HSCR [102], clinically but not genetically similar to MWS.

The gene, mapped in 10q21.3-q22.1, encodes a protein with a still-unknown function, predicted to contain two tetratrico peptide repeats (TPRs) and likely involved in a variety of biological processes. In the two families, HSCR is present as a variable feature, while bilateral generalized polymicrogyria (PMG, a neuronal migration disorder resulting in malformation of the cerebral cortex) is constantly associated with the affected status. This has led to the hypothesis that the protein plays a central role in the development of both the peripheral and central nervous systems, possibly through binding HSCR- or PMG-associated susceptibility factors [101].

5.3.7 Interactions Between Pathways

Formation of the mammalian ENS requires a coordinated and balanced interaction of signaling molecules and transcription factors which play a critical role in the formation of normal enteric ganglia (Fig. 5.4). Failure of this integration leads to the absence of enteric ganglia and therefore to the HSCR phenotype. Two signaling systems mediated by *RET* and *EDNRB* have been identified as critical players in enteric neurogenesis.

RET and EDNRB signaling pathways were considered as biochemically independent until 1999 when the first genetic evidence was reported that some interaction was going on between the two signaling pathways, namely the description of an HSCR patient, heterozygote for weak hypomorphic mutations of both *RET* and *EDNRB* genes each inherited from one of the healthy parents [103]. Subsequently, two systematic genetic studies have sustained this hypothesis. First, a genome-wide association study in 43 Mennonite family trios and noncomplementation of aganglionosis in mouse intercrosses between *Ret* null and the *Ednrb* hypomorphic piebald alleles suggested the presence of epistasis between *EDNRB* and *RET* [104]. Second, by using two-locus noncomplementation of known mouse *Ret* and *Ednrb* mutations, it was demonstrated that compound genotypes of the two major HSCR genes *Ret* and *Ednrb*, which independently fail to yield intestinal aganglionosis, can result in an enteric defect in mice [105].

Moreover, developmental studies have shown that activation of *EDNRB* specifically enhances the effect of *RET* signaling on the proliferation of uncommitted ENS progenitors and that protein kinase A is a key component of the molecular mechanisms that integrate signaling by the two receptors [106].

SOX10 is involved in cell lineage determination and is capable of transactivating both *RET* and *MITF* synergistically with *PAX3* [107, 108] Moreover, the *Ednrb* transcript is absent or drastically reduced in *Dom*−/− and *Dom*+/− mice, respectively [109], due to either a direct effect of Sox10 or an indirect effect due to the common fate of the NC cell progenitors.

On the basis of the above discussion, a few conclusions can be drawn:

1. *RET* is the major HSCR gene with heterozygous mutations found in 50% of familial cases and 15–20% of isolated cases.
2. Penetrance of *RET* mutations is incomplete and sex-dependent.
3. Genotype–phenotype correlation is poor in isolated HSCR.
4. HSCR is genetically heterogeneous and can arise from mutations in distinct pathways.
5. Some patients with mutations in more than one HSCR susceptibility gene (*RET* + *GDNF, RET* + *NTN, RET* + *EDNRB*) are known.

These and other observations have confirmed the complex inheritance of HSCR disease. In this respect, some data have already been collected and used to reconstruct a preliminary picture of the different genetic components involved, as shown in the following paragraphs.

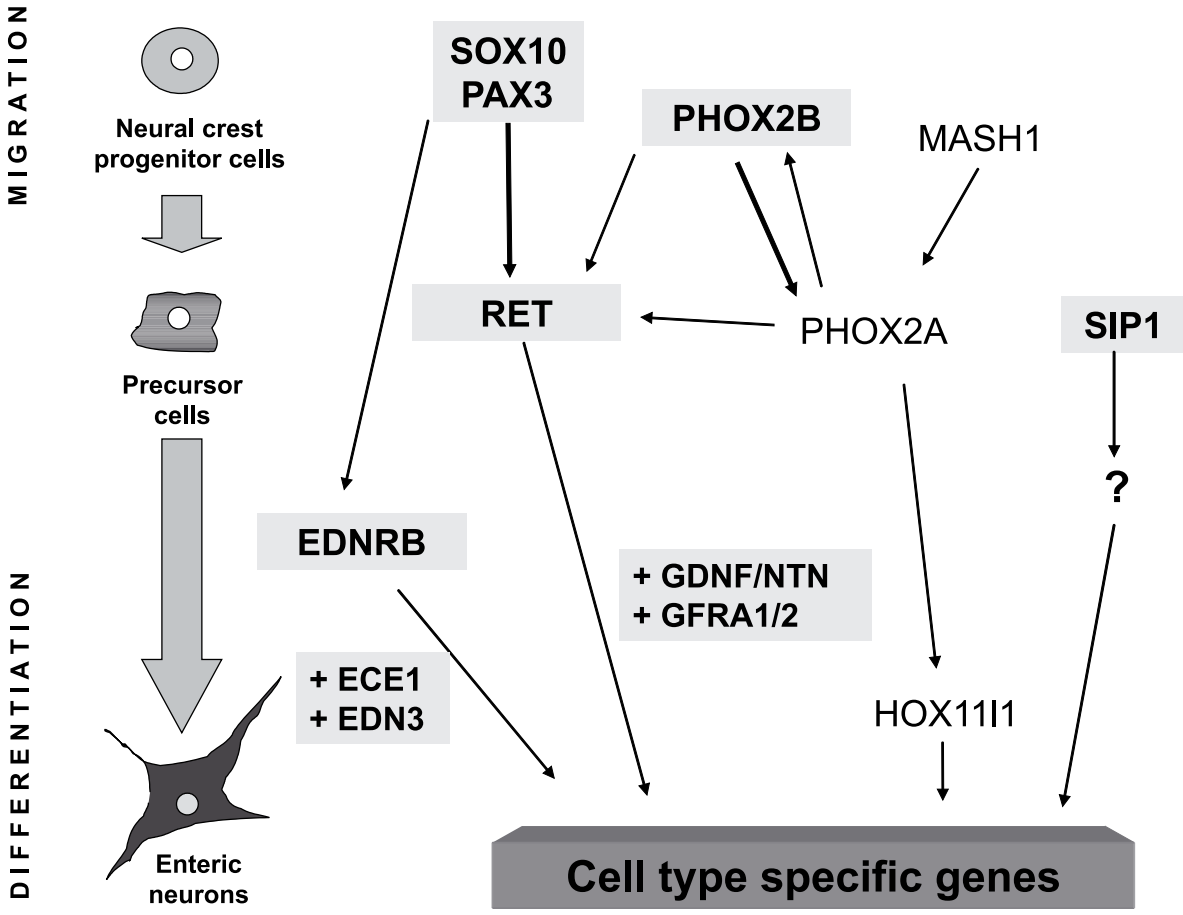

Fig. 5.4 Cascade of transcriptional activation in precursor cells of the ENS, as suggested by in vivo expression studies. *Bold arrows* indicate direct interaction demonstrated by specific functional assays

5.4 Genetic Analysis to Identify Other HSCR Loci

Linkage analysis

Linkage analysis in 12 vertical HSCR families with a large predominance of L-HSCR has shown linkage to the *RET* locus in all but one family [110]. Mutational analysis identified nonsense or missense mutations at highly conserved residues in six families, splice mutations in two families and noncoding sequence variations in three families. Linkage to a novel locus in 9q31 was identified only in families with no or hypomorphic *RET* gene mutations. Therefore, a severe *RET* mutation may lead per se to phenotypic expression by haploinsufficiency, while hypomorphic *RET* mutations would require the action of other mutations, probably located in an undiscovered gene in 9q31.

Sib-pair analysis

A sib-pair analysis in 49 families with S-HSCR probands [111] has shown that three loci, located on chromosomes 3p21, 10q11 and 19q12, are both necessary and sufficient to explain the incidence and sib recurrence risk in HSCR. A multiplicative risk across loci, with most affected individuals being heterozygotes for all three corresponding genes, seems the best genetic model. The HSCR susceptibility gene at the 10q11 locus is *RET* and the two other genes in 3p21 and 19q12 remain to be identified. Interestingly, marker analysis showed a significant parent-of-origin effect at, and only at, the *RET* locus, 78% of shared *RET* alleles being maternally derived, which could explain the sex difference in HSCR expression.

5.5 Additional Contribution of the *RET* Gene: SNPs and Haplotypes

The *RET* protooncogene is mutated in 50% of familial cases, 7–35% of sporadic cases, and up to 75% of L-HSCR, while other genes account for less than 5% of cases, either in syndromic patients or in combination with *RET* mutations in a few isolated HSCR cases. Moreover, as already anticipated, several genetic linkage analyses have shown that in over 90% of families HSCR is linked to the *RET* gene, even in the absence of clearly functional mutations of the coding region of the gene [110, 111]. This and other observations opened the new perspective of a different additional role of *RET* in HSCR onset, sustained by a still-unknown mechanism.

Several hypotheses have been proposed, such as an epistatic regulation of *RET,* requiring the interaction of several genes to produce the phenotype [20, 104], and/or specific *RET* SNPs (single nucleotide polymorphisms) or *RET* haplotypes, acting as either low penetrant alleles themselves or in linkage disequilibrium (LD) with an unknown susceptibility locus [112–116].

This latter possibility, in particular, has received increasing attention in the last 6 years, as attested by a new course of studies. Since 1999, several SNPs in the coding region of *RET* have been described as under- or over-represented in patients compared to controls [113, 114, 117–119], allowing the hypothesis to be advanced that common polymorphisms present in the general population and subsequently considered innocuous could be implicated in the pathogenesis of HSCR. Moreover, the involvement of *RET* polymorphisms has prompted the reconstruction of haplotypes and the study of their distribution within and among populations [104, 112, 116, 120–123]. In particular, a synonymous SNP in exon 2 (c.135G>A, A45A) and haplotypes comprising such a SNP, have repeatedly been shown in association with HSCR, and thus represent a sort of genetic marker of disease predisposition or increased recurrence risk [112, 116, 121, 122].

Recently, attention has focused on the 5' portion of the *RET* gene. Borrego et al., on the basis of the LD observed at several *RET* markers, have suggested the existence of a susceptibility variant in intron 1, in LD with an ancient low penetrant founder locus 20 to 30 kb upstream of SNP2 and related to the transcriptional activity of *RET* [115]. At the same time, Sancandi et al. described a three-locus haplotype named ACA, including the A variant allele of SNP2 and the A and C alleles of two novel SNPs identified in the *RET* promoter region, at −5 and −1 nucleotides from the transcription start codon, respectively. The ACA haplotype accounted, in the Italian population, for 62% of HSCR patients and only for 21.8% of healthy individuals [116] and, spanning from exon 2 up to the promoter, was defined by markers at the opposite end of the 23.5 kb long intron 1, and was thus consistent with the founding locus hypothesis just proposed. The association of this haplotype with the disease has been independently confirmed in other populations [121, 123], including the Chinese population, where the only haplotype found to be over-represented in sporadic HSCR patients included SNP2 [122] and the already known HSCR associated alleles of the promoter SNPs [124]. Notably, the SNP2 variant allele seems to display frequencies that correlate with the incidence of the disease in the different populations, being more frequent in Asiatics, lower in Caucasians and even lower in Hispanics, though no data are available yet to describe the distribution of the ACA haplotype in this latter population. The ACA haplotype probably extends from the 5'-UTR to at least intron 5 [115, 125], and has been suggested to act through an autosomal recessive or a dosage-dependent mechanism [123–127].

The direct role of the ACA haplotype, and especially of its single variants, is still disputed. Fitze et al., following in vitro experiments, hypothesized a direct role of the variant at −5 in determining a low level of *RET* expression [121]. In contrast, using a similar experimental approach, Griseri et al. showed that none of the promoter variants was functionally responsible for the association with HSCR and excluded a role of SNP2 in aberrant splicing. On the other hand, the same authors found that the whole ACA haplotype was associated with low in vivo *RET* gene expression, altogether denoting that the ACA haplotype could be in LD with a low-penetrance susceptibility locus, probably located in intron 1 [126]. Garcia-Barcelo et al. suggested that such discrepancies could be due to the different cell lines and lengths of promoter used in the transfection experiments, and, assuming that the promoter SNPs are located in a putative binding site for the transcription factor TTF-1, they found that the AC allele could decrease *RET* promoter expression by preventing TTF-1-induced trans-activation [124].

At present, the common belief that a frequent susceptibility variant could lie inside the long intron 1, in LD with the known HSCR predisposing *RET* haplotype is under investigation by several groups. To this purpose, the current approach involves a combination of comparative genomics between different species, to identify evolutionarily conserved regions likely relevant in gene expression, and genetic association studies [128]. Following such a strategy, Emison et al. have recently identified a common variant inside intron 1, named RET+3, which they propose as a major HSCR susceptibility mutation. RET+3 shows low penetrance, but still accounts for a 10–20-fold greater contribution to disease susceptibility than all the other known *RET* mutations, and exerts different genetic effects in males and females. The variant is located in a region, conserved among multiple vertebrate species, demonstrated to act as a cell-dependent expression enhancer, with the HSCR-associated allele significantly reducing the enhancer activity. RET+3, as well as the SNP2 discussed above, is more frequent in Asiatics,

and lower in Europeans. Moreover, it is almost absent in Africa. To explain such findings, the authors speculate about a selective advantage of the mutation in heterozygotes, and its possible protective role for another disease, as happens with malaria and microcytemia [129]. Such a hypothesis supports the "common variant–common disease" model of genetic disease which can also therefore be proposed for HSCR.

5.6 Genetic Counseling

HSCR is a sex-modified multifactorial congenital malformation with an overall recurrence risk in sibs of 4% (relative risk 200). In isolated HSCR, adequate relative risk figures can be provided by taking into account the sex and length of the aganglionic segment in the proband and the gender of the sib (2–33%), with the highest recurrence risk being for a male sib of a female proband affected with L-HSCR (Carter's paradox, see Table 5.1). In view of the poor genotype–phenotype correlation, and the low *RET* mutation rate in HSCR patients, thus far the benefit of mutation screening appears limited, except for systematic testing of exons 10 and 11. Special attention should be paid to patients who carry mutations of one of the critical cysteine residues of these exons, known to predispose to MEN2A [17, 22, 34]. In these patients, HSCR can be associated with development of neuroendocrine tumors such as MTC, for which a prophylactic thyroidectomy is advisable in the presence of a tumor causing *RET* mutation.

In particular patients HSCR is associated with other congenital anomalies. In these patients, the long-term prognosis is highly dependent on the severity of the associated anomalies. Several known syndromes have straight Mendelian inheritance. This emphasizes the importance of careful assessment by a clinician trained in syndromology of all newborns diagnosed with HSCR. The success in identifying specific genes for various syndromic and isolated forms of HSCR suggests that mutation detection in familial cases may be warranted. However, with few exceptions, the penetrance of single-gene mutations may be less than 100% so that genetic counseling in HSCR families is usually problematic and performing prenatal diagnosis cannot be advised. In addition, genetic counseling should take into account the great improvement of surgical management of HSCR achieved during the last decades.

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Normal Colonic Motor Function and Relevant Structure

J. Christensen

6

6.1 Introduction

The high degree of interest in the function of the large intestine does not reflect a similar degree of knowledge. Until relatively recent times, many widely held views lacked a basis in facts established by observation or experimentation. But a serious science finally emerged, one that now defines some unique properties of this part of the gut [1].

In all mammals (in virtually all multicellular animals, in fact) a segment at the caudal end of the gastrointestinal tract exhibits morphological and functional distinctions that justify its designation as a structure fundamentally different from the other parts of the tract [2]. Its special functions seem to relate to three particular needs of the bodily economy: for the conservation of water; for the maximal utilization of nutrients; and for the voluntary control of defecation.

The need for the conservation of water must have originated, along with specialization in the kidney, in the adaptation of mammals to terrestrial life. The necessity to maximize the utilization of nutrients arose with the adaptation of mammals to herbivorous diets where intraluminal bacteria came to provide for the digestion of substances from plants that resist mammalian digestive enzymes. The ability to voluntarily control defecation may have evolved in response to animal predation both as a means for predators to identify hunting territories and as a way for those who are hunted to escape tracking.

All three needs, met by functions of the large intestine, derive especially from the unique motility of that organ. Throughout the whole organ, contractions produce very slow antegrade flows that facilitate the mucosal extraction of water from the fecal mass and allow bacterial prolifera-

tion. In the most distal part of the large intestine, there are few spontaneous contractions and fecal flow can be suspended at will to provide for the voluntary control of defecation.

Such specialized motor functions, so distinct from those of the small intestine and other parts of the gastrointestinal tract, require specialization in the nerves and muscles of the gut, the structures responsible for contractions and flow. Thus, this chapter describes both the special morphology and the special motor functions of the large intestine.

Clinicians especially wish to understand the large intestine of humans, yet most experimentation must be done in other species. This fact would present no problem were it not for the enormous variations in gross structure of the large intestine among mammals. Such variation in structure implies variation in function. The differences are probably quantitative rather that qualitative. Still, this problem must be considered even though the large intestine seems to provide fundamentally the same functions in all species.

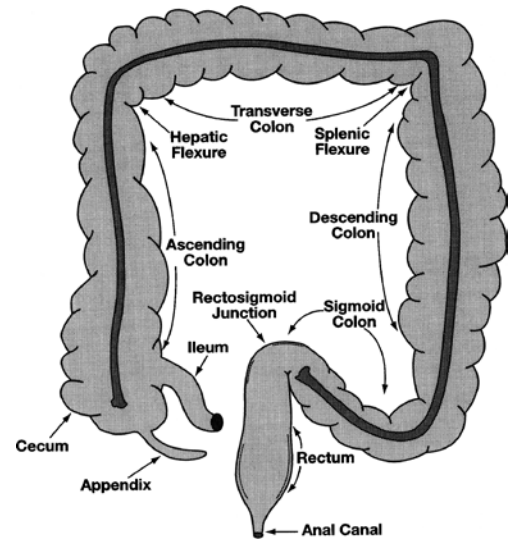

Fig. 6.1 The human large intestine with its parts and landmarks

6.2 Morphology

6.2.1 Gross Anatomy

6.2.1.1 Structure of the Human Large Intestine

The human large intestine, 1.5 m long, forms an arch in the abdomen, beginning in the right iliac region, running cephalad to the caudal surface of the liver, passing across the midline to the left hypochondrium, descending into the left iliac region and then curving to the midline to pass along the posterior pelvic wall to the anus. This configuration provides a set of terms used for the various parts of the human large intestine (Fig. 6.1). The organ has five parts: the appendix, cecum, colon, rectum and anal canal. The colon itself has four parts named for their positions in the abdomen: the ascending (or right) colon, the transverse colon, the descending (or left) colon and the sigmoid colon. Certain landmarks along the organ are called the ileocecal junction, the hepatic flexure, the splenic flexure and the rectosigmoid junction. All these regions and loci appear in Fig. 6.1. The same terms are applied, with variable adequacy, to the large intestine in many other species.

The *ileocecal junction*, sometimes called the *ileocecal valve*, separates the terminal ileum from the large intestine and delineates the cecum from the ascending colon. The *cecum* is the blind pouch of the large intestine that extends upstream from the ileocecal junction. It usually contacts the iliac muscle posteriorly and the abdominal wall anteriorly but the breadth of its mesentery allows great variation in its exact position within the abdomen. The cecum may extend into the pelvis to contact the rectum, extend across the midline into left iliac fossa, or even extend cephalad to the iliac fossa. The *appendix* is the worm-like (vermiform) blind tube, about 20 cm long and 10 mm in diameter, that extends from the apex or blind end of the cecum. Its position in the abdomen also varies greatly because of its generous mesentery.

The *ascending colon*, extending from the ileocecal junction to the hepatic flexure (the angulation formed by the colon in the colic impression on the caudal surface of the liver), lacks a mesentery. The investing peritoneum holds the ascending colon fast against the dorsal structures, mainly the ileopsoas and quadratus lumborum muscles, the aponeurotic origin of the transverse abdominal muscles and the ventrolateral surface of the right kidney. The *transverse colon*, extending from the colic impression on the caudal surface of the liver to the spleen, droops caudad as it crosses over the midline, often extending well below the interiliac line. The breadth of the mesocolon (the mesentery) in this region allows extreme mobility of the transverse colon so that its position varies greatly. The acute angulation formed by the colon just below the spleen, the *splenic flexure*, demarcates the beginning of the *descending colon*. The splenic flexure of the colon usually touches both the spleen and the tail of the pancreas. The splenic flexure shows relatively little mobility, being held in place by the *phrenicocolic ligament*, a peritoneal fold that attaches both the splenic flexure and the spleen to the diaphragm opposite the tenth and eleventh ribs. The descending colon, running caudad from the spleen to the pelvis, lacks a mesentery, the investing peritoneum holding it close against the ileopsoas and quadratus lum-

borum muscles, the left kidney and the aponeurotic origin of the transverse abdominal muscles. The descending colon curves medially in the left iliac fossa ventral to the ileopsoas muscle to form a loop, the *sigmoid colon*. This loop, beginning at about the level of the upper aperture of the lesser pelvis, is suspended from a mesentery, the *sigmoid mesocolon*, whose breadth lends the sigmoid colon such great mobility that it may even extend high in the abdomen or cross the midline into the right iliac fossa. The sigmoid colon ends in the midline on the ventral surface of the sacrum at the level of the third sacral vertebra where it forms the *rectosigmoid junction*.

The relatively straight course of the part of the large intestine that lies between the level of the third sacral vertebra and the pelvic floor dictates its name, the *rectum*. The segment, about 12 cm long, actually exhibits a slight dorsoventral curvature. It widens a little just above the pelvic floor to form the *rectal ampulla*. Three fixed semilunar folds, the *valves of Houston*, indent its lumen. The rectum lies within the peritoneal cavity at its rostral end, lacking a mesentery but invested by peritoneum. This peritoneal covering is reflected parietally at about 7.5 cm above the anal canal in men and 5.5 cm above it in women. The extraperitoneal part of the rectum below that point lies next to the sacrum and coccyx dorsally and next to the bladder and vagina ventrally in women and to the bladder, prostate, and seminal vesicles in men.

The anal canal extends from the end of the rectal ampulla (at about the apex of the prostate in men) to the external anal orifice. The rectum joins the anal canal at an obtuse angle pointing anteriorly. That is, the axis of the anal canal points ventrally toward the umbilicus while the axis of the rectum points dorsally toward the sacroiliac joint. The anal canal 2.5 to 4 cm long, lies wholly within the pelvic floor, surrounded by the *levator ani muscle* and the *external anal sphincter*. A fibromuscular wedge, the *perineal body*, separates the anal canal from the urogenital structures ventrally. A similar structure, the *postanal plate*, separates it from the coccyx dorsally. The mucosa of the anal canal lies in longitudinal folds, the *columns of Morgagni*, separated by valleys, the *rectal sinuses* (Fig. 6.2). Each column ends at the external anal orifice as a triangular nipple. These nipples, the *anal papillae*, covered with squamous epithelium, form a row which marks the squamocolumnar epithelial border (pectinate line). Thin epithelial folds, the *anal valves*, link the adjacent anal papillae and form a row of tiny pockets, the *anal crypts* (or *sinuses)* between the papillae.

Most of the large intestine presents the appearance of an irregular or sacculated tube in contrast to the small intestine which looks like a smooth and uniform cylinder (Fig. 6.3). This irregular configuration arises from the thickening of the longitudinal musculature of the large intestine into three bundles, the *teniae coli*, one lying along the mesenteric insertion and the other two approximately equidistant from it. Between the three teniae,

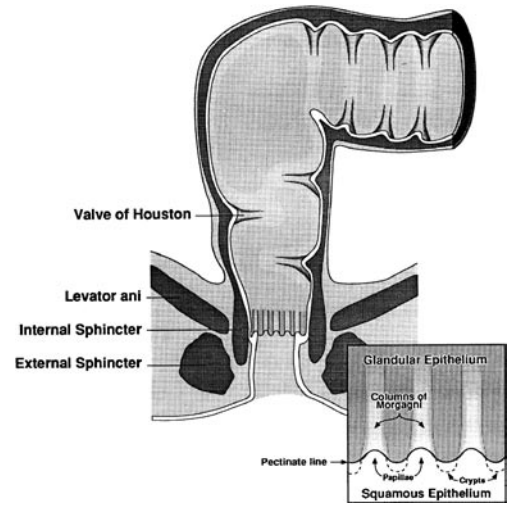

Fig. 6.2 The rectum and anal canal

the walls of the colon are elongated and bulge. Thin rings of the circular muscle layer interrupt the bulging walls at intervals to form the lumen into a chain of saccules or pockets, the *haustra*. This sacculated appearance characterizes the cecum and the colon as far as the rectosigmoid junction. The rectum itself has more the shape of a uniform cylinder, except for the indentations produced by the valves of Houston.

6.2.1.2 Comparative Anatomy of the Large Intestine

The form of the large intestine varies enormously among mammals [3, 4] especially in respect of the size of the cecum and to the extent and distribution of sacculation or haustration. The simplest mammalian large intestine, as seen in the mink and similar animals, possesses no cecum at all, and the colon is quite smooth and cylindrical, with no redundancies or pockets. Complex large intestines, like that of the horse, for example, possess a voluminous cecum with haustration extending all the way to the rectum. These variations in complexity seem to be related to diet, herbivores having complex colons, carnivores simple ones, and omnivores colons of intermediate complexity. But some omnivores (such as the pig) have very complex colons while others (such as the rat) have very simple ones. The reason for this may be that the diet changed more easily than did colonic morphology in mammalian evolution. Those omnivores with simple colons probably evolved from carnivorous antecedents while the omnivores with complex colons had herbivorous ancestors.

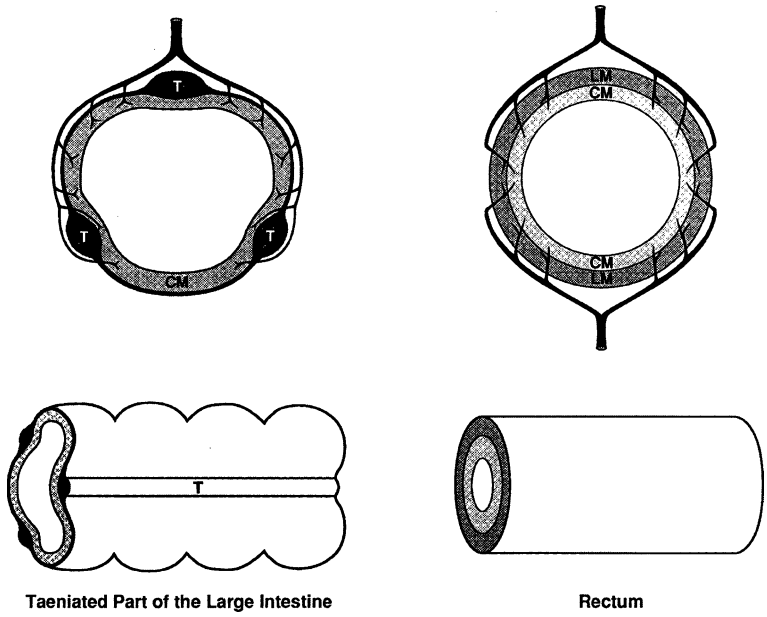

Fig. 6.3 **a** The sacculated configuration that characterizes most of the large intestine in humans. **b** The cylindrical configuration that characterizes the rectum

6.2.2 Histology

6.2.2.1 Structure of the Wall of the Large Intestine

The colon, like the rest of the gastrointestinal tract, is composed of various tissues organized into four layers: the mucosa, the submucosa, the muscularis propria and the serosa. The mucosa itself contains three layers: the epithelium, the lamina propria and the muscularis mucosae. The muscularis propria also contains three layers: the circular muscle layer, the intermuscular space and the longitudinal muscle layer.

The *epithelium* comprises a single layer composed mainly of columnar absorptive cells and goblet cells. The columnar absorptive cells, the principal cells, resemble those of the small intestine except that their microvilli are rudimentary. The goblet cells also resemble those of the small intestine except they are somewhat more numerous. The colonic enteroendocrine cells resemble those of the small intestine except that they are usually solitary rather than aggregated. All three kinds of cells develop from undifferentiated cells in the depths of the colonic epithelial crypts.

The *lamina propria*, filling the space between the epithelium and the muscularis mucosae, comprises mainly a loose stroma of fine collagen fibers. The stroma contains fibroblasts, lymphocytes, plasma cells, macrophages, and eosinophils. Many large lymphoid nodules lie in the colonic lamina propria some of them so large that they protrude into the submucosa.

The *muscularis mucosae*, a continuous thin sheet of visceral muscle at the base of the lamina propria, comprises a network of collagen and elastin fibers that supports about four to six layers of smooth muscle cells. Most of these muscle cells lie with their long axis in the longitudinal axis on the serosal side of the layer and in the circular axis on the mucosal side.

The *submucosa* in the large intestine makes up about half the total wall thickness, a proportion that exceeds that found in other parts of the gut. The loose stroma of collagen and elastin fibers in the submucosa contains fibroblasts, lymphocytes, plasma cells, macrophages, and eosinophils. Arterioles and venules abound, their branches extending into the adjacent mucosa and muscularis propria. The submucosa also contains the submucosal plexus of nerves in which nerve fascicles, bundles of nerve processes, interconnect ganglia, clusters of nerve cell bodies.

The *muscularis propria*, the main coat of muscle of the large intestine, comprises two distinct layers of smooth muscle with an intervening space, the intermuscular space. The inner (or circular) layer of muscle comprises bundles of smooth muscle cells oriented so that their long axes follow the circumference of the organ. The circular muscle layer is uniformly thick along most of the organ. It thickens at the anal canal to form the internal anal sphincter. The submucosal (inner) surface of this muscle layer is covered by a special structure called by various terms but most easily called Stach's plexus after its discoverer. This is a dense network of mesenchymal cells called interstitial cells of Cajal that form a network, essentially a two-dimensional mat, over this surface. Numerous small nerve fiber bundles that arise from ganglia of the submucosal plexus overlie this layer of interstitial

cells. This plexus is described more fully later in this chapter.

The *intermuscular space*, about 100 μm thick, contains the myenteric plexus, large ganglia joined together by interganglionic bundles of fascicles of nerve processes. The space also contains a scattering of interstitial cells of Cajal and a sparse network of blood vessels.

The longitudinal outer muscle layer, a loose stroma of collagen and elastin fibers containing smooth muscle cells, is, overall, much thinner than the circular muscle layer. As described above, in most of the human colon the muscle of this layer is clustered mainly in three major bundles, the tenia coli. It is very thin in the spaces between the teniae.

The *serosa*, a continuous sheet of squamous epithelial cells, invests the colon in whole or in part, being separated from the longitudinal muscle layer by a thin connective tissue stroma. This stroma contains blood vessels and a few nerve fibers. It also contains fatty nodules, the *appendices epiploicae*, which can be quite large.

6.2.2.2 Structure of Gut Smooth Muscle

Gastrointestinal visceral (or smooth) muscle closely resembles vascular and other smooth muscle [5]. Each fusiform cell, about 500–700 μm long and 5–15 μm wide, contains a single elongated nucleus near the midpoint of the cell (Fig. 6.4). The nuclear silhouette usually appears smooth and uniform because of the stretch applied to tissue before fixation, but inspection of unstretched tissues reveals wrinkling of the nucleus. Usually two or more nucleoli stand out in the nucleus, lying within a delicate aggregation of nuclear chromatin fairly uniformly dispersed in the nucleoplasm with some condensation just inside the nuclear envelope.

One muscle cell overlaps the next so that the thickest part of one cell lies next to the thin extremities of its neighbors. Thus, the nuclei in the smooth muscle mass appear to be staggered rather than aligned. The muscle cell cytoplasm or sarcoplasm, viewed by light microscopy, seems essentially devoid of structure, hence the adjective "smooth". The muscle cells lie closely apposed in bundles, delineated and bound together by bands or sheets of connective tissue. Thus, a cross-section shows the muscle as a series of tightly packed palisades of muscle cells separated by connective tissue septa. The muscle bundles join, separate, and join again along their course throughout the muscle. This arrangement ensures the uniform transmission of contractile forces throughout the whole mass in a smooth muscle.

Gut smooth muscle is a relatively dense tissue, the proportion of tissue volume that is extracellular space being about 10–30%. Most of the mass comprises muscle cells, for the other constituents, fibroblasts and nerve processes, make up a very small proportion. The size and spacing of the muscle cells makes for a high surface-to-volume ratio of the muscle cells. About 1.5 m^2 of muscle cell surface is available for exchange with the extracellular space in each gram of muscle tissue.

The cell membrane in smooth muscle possesses many invaginations, the *caveolae*, each about 70 μm in diameter and 120 μm deep, opening to the surface through narrow necks. The caveolae increase the cell surface by 50–70%. More than one-third of the surface area of a cell enters into the formation of caveolae. The basal lamina, an amorphous layer that covers the outer surface of a muscle cell, does not enter the caveolae. Elements of the smooth sarcoplasmic reticulum lie just beneath the caveolae. This juxtaposition supports the concept that the caveolae function like the t-tubules of skeletal muscle in facilitating transmembrane calcium flux to activate contraction.

Dense bands, aggregates of amorphous material, cover the inside of the cell membrane between the caveolae. These dense bands anchor the contractile and structural filaments of the sarcoplasm to the cell membrane. Both the abundant contractile filaments, actin and myosin, and the less abundant structural filaments, desmin, also attach to *dense bodies*, aggregates of amorphous material scattered throughout the sarcoplasm that resemble the membrane-associated dense bands. The dense bands and the dense bodies, providing points for the union of contractile and structural filaments both throughout the sarcoplasm and all along the cell membrane, ensure the uniform distribution of forces throughout the cell.

Cell-to-cell junctions between adjacent smooth muscle cells provide linkages that ensure the integration of muscle cell movements. Anatomists describe *intermediate junctions* as structures that possess features suggesting a role as mechanical linkages. The *gap junctions* provide a physiological linkage through the cell-to-cell transmission of electrical currents and small molecules.

6.2.3 Nerves of the Large Intestine

6.2.3.1 Extrinsic Nerves

The large intestine receives its extrinsic nerve supply through the vagus nerves, from the pelvic nerves and from the mesenteric nerves (Fig. 6.5) [6–8]. The vagus nerves provide a parasympathetic innervation, the cranial part of the craniosacral outflow, to the whole gastrointestinal tract and to the rostral end of the large intestine. The pelvic nerves, arising from the sacral cord, also distribute parasympathetic fibers, the sacral component of the craniosacral outflow, to the whole of the large intestine. The mesenteric nerves emerge from the prevertebral ganglia. The three prevertebral ganglia send branches alongside the corresponding three arteries to the gut. These are sympathetic nerves, elements of the thoracolumbar outflow from the central nervous system.

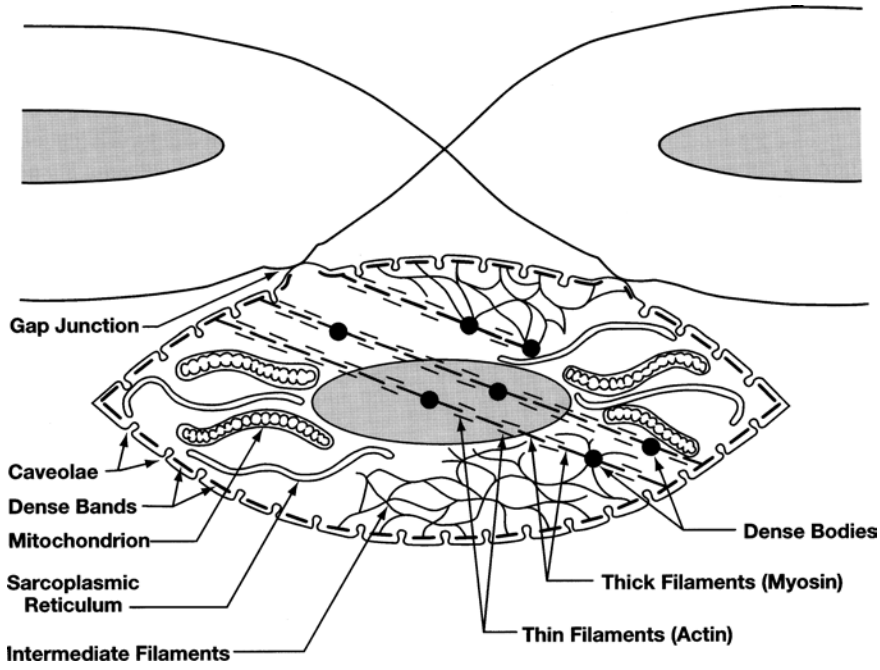

Fig. 6.4 Diagram of a smooth muscle cell to show the ultrastructure

The magnitude of the mass of the large intestine relative to the small number of the extrinsic nerves makes it difficult to trace the distributions the branches of those nerves within the organ. Both physiological and anatomical observations suggest that vagal branches extend no farther than about the middle of the transverse colon. The pelvic nerves distribute nerve fibers through the pelvic plexus to the remainder of the large intestine. The colonic branches from the pelvic plexus pierce the longitudinal muscle layers at about the rectosigmoid junction and then ramify in the intermuscular space through the rectum [9–11]. Branches of these colonic nerves extend rostrally in the myenteric plexus as far as the transverse colon. These branches have the characteristic morphology of extrinsic nerves. That is, they possess a perineurium and a dedicated blood supply. They lie within the myenteric plexus where there is no perineurium or dedicated blood supply. They are called the ascending nerves of the colon (Fig. 6.6). Nerve fibers depart from these ascending nerves to enter into the surrounding myenteric plexus. The domain of the pelvic nerves may well overlap to some degree with that of the vagus nerves.

6.2.3.2 Intrinsic Nerves

The myenteric plexus, the major intrinsic innervation of the large intestine, occupies the intermuscular space between the two muscle layers of the muscularis propria. The ganglia, nodes of closely apposed nerve cell bodies and enteroglial cells, lie in this plane with quite a regular and uniform distribution, joined by interganglionic fascicles. Ganglia in the myenteric plexus lie somewhat closer together beneath the teniae than between them. The mesh formed by the ganglia and the interganglionic fascicles, the primary plexus, delineates irregular polygonal spaces which themselves contain a secondary plexus composed of smaller fascicles that branch from the primary plexus. Still smaller bundles of nerve fibers form a tertiary plexus within the spaces of the secondary plexus.

The density of distribution of ganglia (and of nerve cell bodies) in the myenteric plexus declines along the large intestine [12]. This change represents a decline both in ganglion size and in ganglionic density of distribution. The decline is such that the nerve cell body density in the rectal myenteric plexus is lower than that in any other part of the gut except at the level of the lower esophageal sphincter.

The plane of the myenteric plexus in the large intestine also contains interstitial cells of Cajal. These cells lie in the polygonal interstices of the plexus rather than in the substance of the ganglia and fascicles.

The ganglia of the submucosa form a plexus that differs from the myenteric plexus in gross appearance [13]. The submucosal plexus ganglia are smaller, farther apart and less regularly distributed. The ganglia and fascicles of the submucosal plexus do not form a regular polygonal pattern and there is no subdivision into secondary and tertiary plexus. Neuronal density in the submucosal plexus is much lower in the large intestine than in the small intestine and it declines towards the anus.

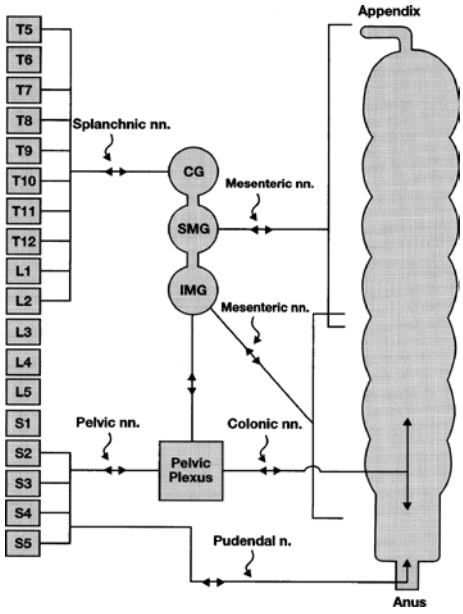

Fig. 6.5 The extrinsic innervation of the large intestine (*CG SMG IMG* three prevertebral ganglia). The blocks at the left represent the levels of the spinal cord

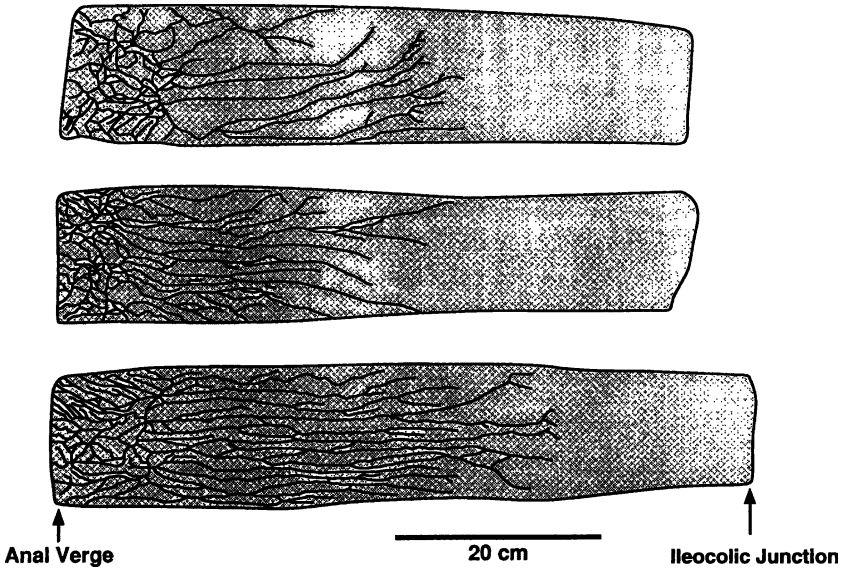

Fig. 6.6 Drawings of the ascending nerves of the colon in three cat colons

The submucosal plexus in the large intestine actually contains two layers of ganglia and interconnecting nerve fascicles. One layer of ganglia, sometimes called *Meissner's plexus*, lies just beneath the muscularis mucosae. The other, *Henle's plexus*, lies close to the surface of the circular muscle layer. The two layers of the plexus, though distinct morphologically, cannot be considered as separate structures since interganglionic fascicles join them together (see also Chapter 3).

Henle's plexus of ganglia in the submucosa gives off bundles of nerve fibers which descend to the underlying surface of the circular muscle layer and ramify there. The branching continues to the point where bundles of nerve processes may contain only one or two nerve fibers. These tiny bundles do not lie directly on the surface of the circular muscle layer but instead lie on an intervening monolayer of specialized mesenchymal cells, the *interstitial cells of Cajal*. These mononuclear cells give off long branching processes which intersect abundantly to form a mat interposed between the nerve fibers and the smooth muscle cells and closely contacting both. This whole laminar structure, interstitial cells with nerve fiber

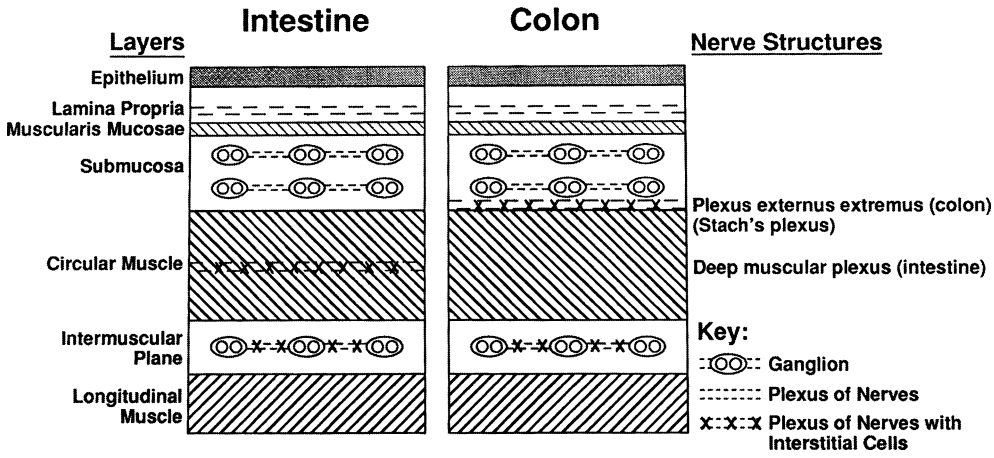

Fig. 6.7 Diagrammatic cross-sections of the wall of the small intestine and the large intestine

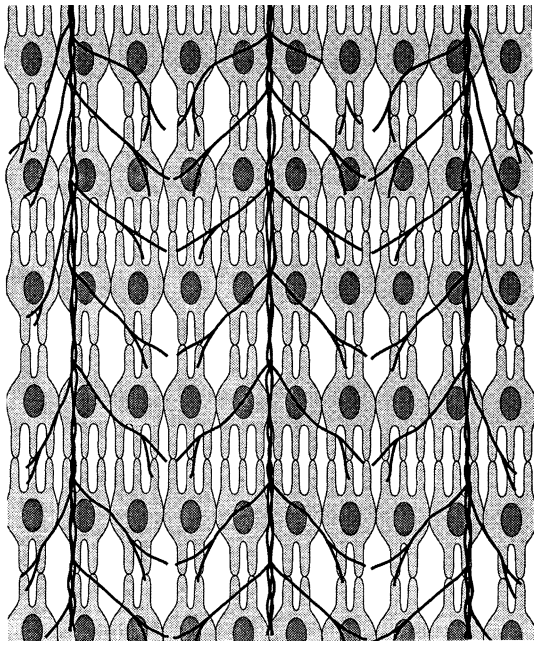

Fig. 6.8 Diagram of Stach's plexus (the plexus *submucosus extremus*) showing the mat of interstitial cells with overlying nerve fibers

bundles, has been called the plexus submucosus extremus, the plexus externus extremus, and other complex Latin names [14, 15]. It is much more easily called *Stach's plexus*, after its discoverer. Stach's plexus is unique to the colon. It is probably analogous to the deep muscular plexus of the small intestine (Figs. 6.7 and 6.8).

6.3 Motor Functions of the Large Intestine

6.3.1 Component Processes of Motor Functions

"Motility" and "motor function", terms widely used to describe the actions of the visceral muscle of the gut, can mean several different things according to context. The terms can refer to any or all of three processes: (1) the flow of luminal contents in the gut; (2) the contractions and relaxations of the muscular walls of the gut that create these flows; and (3) the physiologic functions that control the force of contractions and their distribution in time and in space.

All three kinds of processes—flows, contractions and controlling functions—themselves constitute complex categories of events. For example, gas, mucus, chyme, and stool certainly all flow but they must flow quite differently because they are non-Newtonian fluids with different physical characteristics. As for contractions, they can occupy any or all of the three muscle layers in the large intestine with an enormous range in possible forces and spatiotemporal distributions. As for the controlling processes, physiologist think of them as neural controls, hormonal controls, and controls that arise within the muscle itself, but each of these three broad categories of control functions includes many different processes.

"Motility" thus encompasses a great many different processes. The clarity of our understanding of motility rests in the ability of our methods to reveal these individual processes. The deficiencies of the methods available still restrict our understanding.

6.3.2 Gross Patterns of Contraction and Flow in the Large Intestine

6.3.2.1 Functional Parts of the Large Intestine

The large intestine comprises three functionally distinct units arranged in series. It resembles the stomach in this respect where the difference in behavior of the proximal and distal parts is well known. The functional differentiation of the colon into parts, however, still seems to be unfamiliar to many, although it was first observed nearly a century ago.

In studies of animals by radiography and by inspection of the organ exposed at laparotomy, both American and British investigators, at about the same time, saw different patterns of contractions and flow in the different parts of the large intestine [16, 17]. From their descriptions, one can discern three regions, the right colon, the mid-colon and the distal colon. These three segments are not sharply delineated but merge gradually into one another. Nonetheless, their patterns of contraction and flow seem to be quite distinct.

6.3.2.2 Right Colon

"The usual movement of the transverse and ascending colon is antiperistalsis", wrote Cannon [16]. By "antiperistalsis" he meant a pattern in which ring contractions of the circular muscle layer move retrograde, toward the cecum, rather than caudad like those of the stomach and small intestine. Cannon observed the large intestine of cats radiographically. Elliott and Barclay-Smith [17] studied a variety of small mammals—cats, rats, guinea pigs, rabbits, dogs, ferrets and hedgehogs—by direct observation of the colon exposed at laparotomy and also described antiperistalsis as the dominant pattern of contraction in the ascending colon. How commonly antiperistalsis occurs in humans remains to be discovered. Antiperistalsis is not obvious clinically under the conditions of the barium enema examination, and this may explain the fact that its very existence in humans is not currently acknowledged.

6.3.2.3 Mid-Colon

Both Cannon and Elliott and Barclay-Smith described contractions beyond the level of the hepatic flexure as coordinated antegrade peristalsis, contraction rings of the circular muscle layer moving caudad. This pattern occurred elsewhere too, but it dominated in the mid-colon. Antegrade peristalsis increased with colonic distension.

6.3.2.4 Distal Colon

A still more caudal part of the colon shows very little spontaneous activity but it responds much better than other parts of the colon to stimulation of the pelvic nerves. Such stimulation excites powerful occlusive ring contractions moving caudad.

6.3.2.5 Colonic Motility in Humans and the Mass Movement

The human colon has not had the attention from experimentalists that animal colons have received, but the existing literature tends to suggest that functionally distinct divisions can be distinguished on the basis of the observed behavior. Thus, although antiperistalsis has never been demonstrated in humans, radiographic transit studies suggest that a major delay in mouth-to-anus transit occurs in the proximal colon. Also, extensive mixing occurs there. Something must happen in the proximal colon to hold up flow, and antiperistalsis would do that.

The antegrade orientation of ring contractions in the middle parts of the large intestine seen on radiography in humans agrees with the orientation of contractions seen in animals. One major manifestation of such antegrade contraction is the *mass movement*, first described over a century ago [18] and widely confirmed since. The mass movement appears to be a powerful lumen-occluding contraction ring that develops in the middle or distal parts of the colon after a short period of inhibition. It involves only a relatively short segment of the large intestine. First, haustral indentations disappear. Then, the powerful contraction ring sweeps the segment carrying its contents forward. Then, the haustral indentations reappear. This phenomenon occurs only a few times daily and seems to be precipitated especially by eating.

There is much we do not know about the mass movement. Why does it only sweep a short part of the colon? What determines its location? What are the events or mechanisms that precipitate it? These are important questions because this phenomenon seems to be the principal means by which the fecal mass advances through most of the large intestine beyond the hepatic flexure.

Mass movements have had little experimental study in humans because no one has found a means to reliably precipitate them. They have been seen mainly radiographically, but they also have been observed manometrically and electromyographically.

6.3.2.6 Feeding, Fasting and Sleep

Feeding, fasting and sleep all affect colonic motility, and the magnitude of the effect seems to be considerable. With regard to sleep, colonic motility indeed diminishes greatly in sleep from the level seen in the waking state. Most observers imply or assume that the effect is neurally mediated but, in fact, no one has investigated the possibility that it is not.

Feeding considerably increases motility in the large intestine after a short delay, an effect which is often inaccurately called the gastrocolic reflex. It is a transient effect but one which produces considerable antegrade propulsion of stool.

Fasting also affects motility in the large intestine. The pronounced cycling of contractions in the small intestine that occurs in fasting has received a great deal of study. A somewhat similar cycling occurs in contractions in the large intestine, but the period of the cycle differs from that found in the small intestine. The colonic cycle has a 32-minute period in the dog, whereas the small intestinal cycle has a period about twice that. Both the purpose and the mechanism of this cycling of activity in the large intestine in fasting remains unknown.

6.3.2.7 The Anorectum

It now seems clear from studies done mainly in humans that the motility of the anorectum differs greatly from that of the rest of the large intestine. The rectum is inactive and empty for most of the time. After its evacuation in defecation, the rectum fills very slowly with feces that are delivered to it in rather small amounts at long intervals, presumably by mass movements. The rectal retention of this fecal mass is facilitated by the receptive relaxation of the rectum, which probably resembles that of the gastric fundus, and by the contraction of the anal sphincters. The internal anal sphincter remains contracted involuntarily as the rectum fills. The rectum exhibits brief powerful contractions at long intervals as it fills, especially at night, independent of contractions of the colon and not evacuative. When the degree of rectal filling is sufficient, the internal anal sphincter relaxes as the result of activation of the rectoanal reflex and a powerful and evacuating peristaltic contraction sweeps the rectum. This is the defecation reflex (see also Section 12.4.4).

6.3.2.8 Summary of Contractions and Flow in the Large Intestine

Material entering the large intestine from the ileum tends to pool and to remain in the area of the cecum and ascending colon where there seems to be recirculation and mixing. This is, in part, the result of retrograde peristaltic contractions (antiperistalsis) in this proximal region of the large intestine. Antegrade flow is accomplished in part by the rhythmic peristaltic contractions which predominate in the middle regions of the organ. These antegrade peristaltic movements may be quite infrequent except as they occur during the special phenomenon called the mass movement. This mass movement is a complex motor event occurring at long intervals of time, occupying only a part of the colon and involving first inhibition of the muscle and then peristalsis. This complex event is the principal means for the antegrade flow of luminal contents in the large intestine. The rectum exhibits little activity at rest but simply expands to accommodate the fecal mass delivered to it by successive mass movements. At long intervals, the mechanoreceptive defecation reflex initiates a complex pattern of stereotyped actions associated with defecation. This includes relaxation of both anal sphincters and the evacuation of the rectum (and sigmoid colon) by a powerful and expulsive peristaltic contraction.

6.3.3 Pacemaking System in the Large Intestine

6.3.3.1 Electrical Slow Waves of the Large Intestine

The musculature of the large intestine wall generates pacemaking electrical signals that resemble those of the heart both in form and in function [19]. Similar signals arise as well in the musculature of the gastric antrum and in that of the small intestine.

The electrical signals generated by the large intestine differ from those of the other gastrointestinal viscera in some details, but the processes in signal generation and the functions of the signals seem to be fundamentally the same in all the gastrointestinal organs that manifest them. The unique features of the electromyogram of the large intestine relate to the patterns of spread of the pacemaking electrical signals and to the precise layers in the wall that generate them. Whereas the pacemaking signals in the stomach and small intestine spread antegrade, they spread retrograde in the proximal large intestine. And, whereas they seem to arise in close relationship to the outer longitudinal muscle layer in the stomach and small intestine, they arise in close relationship to the circular muscle layer in the large intestine.

6.3.3.2 Function of Electrical Slow Waves

When electrical events and mechanical events are recorded simultaneously from a single point in the large intestine (or one in the small intestine or gastric antrum) one can see an electrical transient that recurs continuously with a fixed configuration. From baseline, a relatively rapid depolarization occurs, followed by a plateau

and ending in a slower depolarization. Such signals recur at a highly constant frequency that is characteristic of the locus of the recording. This electrical transient, called the *electrical slow wave* (or formerly the basic electrical rhythm or electrical control activity) continues whether or not the muscle at the recording site is contracting. When a contraction of the musculature occurs, the onset of that contraction is signaled by the appearance of one or more much more rapid electrical transients on the plateau of the electrical slow wave. That is, the beginning of a transient or phasic contraction can only occur during an interval of time which is governed by the period of the cycle of the electrical slow wave. The slow wave paces or governs the timing of rhythmic contractions.

If the electromyogram is recorded simultaneously from a series of electrodes aligned along the long axis of the large intestine (or small intestine or gastric antrum), the slow waves can be seen to appear to spread from one end of the electrode array to the other, migrating or propagating in one direction at a fixed velocity. Since the initiation of a contraction is phase-locked to the electrical slow wave, this relationship means that the electrical slow wave also governs the location of rhythmic contractions. Thus, the electrical slow waves establish the frequency, velocity, and direction of spread of rhythmic peristalsis.

6.3.3.3 Origin of the Electrical Slow Waves of the Large Intestine

For a long time, investigators interpreted the experimental evidence as indicating that the electrical slow waves originate in the smooth muscle of the gut, in the longitudinal muscle layer in the small intestine and gastric antrum and in the circular muscle layer in the colon. Now we know that the electrical slow waves arise in relation to the special class of mesenchymal cells called the interstitial cells of Cajal [20]. The evidence from the large intestine is the strongest [21, 22], but it seems most likely that these cells are involved in the generation of pacemaker signals in all other gastrointestinal viscera where such signals occur.

There are two different kinds of electrical slow waves in the colon [23, 24]. One kind arises in Stach's plexus in the inner surface of the circular muscle layer and the other, sometimes called the sinusoidal oscillations, arises in relation to the myenteric plexus. There can be little doubt that the interstitial cells of Cajal generate both. Those interstitial cells that lie in Stach's plexus give rise to the major set of signals, the electrical slow waves, though it is not yet clear how the abundant nerve fibers in that plexus may also participate, or how the underlying circular muscle layer is involved in the generation process. Probably, the nerves at least regulate the signals, controlling their frequency and amplitude. Certainly, the circular muscle layer receives the signals, transmitted through specialized junctions between the interstitial cells and the muscle cells.

6.3.3.4 Spread of the Electrical Slow Waves

The first studies of the electrical slow waves of the large intestine, done in the cat, revealed a pattern of spread or migration that was consistent with the previously observed pattern of retrograde peristalsis in the proximal colon in that species. The site of the dominant pacemaker along the large intestine varied in position from time to time but it was almost always located in a place such that slow waves spread retrograde in the proximal part of the organ and antegrade in more distal sites. The location of the dominant pacemaker shifts along the colon so that patterns of spread may change from retrograde to antegrade in the right colon, but the factors that govern its position remain unknown.

6.3.3.5 Sinusoidal Oscillations

The colonic electromyogram also contains another set of electrical signals besides the electrical slow waves, a sinusoidal oscillation that occurs intermittently rather than continuously. The oscillation is much more rapid than the slow waves. These signals are associated with contractions of the circular muscle layer, contractions that can span several slow wave cycles. It now appears that these rapid sinusoidal oscillations arise in the plane of the myenteric plexus, probably in relation to the interstitial cells of Cajal that are located there. They begin just before a prolonged contraction begins and they end as the contraction ends. The sinusoidal oscillations can be seen to be related to the contractions called mass movements when they occur in the organ in situ. Thus, they seem likely to be both excited and inhibited by intrinsic nerves in the large intestine.

6.3.4 Neurogenic Factors in Large Intestinal Motility

6.3.4.1 Kinds of Nerves in the Colon

There is no reason to suppose that the colonic innervation differs substantially from that of the small intestine in respect to the kinds of intrinsic nerves present. Still, really thorough comparative studies remain to be made. Adrenergic nerve fibers, mainly identified by catecholamine fluorescence staining, end chiefly in relation to ganglion cells of the myenteric plexus in this organ, very few entering the substance of the muscle layers. Cholinergic nerve fibers, also largely identified by histochemistry, vary somewhat in staining intensity and seem to be

the principal excitatory nerve fibers present. Nerve fibers also contain various peptides and other potential neurotransmitters, including VIP, GABA, somatostatin, serotonin, and nitric oxide. Both the classification of neurons on the basis of the colocalization of such substances and the mapping of nerve fibers classified in that way have not been done so carefully in the large intestine as in the guinea pig small intestine. Likewise, comparisons between the myenteric and submucosal plexuses remain to be made. From the point of view of function, however, the principal excitatory motor nerves in the colonic musculature seem to act by the release of acetylcholine and the principal inhibitory motor nerves seem to act by the release of nitric oxide.

6.3.4.2 Intrinsic Reflexes in the Large Intestine

The existence of a peristaltic reflex in the colon was claimed by Bayliss and Starling [25], a response like that which they had seen in the small intestine. They found the reflex, ascending excitation and descending inhibition in response to mucosal stimulation, to be more readily evoked in some species than in others. Other investigators subsequently reported difficulty in demonstrating the reflex as initially reported [26, 27]. Thus there are doubts about its universality as well as about some of the details of its nature. It is not clear that the intestinal peristaltic reflex can be invoked at all to explain the motor functions of the large intestine.

One reflex, however, is clearly demonstrated and clinically useful, the rectoanal inhibitory reflex [28] characterized by the relaxation of the internal anal sphincter in response to rectal distension. The distension of a balloon in the sigmoid colon or rectum induces relaxation of the internal anal sphincter by extrinsic pathways. The reflex is part of the defecation reflex. The morphology of the responsible receptors remains to be discovered. Nerves that act by the release of nitric oxide serve the efferent limb of the reflex arc.

6.3.4.3 Extrinsic Nervous Control of Large Intestinal Motility

The large intestine resembles the rest of the gut in respect the effects of stimulation of the extrinsic nerves. Thus, the lumbar sympathetic nerves carry both excitatory and inhibitory fibers to all parts of the large intestine. The splanchnic nerves are mainly inhibitory. The vagi are excitatory mainly in the proximal colon. The pelvic nerves are excitatory through cholinergic and other mechanisms. Although common thinking credits the brain, especially the psyche, with major effects on the motility of the large intestine in humans, actual evidence indicating any major difference between the large intestine and other gut viscera in respect to the autonomy of motor function remains to be advanced. That is, the colon seems to be no more subject to voluntary control than any other part of the gut, so far as the evidence goes.

The extrinsic nerves can mediate the effects of stimulation in the central nervous system. The stimulation of the hypothalamus and mesencephalon both alter colonic motor function. The existence of such effects, however, does not establish the physiological importance of such central nervous controls. Certainly, the initiation of defecation seems always to be partly voluntary and this implies some importance of the extrinsic nerves. This voluntary control involves, however, the anorectum and pelvic floor more than the whole large intestine. But the area remains obscure. The borderline between voluntary and involuntary functions is even more mysterious at the caudal end of the gastrointestinal tract than it is at the rostral end, in the oropharynx.

6.3.5 Myogenic Factors in the Motility of the Large Intestine

Clearly, tonic contraction is important in large intestinal motility. Tone, a stable or sustained contraction, is much more difficult to assess both in vivo and in vitro than rhythmic or periodic contractions. Still, tone can be seen to exist when circumstances temporarily abolish it. Thus, the tonic contraction of the internal anal sphincter disappears with the excitation of mucosal mechanoreceptors in the rectoanal inhibitory reflex [29]. The other major manifestation of tone in the large intestine is the haustral indentations. These narrow rings indent the lumen of the large intestine at rest at fairly regular intervals to produce the sacculated appearance of the herbivore colon. They were once considered to be fixed and fibrous structures, septa. Their disappearance as a part of the change that occurs in the mass movement, however, indicates that they must be, at least in large part, tonic contractions.

The origin of tone in muscle is certainly not the same in all cases. Tone can reflect the tonic excitation of the muscle by excitatory motor nerves, which is the cause in the somatically innervated striated muscle. Tone could also be the result of hormonal factors. Or, it could represent some special property of the muscle itself. The origin of tone in much of the smooth muscle of the gut has not been investigated carefully. In the lower esophageal sphincter, however, it has. Here, tone persists when that sphincter muscle is isolated in vitro, and after it is treated with tetrodotoxin. This and other evidence indicates that tone in the lower esophageal sphincter is partly, if not largely, myogenic. There is no reason to assume that tone in the large intestine has a different origin. In fact, tone in the internal anal sphincter is very much like that in the lower esophageal sphincter. Still, experiments to establish this point about tone convincingly for the bulk of

the large intestine remain to be done. Of course, tonic contraction in the visceral musculature may have more than one origin.

6.3.6 Some Integrated Motor Functions

6.3.6.1 Continence

Clearly, the capacity to retain feces in the rectum is important, for most mammals possess that capacity and use it most of the time. This capacity for continence involves mainly the anorectum, so far as we know. It involves two distinguishable functions: the reservoir function of the rectum and the closure of the anal canal. The voluntary control of defecation seems to be exerted mainly at the level of the external anal sphincter.

The reservoir function of the rectum is sometimes said to resemble that of the proximal stomach, but actually it differs in several ways. The process of rectal filling is slower and more continuous. There is a continuous concentration of the rectal contents rather than continuous dilution as occurs in the stomach. The stomach empties slowly and incompletely but the emptying of the rectum is abrupt and complete. Still, the rectum seems to share with the gastric fundus some capacity for accommodation or receptive relaxation.

Probably, however, continence depends far more upon the closure of the anal canal provided by the two anal sphincters than upon the receptive relaxation of the rectum. The two sphincters, internal and external, operate in quite different ways. The internal anal sphincter, a thickening of the circular layer of visceral muscle at the end of the rectum, maintains its tone constantly except at times when the rectum has become so full as to initiate the rectoanal inhibitory reflex. The contraction of the internal anal sphincter contributes most of the anal canal pressure that is measured at rest. That is, the internal anal sphincter is the major determinant of continence at rest. The external anal sphincter, a striated musculature derived from the striated muscle of the pelvic floor and sharing the same somatic innervation, is important in continence mainly when sudden rectal distension has abolished the tonic contraction of the internal anal sphincter. The external anal sphincter exhibits a fairly constant resting tone. It can be further contracted by volition. That is normally the means used to abort defecation when a rise in rectal pressure and a relaxation of the internal anal sphincter have occurred as the first steps in the sequence of events that lead to defecation. Thus, the external anal sphincter maintains continence mainly when defecation is imminent. Contraction of the external anal sphincter is not wholly volitional. Involuntary contractions of that sphincter can occur with sudden rectal distension and in response to stimulation of the perianal skin. Continence attributable to the external anal sphincter also requires normal anal and rectal sensation. Both hypersensitivity of the anorectal area and hyposensitivity can lead to incontinence, the former because of exaggerated reflexes and the latter because of the imperception of the imminence of rectal evacuation.

6.3.6.2 Defecation

Many different actions take place in a close temporal sequence in defecation, indicating that the central nervous system clearly participates. Some actions are involuntary, others voluntary. Some of the voluntary actions are necessary to raise the intra-abdominal pressure. They include the closure of the airway, the descent of the diaphragm and the contraction of the abdominal muscles. The involuntary actions include the relaxation of the internal anal sphincter and the peristaltic contraction that empties the rectum.

The process of defecation begins with the excitation of sensations and reflex mechanisms in the anorectum. The anorectal receptors are excited by mechanical stimulation of the rectum. Their exact location remains unknown but much evidence suggests that they may be located in the mucosa very near to the squamocolumnar mucosal junction. In fact, this region contains a profusion of sensory nerve endings. It seems likely that various mechanical and chemical stimuli can excite these receptors to induce, first, the urge to defecate and, second, the reflex relaxation of the internal anal sphincter. A third reflex function follows a little later. This is the powerful peristaltic contraction that evacuates the anorectum. It may involve much of the left colon, even up as far as the splenic flexure. The determinants of its location, velocity and force remain to be investigated. It may well be only a special manifestation of the mechanisms involved in the mass movement.

6.3.6.3 Response of the Large Intestine to Eating

The *gastrocolic reflex*, a term that has broad currency, refers to the association of defecation with eating. The term is in error, for the stimulus is not confined to the stomach, the response is not confined to the colon and the mechanism is not clearly established to be a reflex.

The general nature of the effect is clear. Eating increases the frequency and amplitude of contractions in both the right and left colon (and in the ileum) and this increase may be followed by defecation. The effect takes a little time to start, 20 minutes or more, and it lasts about 20–30 minutes.

Various studies have sought to define the nature of the effective stimulus. Efforts to demonstrate a "cephalic" mechanism, in which the sight, smell or thought of food excites the effect, have failed to produce convincing evi-

dence. Likewise, attempts to establish that the stomach is the sole source of the effect have failed. The entry of nutrients into the duodenum, however, seems to be a highly effective stimulus and the response has been found to be mediated by chemoreceptors in the duodenal mucosa.

Although nervous mechanisms in the gut seem likely to participate to some extent in the effect, the pathways remain obscure. Even the nature of the motor nerves mediating the response is obscure, whether they are adrenergic, cholinergic or nitrergic. Some evidence suggests that the effect is mediated in part by hormones release from the upper gastrointestinal tract by nutrients. There are many such hormones to investigate, including especially gastrin, cholecystokinin and motilin. It may be pointless to try to choose between neural and hormonal mechanisms for the effect because several mechanisms may be involved. Redundancies in mechanisms for important functions are commonly found in animal biology.

6.3.6.4 Effects of the Emotions on Motility in the Large Intestine

Everyday experience suggests that acute anxiety can affect motor function in the large intestine. Involuntary defecation commonly accompanies fright and panic. Past investigations [30–32] have confirmed the general impression of a relationship between acute anxiety and bowel function without yielding much hard information about the mechanism or the significance of the effect. This idea has been extended to advance the idea that bowel motor function is chronically affected in chronic anxiety states. The conviction that bowel motor dysfunction correlates with various personality disorders is firmly entrenched in the folklore of medicine. A great deal of effort continues to be directed to the establishment of a physiological explanation that would provide a foundation for these ideas.

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Pathophysiology of Hirschsprung's Disease

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7

7.1 Introduction

The basic pathophysiological feature in Hirschsprung's disease (HD) is a functional obstruction caused by a narrowed distal aganglionic colonic segment that prevents the propagation of peristaltic waves. Despite extensive research, the pathophysiology of HD is not fully understood. There is no clear explanation for the occurrence of spastic or tonically contracted aganglionic segment of bowel.

The digestive tract is unique among internal organs because it is exposed to a large variety of physiochemical stimuli from the external world in the form of ingested food. As a consequence, the intestine has developed a rich repertoire of coordinated movements of its muscular apparatus to ensure the appropriate mixing and propulsion of contents during digestion, absorption and excretion. The normal motility of the gastrointestinal system is dependent on the interaction of the neural apparatus and the muscular apparatus.

7.2 Organization of the Gut

7.2.1 The Gut Wall

The gut wall comprises two layers of smooth muscles. An outer thin layer of cells arranged along the length of the gut forms the longitudinal smooth muscle layer. A perpendicular, thicker, layer of cells immediately inside the longitudinal muscle forms the circular smooth muscle layer. A well-developed, ganglionated nervous plexus is situated between the two muscle layers, the myenteric plexus. On the luminal side of the circular muscle layer is the submucosa, which contains connective tissue, glands, small vessels and a second ganglionated plexus, the submucous plexus. A thin muscle layer separates the submucosa from the mucosa. The mucosa is densely innervated by sensory nerve fibers from nerve cells in either of the plexuses. Enteroendocrine cells involved in the control of the gut functions are common in the mucosal lining [1–3].

7.2.2 Smooth Muscle Cells

The smooth muscle cells are long thin cells with a large central nucleus. They are interconnected via gap junctions to operate as larger functional mechanical units. Electrical stimuli can spread between the cells through the gap junctions, causing parts of the muscle to act as one single unit [2–4]. The level of muscular activity de-

pends on intrinsic, myogenic activity as well as on the neural apparatus. Electrical slow waves are cyclic changes in membrane potential that are responsible for rhythmic contractions of the muscles. The factors that trigger these slow waves are a network of pacemaker cells called interstitial cells of Cajal (ICC) [2–4].

7.2.3 Interstitial Cells of Cajal

The ICC are mesenchymal cells, spindle shaped or with several processes that form networks that are widely distributed within the submucosal, intramuscular and intermuscular layers of the gastrointestinal tract from the esophagus to the internal anal sphincter [5–7]. Immunohistochemically, they can be localized by the expression of c-Kit, a trans-cell membrane tyrosine-kinase receptor. ICC act as pacemakers in the gut wall, by developing spontaneous slow waves, which spread to the smooth muscle cells. Recent studies have demonstrated that ICC also mediate enteric motor neurotransmission via synaptic-like contacts that exist between varicose nerve terminals and intramuscular ICC [6]. However, the integrative role of the ICC and the enteric nervous system (ENS) in the control of gastrointestinal function is still unknown [8].

7.2.4 Extrinsic Innervation

In addition to intrinsic myogenic activity and the involvement of ICC discussed above, the autonomic nervous system controls gut motility [9]. The autonomic nervous system controls several visceral functions that are not under conscious control. It can be divided into three main divisions: the cranial (parasympathetic) and the spinal (sympathetic and parasympathetic) systems, which relay extrinsic control, and the ENS, which is the intrinsic nervous system of the gut and not only regulates the intestinal motility but also secretions, blood flow, immune and endocrine functions [2, 10]. The extrinsic innervation of the gut involves the vagus nerve and splanchnic nerves to the stomach and upper intestine and the pelvic nerves supplying the distal intestinal segments. Parasympathetic fibers running in the vagus nerve innervate the stomach; however, the majority of the fibers in the vagus are sensory fibers with their nerve cell bodies in the nodose ganglion. These fibers convey information from the stomach and other peripheral organs to the central nervous system [11]. The splanchnic nerves are sympathetic, while the pelvic nerve contains both parasympathetic and sympathetic fibers. Sensory nerve fibers within the spinal nerves, running from the gut to the central nervous system, have their cell bodies located in the dorsal root ganglia [11].

7.2.5 Intrinsic Innervation: the Enteric Nervous System

The ENS is the system of neurons and their supporting cells that is present within the wall of the gastrointestinal tract. It may act independently of extrinsic input but both sympathetic and parasympathetic nerves can influence gut motility via enteric nerves. The ENS is the largest division of the autonomic nervous system, it contains about 100 million neurons, only comparable to the ones of the spinal cord [12, 13]. The neuron cell bodies are clustered together in ganglia. The ENS has two ganglionated plexuses, the myenteric and submucosal plexuses [14]. The myenteric plexus (Auerbach plexus) is positioned between the outer longitudinal and circular muscle layers throughout the digestive tract, from the esophagus to the anus. The submucous plexus is subdivided into separate plexuses: the inner submucous plexus (Meissner plexus) directly below the muscularis mucosae and the outer submucous plexus (Schabadasch or Henle plexus) directly adjacent to the circular muscle layer [13]. The submucosal plexus is absent from the esophagus and stomach, being only prominent in the intestines [3]. This topography has functional relevance is that the myenteric plexus mainly regulates motor function whereas the submucous plexus is mainly involved in control of blood flow, secretion and absorption [13]. The density of neurons varies between myenteric and submucosal ganglia and between gut regions. Typically, myenteric ganglia are considerably larger than submucosal ganglia. The ENS neurons, although clustered into ganglia, do not form nuclei of morphologically similar neuron types as occur, for example, in the brain. Instead, each enteric ganglion contains many different neuron types and neighboring ganglia will contain similar types of neurons although not always in the same proportions [12].

7.2.5.1 Classification of the Neurons of the ENS

Neurons of the ENS can be classified according to their morphological, neurochemical or functional properties. These properties have been disclosed by different methods including light and electron microscopy, immunohistochemistry, electrophysiological analysis, intracellular dyes and retrograde tracing of neuronal projections [3, 15]. In the small intestine 17 different neuronal types, only 14 of which are functionally important, have been identified [14].

Morphology

According to their morphology, neurons are classified into Dogiel type I to type VII and giant neurons. Most neurons are Dogiel types I–III [14]. Dogiel type I neu-

rons have flat cell bodies with many short, lamellar dendrites and a single long axon, and they are considered as enteric motor neurons. Dogiel type II neurons have relatively smooth cell bodies with short and long processes arising in a variety of configurations. The long processes may extend through interganglionic fiber tracts across several rows of ganglia. Shorter processes may project only within the home ganglion. Dogiel type III neurons are similar to type II neurons except that they have more processes and more of the processes are shorter in length [16].

Neurochemistry

Neurons usually express a combination of different neurotransmitters, a phenomenon known as *chemical coding* [17]. The chemical code depends on the type of neuron and the intestinal segment. The general mechanism of chemically mediated synaptic transmission is the same in the ENS as elsewhere in the body, and seemingly as complex as in the central nervous system. More than 30 neurotransmitters have been identified in the ENS, which are usually colocalized according to their function, as shown in Table 7.1 [3, 14]. Enteric neurotransmitters are either small molecules (norepinephrine, 5-HT), larger molecules (peptides) or gases including nitric oxide (NO) and carbon monoxide.

Functional Classification

Neurons are classified according to their function into sensory neurons, interneurons and motor neurons.

Sensory neurons: The sensory neurons are a dense network of extrinsic (vagal and spinal afferents with their cell bodies outside the gut wall) and intrinsic primary afferent neurons (IPAN, with their cell bodies within the gut wall) [18]. They communicate with each other and function together with enteroendocrine and immune cells. Whereas IPAN are essential for the control of the digestion by the ENS, extrinsic afferents notify the brain about processes that are relevant to energy and fluid homeostasis and the sensations of discomfort and pain [19]. Sensory neurons include mechano-, chemo- and thermoreceptors. Mechanoreceptors are activated by distension and generate tonic muscle contractions, but if distension is maintained, they respond by generating peristaltic activity (Fig. 7.1) [10].

Besides direct activation of the IPANs, there are other specialized transducers, the enteroendocrine cells [20]. These cells are strategically positioned in the mucosa to "taste" and sense luminal contents and release their mediators at the basolateral side to activate sensory nerve endings within the lamina propria, which synapse on excitatory or inhibitory motor neurons. While enteroendocrine cells are specialized for luminal nutrient sensing, subepithelial IPANs may also respond to luminal chemicals that freely diffuse across the epithelium [21]. There are regional and topographic differences in the distribution of enteroendocrine cells, with the highest frequency in the duodenum. The major transmitters are cholecystokinin (CCK), secretin, somatostatin, serotonin (5-hydroxytryptamine, 5-HT), and corticotropin releasing factor. Cells containing 5-HT are present in all regions of the intestine and comprise the single largest endocrine cell population [3].

Interneurons: Interneurons are usually Dogiel type II. At least one type of ascending and three types of descending interneurons have been described, most of them being the descending type. The ascending interneurons are mainly cholinergic, whereas the descending ones have a complex chemical coding including acetylcholine, NO, vasoactive intestinal polypeptide, 5-HT and somatostatin (Table 7.1 and Fig. 7.1) [3].

Motor neurons: Motor neurons are Dogiel type I. There are three types: muscle motor neurons, secretomotor neurons that are or are not vasodilators and neurons in-

Table 7.1 Chemical coding of the enteric neurons (5-HT 5-hydroxytryptamine, *Ach* acetylcholine, *Calb* calbindin, *Calret* calretinin, *CCK* cholecystokinin, *CGRP* calcitonin generated peptide, *DYN* dynorphin, *ENK* enkephalins, *GRP* gastrin releasing peptide, *NO* nitric oxide, *NPY* neuropeptide Y, *SP* substance P, *VIP* vasoactive intestinal peptide)

Function	Neurochemical coding
Sensory	Ach, Calb, CGRP, SP
Ascending interneurons	Ach, Calret, ENK, SP
Descending interneurons	5-HT, DYN, GRP, NO, somatostatin, VIP
Short excitatory muscle motor neurons	Ach, SP
Long excitatory muscle motor neurons	Ach, Calret, SP
Inhibitory muscle motor neurons	DYN, ENK, GRP, NO, VIP
Secretomotor neurons	Ach, CCK, CGRP, DYN, NPY, somatostatin, VIP

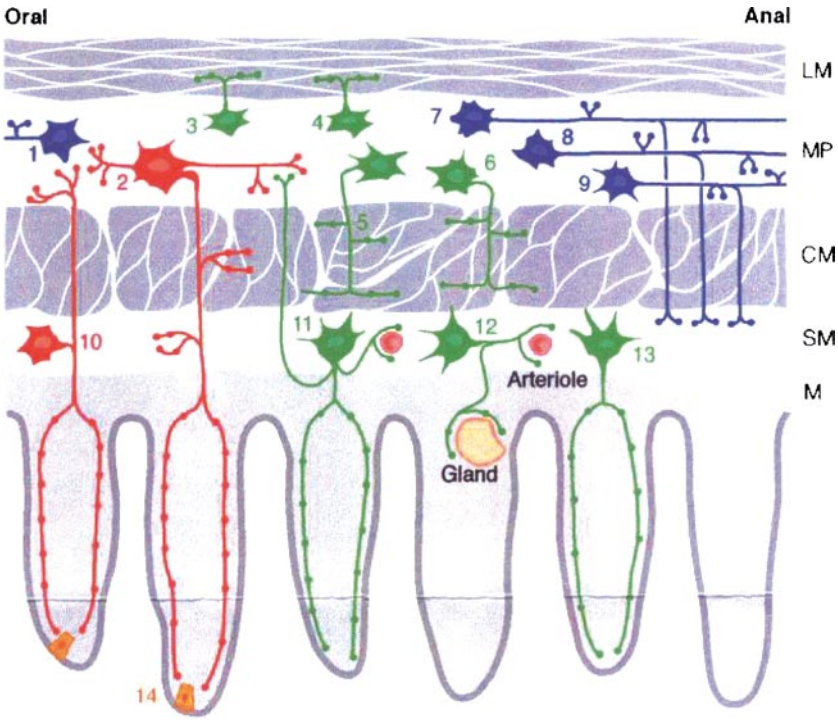

Fig. 7.1 Types of neurons in the small intestine: *1* ascending interneuron, *2* myenteric intrinsic primary afferent neuron, *3* excitatory longitudinal muscle motor neuron, *4* inhibitory longitudinal muscle motor neuron, *5* excitatory circular muscle motor neuron, *6* inhibitory circular muscle motor neuron, *7* descending interneuron (local reflex), *8* descending interneuron (secretomotor reflex), *9* descending interneuron (migrating myoelectric complex), *10* submucosal intrinsic primary afferent neuron, *11* non-cholinergic secretomotor/vasodilator neuron, *12* cholinergic secretomotor/vasodilator neuron, *13* cholinergic secretomotor (non-vasodilator) neuron, *14* enteroendocrine cell; *CM* circular muscle, *LM* longitudinal muscle, *M* mucosa, *MP* myenteric plexus, *SM* submucosal plexus

nervating enteroendocrine cells. Muscle motor neurons innervate the longitudinal and circular muscles and the muscularis mucosae throughout the digestive tract. The muscle motor neurons are either excitatory or inhibitory and release transmitters that provoke muscle contraction or relaxation. For the excitatory neurons, transmission is predominantly muscarinic cholinergic and tachynergic (substance P and neurokinin A). For the inhibitory neurons, the primary transmitter is NO [22, 23], but also vasoactive intestine polypeptide, ATP, pituitary adenylate cyclase-activating polypeptide and carbon monoxide (Table 7.1 and Fig. 7.1) [14].

7.3 Motility of the Gut

Two patterns of activity are recognized in the mammalian intestine, the activity of the interdigestive state and the fed pattern of activity [24].

7.3.1 Migrating Myoelectric Complex

In the interdigestive state, complexes of contractions traveling in an anal direction have been recorded. This is known as migrating myoelectric complex (MMC), which passes along the intestine every 80–110 minutes in humans. The complex takes about 6–10 minutes to pass any point in the intestine, and as it passes, that region undergoes intense rhythmic contractions of the circular muscle [24]. These MMC probably act as housekeepers, to transport waste products in the interdigestive stage and they also control the bacterial flora, preventing overgrowth and returning bacteria to the large intestine [25, 26]. The MMCs disappear soon after a meal is taken, to be replaced by the fed pattern of activity, the peristaltic movements. Both the interdigestive pattern and the fed pattern are generated through the ENS but are modified by the extrinsic nerves. The continuity of the ENS is necessary for the orderly progress of the MMC; if the intestine is interrupted surgically and then rejoined,

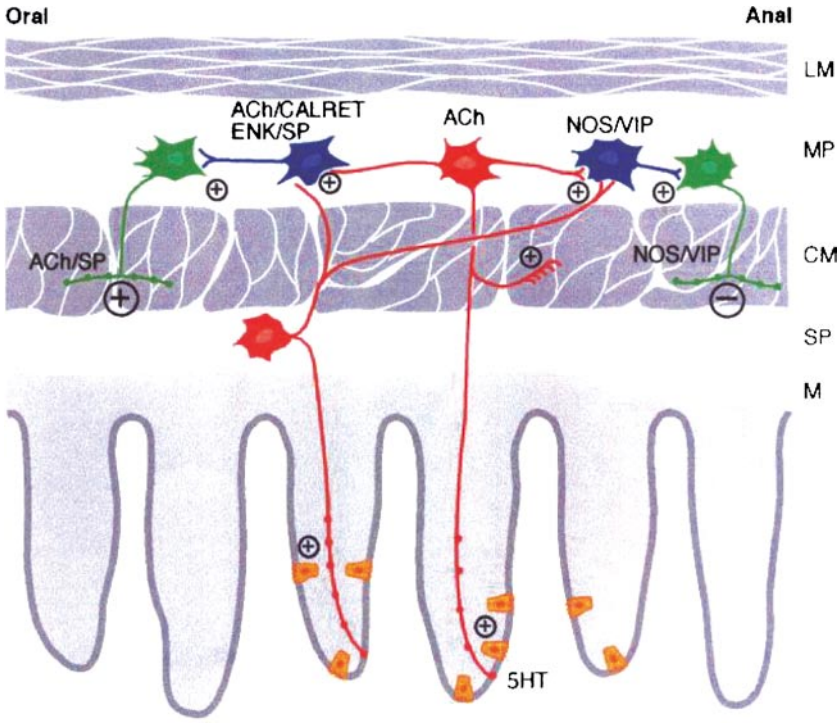

Fig. 7.2 Generalized picture of ascending and descending reflex pathways controlling intestinal peristalsis. The passage of food may cause release of 5-HT from enteroendocrine cells (*yellow*) in the mucosa stimulating sensory nerve endings from IPAN projecting from cell bodies in the myenteric or submucous plexus (*red*). In addition, IPAN may be directly stimulated by distension of the gut wall. The IPAN activate ascending (*oral*) and descending (*anal*) interneurons (*blue*). Orally projecting interneurons release acetylcholine, calretinin, enkephalins and substance P that stimulate excitatory motor neurons innervating the circular muscle, which in turn release acetylcholine and substance P (*green*). Anally projecting interneurons contain NO, and vasoactive intestinal peptide. These interneurons stimulate inhibitory motor neurons that release NO and vasoactive intestinal peptide among other neurotransmitters (*green*) (*5HT* 5-hydroxytryptamine, *ACh* acetylcholine, *CALRET* calretinin, *ENK* enkephalins, *NOS* nitric oxide synthase, *SP* substance P, *VIP* vasoactive intestinal peptide; *CM* circular muscle, *LM* longitudinal muscle, *M* mucosa, *MP* myenteric plexus, *SP* submucous plexus)

the MMC does not always pass the lesion and ectopic MMCs occur on the anal side [24].

7.3.2 Peristalsis

The fed pattern of activity both mixes and propels the contents. In one human study, about 45% of individual contractions did not propagate and about 35% propagated for less than 9 cm [27]. These nonpropagating contractions correspond to the mixing activity. The propagated contractions are peristaltic waves, which consist of contraction of the circular muscle oral to a bolus in the lumen, the ascending excitatory reflex; and relaxation on the anal side, the descending inhibitory reflex. In addition, longitudinal muscle on the anal side may contract while the oral longitudinal muscle relaxes. Total extrinsic denervation of the bowel does not affect peristalsis [24].

All the neural elements for the peristaltic reflex are in the intestine; these are the IPAN, interneurons and motor neurons. Passing the food may cause release of 5-HT from enteroendocrine cells in the mucosa stimulating sensory nerve endings from IPAN projecting from cell bodies in the myenteric or submucous plexus (Table 7.1 and Fig. 7.2). In addition, IPAN may be directly stimulated by distension of the gut wall. The IPAN activate ascending (oral) and descending (anal) interneurons. Orally projecting interneurons release acetylcholine, calretinin, enkephalins and substance P that stimulate excitatory motor neurons innervating the circular muscle, which in turn release acetylcholine and substance P. Anally projecting interneurons contain NO, and vasoactive intestinal peptide. These interneurons stimulate inhibitory motor neurons that release NO and vasoactive intestinal peptide among other neurotransmitters (Table 7.1 and Fig. 7.2)

7.4 The Gut in Hirschsprung's Disease

The characteristic gross pathological feature of HD is a narrowed distal colon with a funnel shaped transition zone to a dilated and hypertrophied proximal colon. However, these features may vary with the duration of untreated disease. In the neonatal period, the intestine may appear normal, but as the child ages, the proximal intestine hypertrophies and becomes thicker and longer than normal. The taeniae disappear and the longitudinal muscle layer seems to completely surround the colon [28]. It has long been recognized that the obstructive symptoms in HD are secondary to the abnormal motility of the distal narrow segment, but there is still no clear explanation for the occurrence of contracted intestinal wall in the distal bowel in HD [29].

7.4.1 Aganglionosis

The most striking finding in the distal intestine in HD is the absence of ganglion cells in the myenteric and submucous plexuses [30]. Aganglionosis typically extends to the rectosigmoid region in approximately 80% of patients. The aganglionosis is continuous and uninterrupted until the proximal transitional zone is reached. The length of this zone may vary and extend for several centimeters and is characterized by hypoganglionosis. Several other abnormalities have been described associated with HD that may contribute to its pathophysiology and may explain the clear discrepancy between the length of the nonfunctional bowel and the degree of obstruction.

7.4.2 Cholinergic Hyperinnervation

In association with aganglionosis, there is a marked increase in cholinergic nerve fibers in the intermuscular zone and submucosa of the aganglionic segment. These fibers appear as thick nerve trunks and correspond to extrinsic preganglionic parasympathetic nerves [31–35]. The continuous acetylcholine release from the axons of these parasympathetic nerves result in an excessive accumulation of the enzyme acetylcholinesterase that is typically found using histochemical staining techniques in the lamina propria mucosae, muscularis mucosae and circular muscle [30]. Both the thick nerve trunks and the increased acetylcholinesterase activity are most pronounced in the most distal aganglionic rectum and progressively diminish proximally as normal bowel is approached [36]. The proximal extent of increased cholinergic activity does not necessarily correspond to the extent of the aganglionosis, which usually extends more proximally to a variable degree. Pharmacological investigations of the colon in HD have demonstrated higher acetylcholine release in the aganglionic segment at rest and after stimulation compared with the proximal ganglionic bowel [37, 38]. Acetylcholinesterase concentrations have also been found to be higher in the serum and erythrocytes from children suffering from HD [39]. Cholinergic nerve hyperplasia has been proposed as the cause of spasticity of the aganglionic segment since acetylcholine is the main excitatory neurotransmitter. However, in the chemical animal model of aganglionosis, after application of benzalkonium chloride or corrosive sublimate, the aganglionic bowel does not show hypertrophic nerve bundles and the bowel still appears narrow, and animals exhibit typical obstructive symptoms [40, 41]. Therefore, the cholinergic hyperinnervation does not seem to be a prerequisite for the appearance of a narrow spastic segment.

7.4.3 Adrenergic Innervation

Fluorescent-histochemical studies for localization of adrenergic nerves have demonstrated that they are increased in number in the aganglionic colon of HD and have a chaotic distribution. They are also present in the circular and longitudinal muscle layers as well as in the mucosa, whereas they are almost absent from normal ganglionic colon [42–44]. However, the sensitivity of the aganglionic bowel to epinephrine is apparently not increased, despite the elevated number of adrenergic fibers [45, 46]. The tissue concentration of norepinephrine is two to three times higher in the aganglionic bowel than in the normal colon; and also there is a corresponding increase in tyrosine hydroxylase, an enzyme that regulates norepinephrine biosynthesis [43]. Because adrenergic nerves normally act to relax the bowel, it is unlikely that adrenergic hyperactivity is responsible for increased tone in the aganglionic colon [47].

7.4.4 Nitrergic Innervation

NO is considered to be one of the most important neurotransmitters involved in relaxation of the smooth muscle of the gut [48]. It is synthesized in a reaction catalyzed by nitric oxide synthase (NOS) and depends on l-arginine and molecular oxygen as cosubstrates to form l-citrulline and NO. NO binds to cytosolic guanylate cyclase and increases the production of 3′5′-cyclic guanosine monophosphate (cGMP) with subsequent relaxation of smooth muscle [49]. NOS has been shown to be colocalized with reduced nicotine adenine dinucleotide phosphate (NADPH) diaphorase, which has been demonstrated to have identical functions [50, 51]. Several investigators have studied NOS distribution in the ganglionic and aganglionic bowel in patients with HD using NOS

immunohistochemistry or NADPH diaphorase histochemistry [52–57]. In normal and ganglionic colon from patients with HD, there is a strong NADPH diaphorase staining of the submucous and myenteric plexuses and a large number of positive nerve fibers in the circular and longitudinal muscle as well as in the muscularis mucosae [49]. In the aganglionic segment of HD patients, there are no ganglia and there is an absence or marked reduction of nerve fibers positive for NADPH diaphorase in both muscle layers and in the muscularis mucosae. The typical hypertrophied nerve trunks appear weakly stained [49]. Kusafuka and Puri [58] examined the expression of neural NOS mRNA in the aganglionic segment from seven patients who had HD and demonstrated that NOS mRNA expression was at least 1/50 to 1/100 of the level expressed in ganglionic bowel. These findings indicate that there is impaired NO synthesis in the aganglionic bowel in HD and this deficiency could prevent smooth muscle relaxation, thereby causing the lack of peristalsis in HD. In an interesting experiment, Bealer et al. [59] compared the effect of an exogenous source of NO, *S*-nitroso-*N*-acetylpenicillamine (SNAP) on the isometric tension of smooth muscle strips from aganglionic bowel and demonstrated a 70% reduction of resting tension. These results suggest that the defective distribution of nerves containing NOS may be involved in the pathogenesis of HD.

7.4.5 Interstitial Cells of Cajal

Abnormalities of ICC have been described in several disorders of human intestinal motility including HD. Vanderwinden et al. [52] using c-kit immunohistochemistry were the first to report that ICC were scarce and the network appeared to be disrupted in aganglionic segments of HD whereas the distribution of ICC in the ganglionic bowel of HD was similar to that observed in controls. Yamataka et al. [60, 61] found few c-kit-positive cells in the muscle layers in HD and a moderate number around the thick nerve bundles in the space between the two muscle layers in the aganglionic bowel. Horisawa et al. [62] found no differences in c-kit immunopositive cells in aganglionic segments compared with the corresponding area of ganglionic bowel. Rolle et al. [63] using whole-mount and frozen sections stained with c-kit immunohistochemistry preparations found an altered distribution of ICC in the entire resected bowel of HD patients and not only in the aganglionic segment. Moreover, gap junctions connecting ICC were immunolocalized by anti-connexin 43 antibody and found to be absent from the aganglionic part of HD bowel and highly reduced from the transitional zone [64]. Rolle et al. proposed that persistent dysmotility problems after a pull-through operation in HD may be due to altered distribution and impaired function of ICC.

7.4.6 Enteroendocrine Cells

Using the generic enteroendocrine cell immunohistochemical markers chromogranin A and synaptophysin, Soeda et al. [65] demonstrated that the number of enteroendocrine cells in the aganglionic colon in patients with HD is significantly increased compared with the number in the normal ganglionic segment. The increase of enteroendocrine cells in the mucosa of aganglionic colon may well influence sustained contraction of the bowel wall mainly mediated by the release of 5-hydroxytryptamine.

7.4.7 Smooth Muscle

Since smooth muscle is the final effector for bowel motility, it is likely that it could also be abnormal in HD. The smooth muscle cell cytoskeleton consists of proteins whose primary function is as a structural framework that surrounds and supports the contractile apparatus of actin and myosin filaments in the body of the smooth muscle cell. Nemeth et al. [66] studied the distribution of cytoskeleton in the smooth muscle of HD bowel by means of immunohistochemistry and found that dystrophin, vinculin and desmin immunoreactivities are either absent or weak in the smooth muscle of aganglionic bowel, whereas they are moderate to strong in the smooth muscle of normal bowel and ganglionic bowel from patients with HD. Neural cell adhesion molecule (NCAM) is a cell surface glycoprotein involved in cell–cell adhesion during development that has been suggested to play an important role in development and maintenance of the neuromuscular system [67–69]. NCAM is present in the innervation of normal infant bowel and, less densely, in some components of the enteric smooth muscle. Contradictory results have been published regarding NCAM expression in the smooth muscle of aganglionic bowel. Kobayashi et al. [53] have described a lack of expression of NCAM in the muscularis propria of the aganglionic bowel compared with the ganglionic segment, whereas Romanska et al. [70] have found an increase in NCAM expression in muscle, particularly in the muscularis mucosae. Anyhow, both authors agree that there is a strong expression of NCAM in the hypertrophied nerve trunks from the aganglionic segment.

7.4.8 Extracellular Matrix

Although extracellular matrix (EM) abnormalities have been described mainly related to the pathogenesis of HD, they could also have an influence on its pathophysiology. The lethal spotted mouse, an animal model which develops aganglionosis in its distal bowel, displays an

abnormal distribution of EM components including laminin, collagen type IV, glycosaminoglycans and proteoglycans in the smooth muscle layer [71, 72]. Parikh et al. [73] have demonstrated that the laminin concentration in aganglionic bowel is twice as high as in the normoganglionic bowel of HD and three times higher than in an age-matched control. Moreover, by means of immunohistochemistry, they found an uneven distribution of laminin and collagen type IV in the muscularis propria of aganglionic bowel, being more intensely expressed in the circular layer than in the longitudinal layer [74]. The same authors have reported that the EM components tenascin and fibronectin are more intensely expressed in aganglionic bowel from HD [75].

7.4.9 Alterations in the Proximal Ganglionic Segment

Several recent studies [76–79] have shown that gastrointestinal motor dysfunction persists in a subset of HD patients long after surgical correction, indicating that morphological and functional abnormalities of the gut are not necessarily restricted to the aganglionic segment. Intestinal neuronal dysplasia (IND) is a malformation of the ENS characterized by the presence of giant ganglia in the submucous plexus, ectopic ganglion cells in the lamina propria of the mucosa and an increased acetylcholinesterase activity in the lamina propria and around submucosal blood vessels [80]. In 1977, Puri et al. reported the first case of IND immediately proximal to a segment of aganglionic colon [81]. Since then, there have been several reports of the combined occurrence of these disorders. Some investigators have reported that 25–35% of patients with HD have associated IND [82, 83] and stress that this could be the cause of persistent bowel symptoms after a pull-through operation for HD [84–86]. Recently, Sandgren et al. [87] have studied in depth the proximal ganglionic bowel in the lethal spotted mouse, a natural mutant model of rectosigmoid HD. They showed that the number of neurons is increased in the submucous plexus from the ileum and colon proximal to the aganglionosis, resembling human IND. They suggested that these findings might explain the persistence of dysmotility after operation for Hirschsprung's disease. Sandgren et al. also demonstrated that the expression of NO and vasoactive intestinal peptide are upregulated in the proximal ganglionic segment, whereas the expression of substance P is downregulated [87].

7.5 Gut motility in Hirschsprung's Disease

In the 1940s Swenson et al. recorded the peristaltic tracings of HD specimens. They found that the progressive contractions of the dilated proximal colon do not enter the more distal narrow segment [88]. These findings provided the evidence for a physiological defect in the distal segment and led to the creation of a novel curative surgical procedure involving the resection of the rectosigmoid in these patients [89]. Kubota et al. [90–94] have studied for many years the electrophysiological and pharmacological characteristics of the different bowel segments in surgically resected specimens of HD. They have found that, while a single pulse stimulation is sufficient to induce a rapid membrane hyperpolarization followed by a spike generation in the majority of cells in the dilated ganglionic bowel, in the transitional region, the amplitude of the hyperpolarization response decreases and repeated stimulations are necessary to induce a response. Even more, in the narrow aganglionic segment, repeated stimulations evoke only a membrane depolarization in about 20% of the cells and spike potentials are generated only when the number of pulses is increased (Fig. 7.3). They have shown that atropine completely abolishes the depolarization response in all the segments and that a membrane hyperpolarization is insensitive to both cholinergic and adrenergic blockers and is completely abolished by tetrodotoxin, demonstrating electrophysiologically the presence of a nonadrenergic non-cholinergic inhibitory innervation. Then, by studying the regional changes in the ampli-

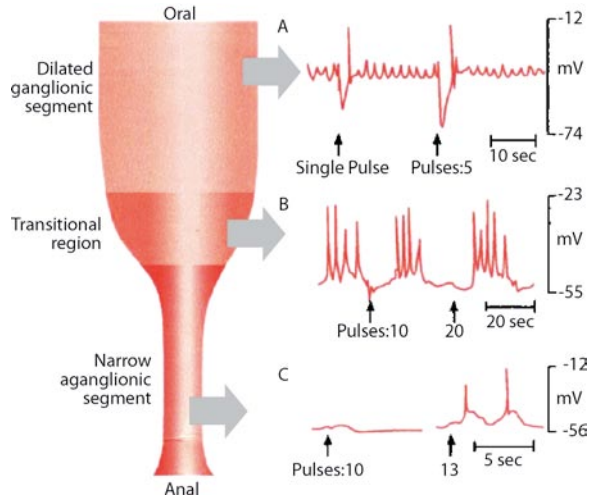

Fig. 7.3 Electrophysiological characteristics of the bowel in Hirschsprung's disease. In the dilated ganglionic bowel, a single pulse stimulation is sufficient to induce a rapid membrane hyperpolarization followed by a spike generation in the majority of cells. In the transitional region, the amplitude of the hyperpolarization response decreases and repetitive stimulations are necessary to induce a response. In the aganglionic segment, repetitive stimulations evoke only a membrane depolarization in about 20% of the cells and spike potential are generated only when the number of pulses is increased

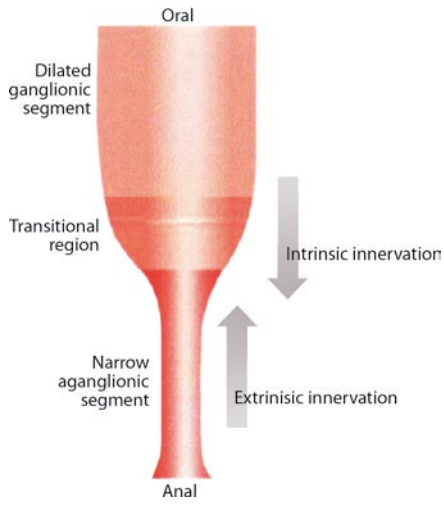

Fig. 7.4 Schematic view of the aganglionic bowel, which receives two nervous flows of different origins: the intrinsic inhibitory nervous flow from the ganglionic segment through the transitional region and the extrinsic excitatory nervous flow from the lower end of the aganglionic segment

tudes of the non-adrenergic non-cholinergic inhibitory junction potentials, they have concluded that the aganglionic segment receives two nervous flows of different origins: one is the intrinsic inhibitory nervous flow from the ganglionic segment through the transitional region, while the other is the extrinsic excitatory nervous flow from the lower end of the aganglionic segment (Fig. 7.4). Since the transitional zone is the place where the stagnation of intestinal contents takes place, they conclude that a decrease in the intrinsic inhibitory nervous flow might be the cause of the intestinal obstruction.

7.6 Final Remarks

Although the more striking histological feature in HD is the absence of ganglion cells, it is unlikely that this is the only cause of the increased intestinal wall tone provoking a functional intestinal obstruction. There are numbers of other histopathological findings both in the aganglionic segment and in the proximal ganglionic segment in HD which may account for the frequent discrepancy encountered between the length of the non-functional bowel and the degree of obstruction and also for the persistent obstructive symptoms after a pull-through operation for HD.

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Hirschsprung's Disease: Clinical Features

P. Puri and S. Montedonico

8

8.1 Introduction

Hirschsprung's disease (HD) is a relatively common cause of intestinal obstruction in the newborn [1]. It is characterized by absence of ganglion cells in the distal bowel beginning at the internal sphincter and extending proximally for varying distances. In the human fetus, neural crest-derived neuroblasts first appear in the developing esophagus at 5 weeks of gestation, and then migrate down to the anal canal in a craniocaudal direction during the 5th to the 12th week of gestation. The absence of ganglion cells in HD has been attributed to a failure of migration of neural crest cells [2, 3]. The earlier the arrest of migration the longer the aganglionic segment is. The absence of ganglion cells results in absent peristalsis in the affected bowel and the development of functional intestinal obstruction.

Although Harald Hirschsprung [4] first described this disease in 1888, the pathological features were not understood until the 1940s when Whitehouse and Kernohan demonstrated that the aganglionosis within the distal colon or rectum was the cause of the functional obstruction [5]. In 1948, Swenson and Bill reported rectosigmoidectomy with preservation of the sphincter as the optimal treatment for HD [6]. In recent years, the vast majority of cases of HD are diagnosed in the neonatal period and many centers are now performing one-stage pull-through operations in the newborn period with minimal morbidity and encouraging results [7].

8.2 Incidence

Several studies on the frequency of HD have been reported. The incidence of HD is estimated to be 1 in 5,000 live births and ranges from 1 in 2,000 to 1 in 12,000 live births (Table 8.1) [8–17]. A large survey of HD cases from the California Birth Defects Monitoring Program (1983–1997) found an incidence of 1.5 in 10,000 live births in whites, 2.1 in 10,000 live births in African-Americans, 1 in 10,000 live births in Hispanic and 2.8 in 10,000 live births in Asians [18]. Recently, a nationwide survey from Japan found an incidence of HD of 1 in 5,343 live births between 1998 and 2002 [19].

8.3 Classification

While the internal anal sphincter is the constant inferior limit, patients can be classified as classical segment HD when the aganglionic segment does not extend beyond the upper sigmoid, long-segment HD when aganglionosis extends to the splenic flexure or transverse colon, and total colonic aganglionosis when the aganglionic segment extends to the colon and a short segment of terminal ileum [20]. Table 8.2 shows the level of aganglionosis in different series with more than 100 patients studied [10, 11, 13, 19, 21–25]. Total intestinal aganglionosis with absence of ganglion cells from duodenum to the rectum is the most rare form of HD [26, 27].

8.4 Sex

It has long been recognized that males are more commonly affected than females with a male:female ratio of 4:1 [10, 11, 13, 17, 19, 21, 22, 24]. The male preponderance is less evident in long-segment HD, where the male:female ratio is 1:1–2:1 [10, 22, 24] and is even reversed in total colonic aganglionosis, where the male:female ratio is 0.8:1 [11]. The reason for these skewed ratios is unclear; no X-linked loci have been described in HD.

Table 8.1 Incidence of Hirschsprung's disease

Year	Reference	Incidence	Area
1962	16	1 in 12,000	Bremen
1963	8	1 in 2,000–10,000	Britain
1964	15	1 in 4,700	Denmark
1967	9	1 in 5,000	Cincinnati
1983	10	1 in 4,500	Southeast Scotland
1984	17	1 in 5,682	Baltimore
1984	11	1 in 4,697	Japan
1985	12	1 in 4,417	British Columbia
1994	13	1 in 7,165	Denmark
1997	14	1 in 3,070	Oman
1998	18	1 in 5,405	California
2005	19	1 in 5,343	Japan

Table 8.2 Classification of Hirschsprung's disease

Reference	Patients (*n*)	Rectosigmoid aganglionosis (%)	Long-segment aganglionosis (%)	Total colonic aganglionosis (%)
21	498	72.5	23.7	3.8
22	998	74	17	9
11	1562	79.4	11.6	12.6
10	103	81.6	18.4	–
23	874	74.6	22	3.5
24	179	88.8	3.9	7.3
13	161	88.2	8.7	3.1
25	105	72.4	19	8.6
19	1103	77.6	13	9.4

Badner et al. [28] demonstrated that recurrence risk to siblings is dependent upon the sex of the person affected and the extent of the aganglionosis. If the index patient is female, the proportion of affected siblings is higher. The recurrence risk to siblings also increases as the aganglionosis becomes more extensive (Table 8.3) [28, 29].

8.5 Race

Recently, the California Birth Defects Monitoring Program have found the highest incidence of HD among Asians with a frequency of 2.8 in 10,000 live births followed by African-Americans with a frequency of 2.1 in 10,000 live births [18]. Goldberg, in a previous epidemiological study, found the incidence of HD among non-white males to be 3.76 in 10,000 live births [17]. In 1979, a survey of the Members of the Surgical Section of the AAP found no differences in the incidence of HD among whites and African-Americans; however, they found that long-segment disease occurs significantly less frequently in nonwhites than in whites [22]. Sherman et al. later confirmed these findings [23]. Although the highest incidence of HD reported in the literature is 1 in 3,070 from a survey in Oman, this is unlikely to be due to racial differences but to a high consanguinity rate [14].

8.6 Heredity

Genetic factors have been implicated in the etiology of HD. HD is known to occur in families. The reported in-

Table 8.3 Recurrence risk to siblings in Hirschsprung's disease

Segment affected	Sex of index patient	Sex of sibling	Recurrence risk (%)
Rectosigmoid HD	Male	Male	5
	Male	Female	1
	Female	Male	5
	Female	Female	3
Long segment HD	Male	Male	17
	Male	Female	13
	Female	Male	33
	Female	Female	9

cidence of familial cases in rectosigmoid HD varies from 3.6% to 7.8% in different series [7]. A familial incidence of 15% to 21% has been reported in total colonic aganglionosis and 50% in the rare total intestinal aganglionosis [28]. Schiller et al. [30] reported 22 infants belonging to four families from Gaza, who had either documented or clinically suspected HD. Of these infants, 13 underwent laparotomy and multiple intestinal biopsies, 10 had total intestinal aganglionosis, 1 had total colonic aganglionosis, 1 had near total colonic aganglionosis, and only 1 had rectosigmoid HD. Engum et al. [31] reported 20 patients with HD in 12 kindreds. The level of aganglionosis was rectal or rectosigmoid in eight, left colon in two, transverse or right colon in two, and total colonic ganglionosis with variable small bowel involvement in eight.

HD occurs as an isolated trait in 70% of patients [32]. A chromosomal abnormality is associated with HD in 12% of patients, trisomy 21 being by far the most frequent (>90%). The relationship with Down's syndrome also tends to suggest a probable genetic component in the etiology of HD. Down's syndrome is the most common chromosomal abnormality associated with aganglionosis and has been reported to occur in 4.5–16% of all patients with HD [24, 33, 34]. Associated congenital anomalies are found in 18% of HD patients and include gastrointestinal malformations, cleft palate, cardiac malformations, craniofacial anomalies, and polydactyly [32]. Other chromosomal abnormalities that have been described in association with HD include interstitial deletion of distal 13q, partial deletion of 2p, reciprocal translation, and trisomy 18 mosaic. A number of unusual hereditary syndromes have been reported in patients with HD. These include Shah-Waardenburg syndrome, multiple endocrine neoplasia (MEN) type 2 syndrome, congenital central hypoventilation syndrome (Ondine's curse), Goldberg-Shprintzen syndrome, Kaufman-McKusick syndrome, Bardet-Biedl syndrome, Smith-Lemli-Opitz syndrome, Cartilage-hair hypoplasia syndrome, and syndromes with HD and distal limbs anomalies (Table 8.4) [24, 29, 32, 34, 35].

The genetics of HD display three characteristics: (1) the penetrance of mutations is generally low, (2) there is a sex difference in the penetrance and expression of mutations, and (3) the penetrance of a gene mutation depends upon the extent of aganglionosis in affected family members [35]. Most identified gene mutations associated with HD are best thought of as susceptibility genes, i.e. the mutation increases an individual's odds of having HD, but is not predictive of the abnormality [35]. So far, eleven HD susceptibility genes have been identified in humans, namely the protooncogene RET (RET), glial cell line-derived neurotrophic factor (GDNF), neurturin (NTN), endothelin B receptor (EDNRB), endothelin 3 (EDN3), endothelin-converting enzyme 1 (ECE1), SOX10, Phox 21, GFRq1 and SIP1 genes [32, 36]. RET mutations account for 50% of familial and 15–35% cases of sporadic HD, whereas EDNRB mutations are found in 5% of HD patients. Disease-associated mutations in the other nine genes are rarer, and in some cases have been documented in only one family [20, 37].

In isolated HD, adequate recurrence risk figures will be provided by taking into account the sex and length of the aganglionic segment in the patient and the gender of the sibling [28, 29]. Risk of recurrence of the disease is greater in relatives of an affected female than an affected male. Risk of recurrence is also greater in relatives of a patient with long-segment compared to short-segment disease. For example, the recurrence risk in a sibling of a female with aganglionosis beginning proximal to the splenic flexure is approximately 23% for a male and 18% for a female, whereas the recurrence risk in a sibling of a male with aganglionosis beginning proximal to the splenic flexure is approximately 11% for a male and 8% for a female. These risks fall to 6% and below for siblings of a patient with short-segment disease (Table 8.3) [29]. The recurrence risk and prognosis of syndromic HD and HD associated with chromosomal abnormalities depends on the recurrence risk of the associated syndrome rather than on the HD [32, 35, 37].

Table 8.4 Partial list of syndromes associated with Hirschsprung's Disease

Syndrome	Features
Neurocristopathies	
Shah-Waardenburg	Pigmentary anomalies, deafness
Congenital central hypoventilation	Abnormal autonomic control of respiration
Multiple endocrine neoplasia 2A	Medullary thyroid carcinoma, pheochromocytoma, hyperplasia of the parathyroid
Non-neurocristopathies	
Goldberg-Shprintzen	Cleft palate, hypotonia, microcephaly, mental retardation
HD with limb anomalies	Polydactyly, brachydactyly or nail hypoplasia with other assorted anomalies
BRESEK	Brain abnormalities, retardation, ectodermal dysplasia, skeletal malformations, Hirschsprung's disease, ear/eye anomalies, kidney dysplasia
Bardet-Biedl	Pigmentary retinopathy, obesity, hypogenitalism, mild mental retardation, postaxial polydactyly
Kaufman-McKusick	Hydrometrocolpos, postaxial polydactyly, congenital heart defect

8.7 Clinical Presentation

Hirschsprung's disease should be considered in any child who has a history of constipation dating back to the newborn period. The median age at which children are diagnosed with HD has progressively decreased over the past decades with greater awareness of the disease. In a survey conducted in 1979 by the Members of the Surgical Section of the AAP, the diagnosis of HD was made in the first month of life in 8% of patients; by 3 months of age the diagnostic rate had risen to 40% [22]. In a nationwide survey from Japan from 1978 to 1982, the diagnosis of HD was made in the first month of life in 48.7% of patients [11]. Recently, the Australian Paediatric Surveillance Unit in a prospective survey from 1997 to 2000 has reported that the diagnosis of HD in the newborn period is made in 90.5% of patients [25]. The neonate with HD is usually a full-term baby [11, 24, 38, 39] and presents with a distended abdomen, feeding intolerance with bilious aspirates or bilious vomiting and classically, with delay in the passage of meconium (Fig. 8.1). In many cases a rectal examination or rectal irrigation causes passage of meconium and relief of acute intestinal obstruction.

Among normal full-term infants, 98% pass meconium in the first 24 hours of life and the remainder will pass their first stool by 48 hours [40]. It has always been said that over 90% of HD infants fail to pass meconium in the first 24 hours of life [1]. However, several authors have found that more than 40% of HD newborns pass meconium in the first 24 hours of life [25, 39]. Thus one should not be dissuaded from carrying out a rectal suction biopsy by the absence of a history of delayed passage of meconium. Diarrhea, fever and abdominal distension in HD are always symptoms of enterocolitis, and this remains the most serious complication of this disease [20]. The reported incidence of enterocolitis ranges from 12% to 58%, and it can be seen before or after a pull-through operation [25, 41–43]. A recent survey has found the incidence of preoperative enterocolitis to be much higher in patients who had the diagnosis of HD established in the postneonatal period, stressing the importance of a prompt diagnosis [25]. A prenatal history suggestive of intestinal obstruction is rare, except in children with total colonic aganglionosis [44]. Occasionally, a diagnosis of HD should be considered in the presence of unexplained perforation of the cecum or appendix, although this is a rare presentation [21, 38, 45]. Some children do not become obstructed in the neonatal period and present later in infancy or in adulthood with severe constipation, chronic abdominal distension and failure to thrive [46, 47]. This is most common among breast-fed infants who may develop constipation around the time of weaning [1]. Rectal examination of patients with HD may show a tight anus [46]; however, some authors think this finding is unreliable [1]. The differential diagnosis for each presentation is shown in Table 8.5. After a careful history and physical examination, the diagnostic steps may include radiographic studies, anorectal manometry and a rectal biopsy.

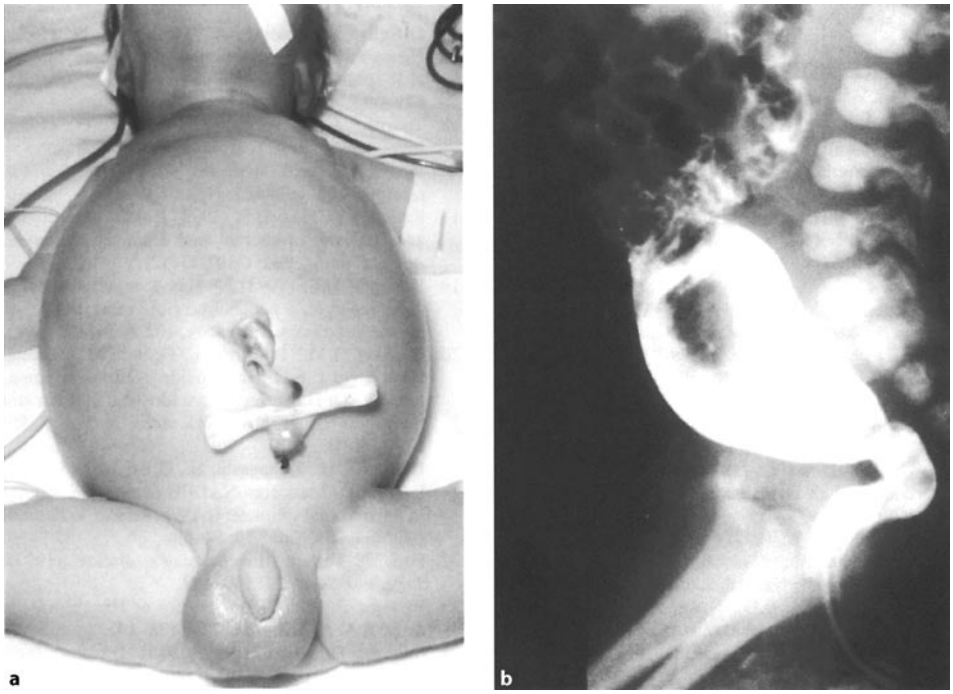

Fig. 8.1 **a** Newborn with Hirschsprung's disease **b** Barium enema in the same infant

Table 8.5 Differential diagnosis of Hirschsprung's disease

Neonatal bowel obstruction	**Meconium ileus resulting from cystic fibrosis**
	Ileal or colonic atresia
	Meconium plug syndrome
	Malrotation
	Congenital band
	Anorectal malformation
	Intestinal motility disorders/pseudo-obstruction
	Necrotizing enterocolitis
	Medical causes: sepsis, electrolyte abnormalities, drugs, hypothyroidism, etc
Chronic constipation	**Functional megacolon**
	Intestinal motility disorders/pseudo-obstruction
	Medical causes: electrolyte abnormalities, drugs, hypothyroidism, etc

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Congenital Anomalies and Genetic Associations in Hirschsprung's Disease

S.W. Moore

9

9.1 Introduction

Although Hirschsprung's disease (HSCR) occurs as an isolated phenotype in at least 70% of cases, a number of associated congenital abnormalities and associated syndromes have been reported. These associations are of significance for at least two reasons. First, the majority may be attributed to abnormal genetic development signaling, yielding clues as to the genetic background of HSCR and its pathogenesis, and second, the influence of associated anomalies on the long-term prognosis.

9.2 Etiology of HSCR

Essentially, HSCR appears to result at a molecular level from disruption of normal signaling during development. As a result, the cues controlling the migration of the neural crest cells go awry resulting in aganglionosis of the distal bowel. The disorder is complex, as is shown by the number of genes implicated in its pathogenesis (at least eight). This is hardly surprising as the signals governing cell migration and development in the embryo are extraordinarily complicated and signaling molecules are notorious for crosstalk and redundancy, as well as having coordinate independent regulation of expression on occasion.

HSCR is therefore characterized as a sex-linked heterogeneous disorder with variable severity and incomplete penetrance [9] giving rise to a variable pattern of inheritance. As a result, dominant, recessive and polygenic patterns are observed.

The genetic influence appears to vary in terms of the length of the affected segment, long-segment HSCR being considered to have an autosomal dominant inheritance pattern with incomplete penetrance (mostly RET), whereas short-segment HSCR appears to be transmitted in an autosomal recessive manner or due to multiplicative effects of a number of involved genes [26]. In addition, several known associated syndromes are also inherited in an autosomal dominant manner.

Known genetic variations have been identified in at least 12% of HSCR patients [2, 16, 26, 29, 132], which is higher than expected in the normal population. In addition, these genetic variations account for more than 50% of the observed abnormalities associated with HSCR. On the other hand, it must also be borne in mind that certain observed associations may not be higher than in the general population and may have little to do with HSCR per se.

The present tendency in the genetic study of a condition is to couple human genetics with genomics to delineate basic methods of development [13]. By looking at associated birth defects of a genetic condition, information can be gleaned on not only how the genes controlling development work but also how they interact and crosstalk by gene–gene interaction whist remaining genetically distinct. The pattern of conditions associated with HSCR have already been of great value in revealing the genetic nature and many of the associations of the disease [34, 173].

9.3 Overview of Associated Anomalies in HSCR

9.3.1 Incidence

Table 9.1 and Fig. 9.1 summarize the collective experience of 18 reported series representing 4,829 individual cases. Only complete personal series were included. Collective series were excluded and in the event of more than one publication from a center, the most representative one was selected. The reported incidence varied between 5% and 32% with a mean of 21.1% [38, 44, 45, 67, 73, 78, 89, 90, 103, 130, 139, 146, 158, 161, 167, 168, 173, 177]. Table 9.2 is a review of Down's syndrome incidence in 5,355 patients with a mean of 7.06% (see Section 9.5.2 Trisomy Chromosome 21).

There are several clear cut associations known or suspected to be related to an increased risk of HSCR which include Down's syndrome [139], dominant sensorineural deafness [192], Waardenburg's syndrome [15, 21, 34, 136, 139, 163, 165], neurofibromatosis [163], neuroblastoma (NB) [139], pheochromocytoma [15, 139, 163], the MEN type IIB syndrome [15, 88] and other abnormalities [139].

9.3.2 Chromosomal Associations of HSCR

Links to specific chromosomes have proved to be of considerable value in the study of HSCR.

9.3.2.1 Chromosome 10 Associations

The segregation analysis of 10q11.2 [116] led to the identification of the RET proto-oncogene and its central role as the major susceptibility gene in HSCR. Since then a number of specific HSCR mutations have been mapped to the RET proto-oncogene, at 10q11.2 [3, 6, 42, 57, 105, 155, 159]. There is now good evidence that RET transduces a signal from the glial cell line-derived neurotrophic factor (*GDNF*) gene [84, 181], which signals through a complex signaling cascade which includes RET and a novel molecule GFRA-1 [84, 181]. Angrist et al. [4] have reported a patient with mutations of both GDNF and RET.

9.3.2.2 Chromosome 13 Associations

Similarly, identification of a deletion at 13q22-32.1 led to identification of the second major susceptibility gene [20, 93, 148, 172] and its association with Waardenburg's and other neurocristopathies. This includes the recessive EDNRB gene, located at 13q22 [6, 30, 148] and much less frequently its ligand endothelin 3 (EDN3) [12]. Other related genes involved in HSCR pathogenesis include the endothelin-converting enzyme 1 (*ECE1*) [72], the sex-dependent Y factor-like homeobox 10 (*SOX10*) gene

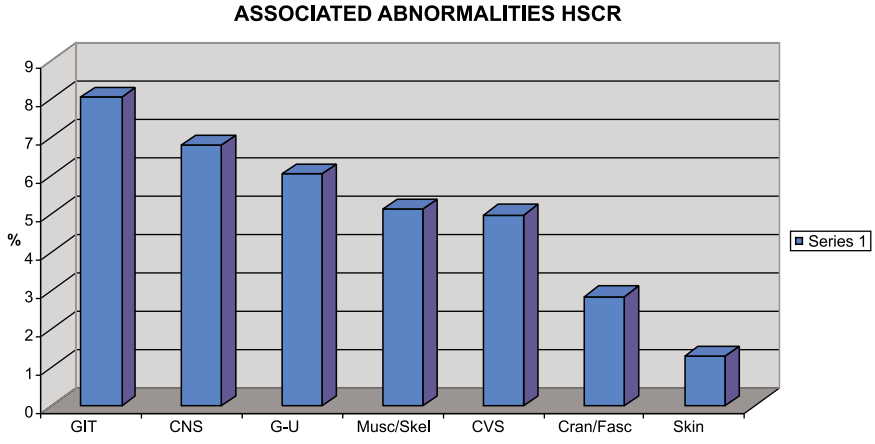

Fig. 9.1 Bar graph showing mean incidence of anomalies associated with HSCR. Based on analysis of 4,366 reported cases in 18 separate series. Only complete series were included, collective series were excluded and in the event of more than one publication from a center, the most representative one was selected

Table 9.1 Congenital anomalies in HSCR

Reference	Year	Location	No. of patients	Associated anomalies (%)
139	1967	USA, Cincinnati	63	11.1
89	1970	USA, Oakland, CA	31	32.2
44	1970	Norway	124	5
173	1985	Canada, Vancouver	178	29.7
146	1986	USA, Ann Arbor	99	26
78	1986	Japan	1628	11.1
167	1990	USA, Detroit	220	19
102	1990	UK, Liverpool	120	20.8
129	1990	South Africa	370	16
177	1990	USA, Chicago	172	26.1
158	1992	USA, Boston	321	22
45	1993	USA, Indianapolis	20	25
90	1993	USA, Detroit	250	18
67	1994	Israel	65	29.2
161	1997	Turkey, Ankara	302	27.4
38	2001	India	35	11.4
73	2003	Germany, Cologne	203	35
168	2003	Australia, Sydney	127	25.9
Total			4366	21.1

[144] and neurturin (*NT*) [40]. SOX8 also appears to be required along with SOX10 to maintain vagal neural crest stem cells [110].

9.3.2.3 Chromosome 21 Associations

The association with 21q22 is derived from the fairly constant clinical association with Down's syndrome and is backed up by other evidence such as the suggestion of a "modifier" susceptibility gene in a Mennonite kindred [148]. Recent work from our laboratory implicates the flanking ITGB2 gene region at 21q22.3, thus implicating the integrin system in HSCR pathogenesis (Zaahl M et al., submitted for publication) (see also Section 9.5.2 Trisomy Chromosome 21)

9.3.2.4 Other Reported Chromosomal Links

Chromosome 2 Associations

Rarer chromosomal associations initially included chromosome 2q37 in association with HSCR and possible homology with the splotch mouse model [53]. Since that time, the SMAD interacting protein 1 gene (SIP-1) at 2q22-23 [187], partial duplication of chromosome 2 [106, 131], and the Mowat-Wilson syndrome with its ZFHX1B mutations and deletions at 2q22-q24 [131], have been associated with HSCR.

Chromosome 9 Associations

Genomic studies [17] re-emphasized the known link to the 9q31 region in sib pairs without significant RET variations. This site has been previously associated with reports of tetrasomy of 9p [122] and the association with Riley-Day familial dysautonomia [8] whose IKB-KAP gene has been linked to 9q31 [169]. In addition, the RMRP gene mutation in the cartilage–hair hypoplasia syndrome relates to a similar area [18].

Chromosome 22 Associations

Interest in chromosome 22 was first raised by Beedgen et al. [11]. Additional associations include the cat-eye syn-

Table 9.2 Down's syndrome associated with HSCR

Reference	Year	Location	No. of patients	Down's syndrome (%)
14	1963	UK, London	220	1.47
139	1967	USA, Cincinnati	63	9.5
89	1970	USA, Oakland, CA	31	16.1
61	1984	USA, Baltimore	33	9
56	1985	USA, Pittsburgh	263	5.9
173	1985	Canada, Vancouver	178	2.8
25	1990	USA, Columbus, OH	80	2.8
125	1990	Sweden, Stockholm	90	2.9
78	1986	Japan	1628	15.5
103	1990	UK, Liverpool	880	4.2
177	1990	USA, Chicago	172	3.2
130	1991	South Africa	370	3.19
158	1992	USA, Boston	321	8.4
157	1994	Denmark	224	2.24
149	1994	Republic of Ireland, Dublin	135	12.59
161	1997	Turkey, Ankara	302	12.5
38	2001	India	35	5.71
73	2003	Germany, Cologne	203	6
168	2003	Australia	127	10.2
Total			5355	7.06

drome associated with trisomy 22pter-q11 [109] and the Di-George velocardiofacial syndrome at del22q11 [87], both of which have been associated with HSCR.

Other Isolated Chromosomal Associations

Additional reports include deletion of 20p [185], 18p monosomy and 18q trisomy [146], and XO/XX/XXX mosaicism [70].

Chromosome 5

More recently, a mutation in the significant cluster region (MCR) of the *APC* gene (E1317Q) has been identified in 1 out of 40 HSCR patients investigated in our unit. This patient had an additional exon 3 V202M *RET* mutation (Zaahl M, unpublished data). This association with chromosome 5 is of considerable interest because the gene for GFRalpha-3 has been mapped to the human chromosome 5q31.1-q31.3 region along with several disease loci, growth factor and growth factor receptor genes [117].

These isolated events are of uncertain significance as yet but may indicate the multiplicative effect of gene–gene interaction.

9.4 Gene-related Associations of HSCR

9.4.1 The Significance of Observed Genetic Variations

It is now clear that many of the genetic variations associated with HSCR relate to the main susceptibility genes and the cluster of related genetic abnormalities associated with them.

Mutations of susceptibility genes have been shown to account for up to 50% of familial cases and possibly as many as 20–30% of sporadic cases [171]. It would appear that the accumulation of mutations/variations in these critical genes contributes towards the resultant Hirschsprung's phenotype. In the light of recent research, we are now in a position to identify many of the relevant genetic links in many of these associations and raise questions as to possible signaling pathways involved in many of the remainder.

There are a number of reported associations and neurocristopathies associated with syndromes, some of which have autosomal dominant inheritance. In many cases, the underlying genetic link is already known or suspected. It seems logical to deal with the known genetic associations giving rise to neurocristopathies and the less frequently associated syndromes on a separate basis.

Interestingly, associated anomalies are not frequently associated with familial transmission. In our study of 407 patients [23, 130], the associated physical anomalies were less frequent in the familial than in sporadic HSCR (9.6% familial vs 16.7% sporadic HSCR). This suggests a possible different pattern of inheritance and/or the involvement of signaling mechanisms possibly outside of the known susceptibility genes [126, 130]

9.4.2 Gene–Gene Interaction and the Role of Flanking Genes

It is now well recognized that whereas major RET mutations may give rise to HSCR by haploinsufficiency, the majority of HSCR arises from the multiplicative combined effects of other susceptibility genes and mutations leading to disturbed signaling pathways [2]. As RET mutations per se are probably only responsible for 20–25% of HSCR, this suggests that in the majority of those with sporadic HSCR, the condition results from the combined cumulative effects of the susceptibility loci at critical genes that control the mechanisms of cell proliferation, differentiation and maturation [119]. It is important to note that the majority of the chromosomal sites outside the major susceptibility genes have been identified in patients without major RET mutations. This has led to the hypothesis that whereas major RET mutations may give rise to the condition by haploinsufficiency, lesser mutations require the multiplicative effects of other disturbed signaling pathways [2].

The early timing of the effects of major susceptibility genes (RET and EDNRB) on intestinal neuroblast development [6], suggests some overlap in function [119] between these particular genes. This is supported by our own work [128] but may be further influenced by the interaction of other mutated susceptibility genes [26].

Genome-wide association study as well as the study of mouse models has assisted the identification of possible interaction sites between signaling pathways in HSCR [26, 119]. Bolk-Gabriel et al. [17] identified at least three critical genetic regions in 106 non-syndromic HSCR patients studied by means of a genome-wide linkage scan (covering more than 92% of the human genome). These included chromosome 10 (RET), chromosome 13 (EDNRB) and chromosome 9 (see below). All three regions seem to be involved and necessary in terms of a multiplicative effect model [140]. This work suggests among other things that major susceptibility genes can interact with each other. Statistically significant joint transmission of RET and EDNRB alleles have been reported to result in interaction between RET and EDNRB pathways in HSCR [5, 26] and this is supported by evidence from mouse models. Both pathways are critical to the normal development of enteric nerve cells and although their signaling cascades appear tissue-specific during development, these findings lend support to the hypothesis that genetic interaction between mutations in RET and EDNRB is an underlying mechanism in HSCR. There would have to be some degree of incomplete penetrance as well as significant phenotypic variation for this to be true and other modifier genes would probably influence the penetrance of the phenotype.

The associations with chromosomal abnormalities and congenital anomalies strengthen the hypothesis of a multiplicative model, implicating the involvement of a larger number of genes, each with a small cumulative effect [9]. The relatively constant association of Down's syndrome (2–15%) [9, 24, 56, 61, 63, 78, 127, 139, 149, 173] with HSCR disease, for instance, is important as it indicates a probable modifying site on chromosome 21 [139, 148]. Although known chromosomal susceptibility genes appear the most important, study of these extra chromosomal sites becomes important in any attempts to understand the interlinking signaling pathways giving rise to congenital aganglionosis.

9.4.3 The Effect of Flanking Regions and Genes on Phenotype

Many of the syndromic features of HSCR may involve flanking genes 10^3–10^4 base pairs either side of the terminal exons of a gene, and these may be involved in the final phenotypic expression. The problem of flanking genes is well known in animal experiments, especially with knockout mice [59]. These do not necessarily need to be large mutations, but fairly short mutations which contain regulatory or promoter sequences may result in a genetic frameshift and affect DNA replication.

9.5 Significant Clinical Associations of HSCR

9.5.1 Neurocristopathies Associated with HSCR

It is clear that some of the syndromic expression of HSCR represents neurocristopathies. They are mentioned here for completeness, but are discussed fully in Chapter 18.

Mutations in the RET proto-oncogene give rise to specific related phenotypes which include neurocristopathies, MEN syndromes and ganglioneuromatosis RET-related neurocristopathies.

Neurocristopathies associated with the endothelin system are mostly related to the EDNRB gene and Sox10 and include the following:

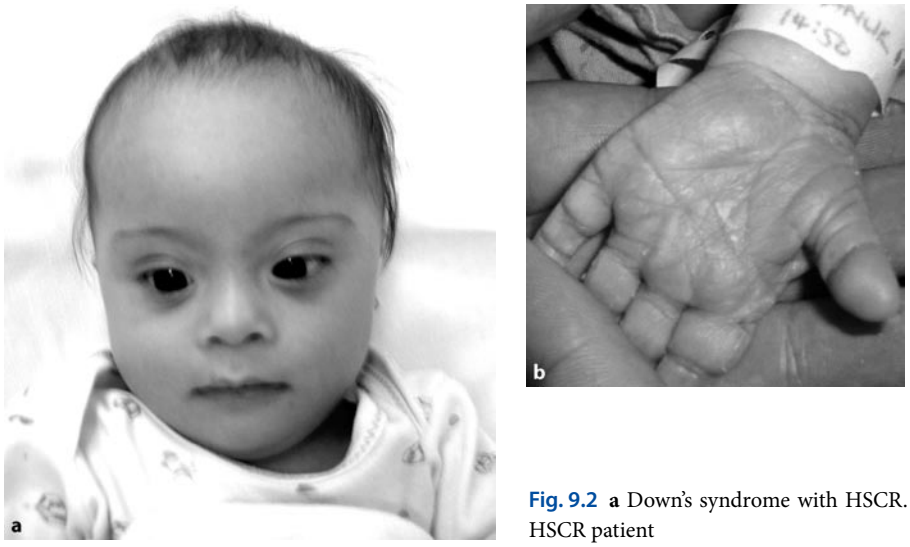

Fig. 9.2 **a** Down's syndrome with HSCR. **b** Simian crease in a Down's-HSCR patient

1. Long-segment Hirschsprung's disease in the Waardenburg-Shah syndrome
2. Congenital hypomyelinating neuropathy, central dysmyelination, and Waardenburg-Hirschsprung disease (phenotypes linked by SOX10 mutation)
3. Waardenburg-Shah (type 1V WS)
4. Certain other forms of sensorineural deafness

Finally, congenital central hypoventilation syndrome (CCHS) may be associated with HSCR.

9.5.2 Trisomy Chromosome 21 (Down's syndrome)

9.5.2.1 Incidence of HSCR-Down's

The association between Down's syndrome (trisomy 21) and HSCR (Down's-HSCR) remains one of the few consistent associations to emerge from any epidemiological study of HSCR and has been confirmed on segregation analysis [9]. Table 9.2 summarizes the reported incidence in 19 reported studies representing a collective experience of 5,355 HSCR patients [14, 24, 38, 56, 61, 73, 78, 89, 101, 125, 127, 130, 139, 149, 157, 158, 161, 168, 173, 177]. The reported incidence among these 5,355 patients ranges from 0.6% to 16.1% with a mean of 7% of reported patients.

Further comparison of the relative incidences of the two conditions, HSCR and Down's syndrome (viz. 1:5,000 and 1:600), indicates that the incidence of HSCR in Down's syndrome is of the order of one patient for every 200–300 Down's syndrome patients [136] which is considerably higher than the expected population incidence of 0.15–0.17.

9.5.2.2 Clinical Associations of Trisomy 21

Apart from its linkage with HSCR and the Down's phenotype (Fig. 9.2), other associations of trisomy 21 with congenital anomalies such as cardiac, anorectal malformations [32] and other atresias, raises interesting questions. In our series [130], none of 3.2% with Down's syndrome had a family history, all had short-segment aganglionosis and there was a male preponderance. Associated abnormalities were present in 85%, six (46%) of whom were cardiac, and three had multiple abnormalities. More than half of our Down's patients died, which is much higher than patients without trisomy 21.The associated high mortality in Down's patients can be attributed to the high incidence of immune disorders and congenital heart disease, but is also associated with a number of gastrointestinal abnormalities and/or enterocolitis.

9.5.2.3 Genetic Associations of Trisomy 21

The observed non-random association of RET and chromosome 21 in the EDNRB-linked Mennonite kindreds suggests a multiplicative form of inheritance [148] demonstrating a mechanism whereby the cumulative effects of multiple mutations appear to represent a likely mechanism in HSCR pathogenesis. In addition to aganglionosis, hypoganglionosis has been demonstrated in an animal model [98] adding further support to the view that chromosome 21 contains an important modifying susceptibility gene on chromosome 21q22 [148]. Although there is sufficient evidence to support this hypothesis, the effect of the extra 21 chromosome on the development of the gastrointestinal tract cannot be ruled out. The latter consideration receives some support from a

hypoganglionic animal model investigated by Leffler et al. [98] as well as reports of an increase in cellular adhesion in Down's syndrome enteric nervous system (ENS), resulting in the prevention of complete caudal ganglion cell migration [193]. It must be noted that the integrin system plays a pivotal role in the cellular adhesive process, particularly in leukocytes and that the identified genetic variations at the ITGB2 site on chromosome 21 may be of considerable significance.

HSCR-Down's does not appear to have major associations with the major susceptibility genes although our own studies have shown the T-allele of the 561C/T polymorphism is over-represented in the HSCR-Down's syndrome patient group (35%) compared to normal controls (6%). The high frequency of this allele in the HSCR-Down's syndrome patient group ($p<0.002$, χ^2 with Yates' correction = 12.14) suggests that variant 561C/T is associated with a low penetrance effect in patients with this complex phenotype [198].

Of particular interest to any research on chromosome 21 in HSCR are alterations in the 21q22 band (the so-called Down's syndrome region of chromosome 21). A partial deletion of the distal part of 21q (21q22.3) has been reported in three unusual cases of phenotypic Down's syndrome, suggesting that this region is not necessary for the pathogenesis of the observed features of Down's syndrome in these patients (facial and hand features, muscular hypotonia, Fallot's tetralogy and some mental retardation) [138]. The 21q22.3 region is the source of a number of proteins coded by chromosome 21 which are important in the development of the nervous and immune systems. These include SOD-1, the amyloid precursor protein (App), protein S-100 beta, and the ITGB2 (CD18) gene, which is responsible for the beta chain of the lymphocyte function-associated antigen (LFA-1). Overexpression of these molecules may contribute to the abnormal development of the immune and nervous systems.

The initial connection of ITGB2 with HSCR was based on individual case reports where leukocyte-adhesion deficiency with ITGB2-defective expression mimicked HSCR [154]. Beta 2-integrins (including ITGB2) and their ligands, the intercellular adhesion molecules, play an important role in adhesion and development [55]. In the fetus, ITGB2 is a surface cell-adhesion molecule (CAM) involved in neural cell migration (along with CD11a, 11b and 11c, and CD62L [141, 174]), and probably works through the pathways dependent on P13K and tyrosine kinases [184]. Our study of this region has shown a high incidence of variations in HSCR with 75% of patients with sporadic HSCR and 33% of those with HSCR-Down's syndrome without associated enterocolitis/cardiac lesions making it an interesting subject for future research.

9.5.3 Congenital Associations of HSCR

HSCR may be associated with brain anomalies, mental retardation and growth, ectodermal dysplasia, skeletal malformations, ear deformity and deafness, eye hypoplasia, and craniofacial and genitourinary abnormalities. A collective review of 4,366 reported cases shows that apart from Down's syndrome, the relative incidence of these anomalies is of the order of 21%. The incidence of individual groups of anomalies varies from 2.97% to 8% (Fig. 9.2), the most frequent being related to the gastrointestinal tract (8.05%), followed by the central nervous system and sensorineural anomalies (6.79%), These are followed closely by anomalies of the genitourinary tract (6.05%), and musculoskeletal (5.12%) and cardiovascular system (4.99%). Although craniofacial and eye abnormalities appear important, they represent only 3%. The skin and integumentary system is a further uncommon association.

The associated anomalies are discussed in the following sections.

9.5.3.1 Gastrointestinal Tract Anomalies

Signaling pathways and disruption of gene expression have been implicated in a number of gastrointestinal conditions which include HSCR, malrotation, anorectal anomalies, pyloric stenosis, Meckel diverticulum, biliary atresia as well as pancreatic agenesis and heterotopia among others. It stands to reason that similar signaling pathways may underlie these congenitally acquired conditions.

Intestinal Malrotation

The association of Hirschsprung's disease and intestinal malrotation appears uncommon [39] although at least 28 infant cases have been reported [91] as well as a number of additional reports [35, 82] and there were 5 additional cases in our series [126, 130] giving a total of at least 37 cases. A case of malrotation and midgut volvulus has been described in association with HSCR [82]. There probably is some measure of under-reporting of these anomalies, although it must be borne in mind that many of the associated gastrointestinal anomalies in HSCR may correspond to the incidence in the general population.

A molecular basis for malrotation has been suggested from animal models where hedgehog signaling cascades have been associated with both malrotation and features of aganglionic colon [151] in mice. The reported association of malrotation and anorectal malformations [145] where the hedgehog signaling systems are known to be affected, strengthens this association.

Anorectal malformations

It is generally accepted that the association between HSCR and anorectal malformations is uncommon [52] but that anal stenosis may be under-reported. In one large collective series, anorectal malformations accounted for 2.5% of more than 1,200 cases [120]. It has been described in nine cases from one center over a 10-year period [189] and has been recorded in two siblings of consanguineous parents [178] as well as in association with trisomy 21 [32]. In our series we encountered only 1 out of 408 cases [126, 130].

Anorectal malformation has been reported in association with malrotation [145], and in the Pallister-Hall syndrome [69], Currarino's syndrome [10] and Down's syndrome [32, 50]. It may lead to diagnostic delay of HSCR because of the initial diagnosis of the anorectal malformation and the fact that the defunctioning colostomy is proximal to the affected bowel.

IntestinalAtresia

Intestinal atresia is an infrequent association with HSCR occurring in 32 previously reported cases. A number of reported series include a higher than the estimated population incidence (1:2,700, 0.04%) [51, 78, 126, 130, 156]. In a national review of 1,628 patients with HSCR, Ikeda and Goto [78] recorded 4 patients with associated intestinal atresia, an incidence of 0.25%. Of those reported, 22 were small-bowel atresia and 8 affected the colon. A further 32 patients with small intestinal atresia and 26 with colonic atresia have been reported.

Intestinal atresia occurs especially in association with long-segment HSCR [37, 58] and in unfixed colon [51]. The danger of a missed diagnosis has been stressed [81] as patients are generally treated for the atresia and the possibility of associated HSCR may be missed until recurrent obstruction or anastomotic dehiscence occurs.

The most plausible explanation for the association between HSCR and intestinal atresia is the tendency of a malrotated, obstructed segment of bowel proximal to the aganglionic segment to undergo volvulus. The debate centers on the role of additional localized abnormalities (e.g. duplication cyst) resulting in intestinal volvulus and atresia [129] and the role of HSCR per se.

9.5.3.2 CNS and Brain Anomalies

A number of reports include the cosegregation of HSCR with mental retardation and various dysmorphic features which include absence of the corpus callosum which may be an isolated feature or in association with the Goldberg-Shprintzen syndrome [166] and other syndromes. Autopsies on 12 consecutive anencephalic newborns showed some degree of aganglionosis in all [118].

The incidence of CNS abnormalities varies but has been reported to be as high as 29% in one large series [161], but was a mean of 8.3% in our overview of 4,366 patients (Table 9.1).

This is not surprising, as brain development is largely controlled by the same neural growth factors as the ENS. Neocortical development arises from progenitor cells (mostly composed of radial glial cells [180]) undergoing neurogenesis under the influence of cell cycle signaling molecules. It is interesting to note that in the mouse model, neurogenesis of the cortex begins on day E11 [179], also a critical time in mouse models of HSCR.

Hydrocephalus and HSCR has been reported in a patient with a mutation of L1CAM [135] and Dandy Walker abnormalities have been described [122, 158, 195]. Meningomyelocele was identified in our series [129] in keeping with previous reports [123]. This is an uncommon association and myelomeningoceles were absent from a number of large series [78, 167]. The association with myelomeningocele may therefore be a chance occurrence, but evidence of one further patient with spina bifida occulta in our series and two cousins of patients with HSCR known to have a myelomeningocele [62] raises speculation as to the part played by the migration of nerve cells from the neural crest cells at the sacral level as a second source of neural precursor cells in the innervation of the hindgut.

9.5.3.3 Genitourinary Abnormalities

Congenital genitourinary anomalies described in association with HSCR include hypospadias as well as undescended testes, congenital kidney anomalies, ureteric duplications, hydronephrosis/hydroureter, and disorders of bladder function [85]. A patient with ambiguous genitalia reported in our series [23, 130] would fit with the reported lack of mullerian inhibiting substance reported by Cass and Hutson [28].

The reported incidence of genitourinary anomalies varies considerably with some reports suggesting a fairly high incidence [44, 49, 161, 176] and others a much lower incidence (2–3%) [2, 127, 130]. The division between congenital anomalies (3%) and functional disturbances [85] seems a reasonable compromise as some may be of a secondary nature due to obstruction of the lower ureters and neck of the bladder.

The association with renal anomalies is of particular interest as these are commonly identified in the ret knockout mouse model [199]. Amiel and Lyonnet [2] have reported a 4.4% renal agenesis plus a further 2–3% incidence of genital anomalies including hypospadias pointing out that there may be some measure of under-reporting.

Certain associated syndromes (e.g. McKusick-Kaufman syndrome [74]) include numerous genitourinary anomalies, which may include bilateral hydronephrosis, hypospadias and prominent scrotal raphe.

9.5.3.4 Skeletal, Muscle and Limb Anomalies

Skeletal, muscle, limb and digital anomalies, represent approximately 4.6%, but the incidence has been reported to be as high as 24% [161]. Skeletal abnormalities include sacral agenesis as well as extremity defects [85].

Muscular anomalies associated with HSCR include muscular dystrophy [114]. In addition, Fryn's syndrome with diaphragmatic hernias [1] and distal limb anomalies [1] may be associated with HSCR.

Distal Limb abnormalities are largely represented by polydactyly and limb hypoplasia but may also be associated with congenital deafness [160] or cardiac anomalies [76, 96]. Polydactyly has been described as part of a syndrome of heart defects, laryngeal anomalies and HSCR [76, 83] and other autosomal recessive syndromes in siblings [160]. Werner mesomelic dysplasia with polydactyly has been associated with HSCR [60], as is short stature [97] and the BRESHEK syndrome [153]. Osteopetrosis has been reported in seven children of consanguineous marriages [41].

9.5.3.5 Cardiac Abnormalities

The incidence of cardiovascular anomalies varies between 2.1% and 8.4% although our survey puts it at a mean of 3.2%, which exceeds the 0.1–0.5% in the normal population. Although a number of the cardiovascular system anomalies have been found in Down's-HSCR patients in our series, they are also present in non-Down's HSCR if slightly less frequently (4.8% Down's syndrome vs 0.3%) [126, 130].

Septation defects (atrioseptal and ventriculoseptal defects) [133] and conotruncal developmental defects [2] appear to be the most frequent. This is understandable as the critical stage of cardiac development occurs at more or less the same time and is dependant on neural crest cell proliferation which then in turn links them to the neurocristopathies. Neural crest cells originating from a specific hindbrain region are essential for the normal development of the cardiac outflow tract and aortopulmonary septum, which is closely related to the cells which proliferate into the primitive gut to form the enteric ganglia [120]. Syndromes which include polydactyly, HSCR and cardiac anomalies [96] as well as the McKusick-Kaufman syndrome [74, 77, 104] probably represent similar underlying genetic mechanisms.

9.5.3.6 Craniofacial Anomalies

Unusual facial appearances such as narrow palpebral fissures, broad nose base, cranial anomalies and developmental delay are not that uncommon in association with HSCR. This may also include cleft palate as well as certain cases as in the Goldberg-Shprintzen syndrome [196]. Cleft palate per se was identified in 1.1% of the series by Spouge and Baird [173] and 0.6% in a large national study by Ikeda and Goto [78]. The Pierre-Robin syndrome has been reported in association with HSCR [45] and there is one further report of a patient with Hanhart's syndrome [146] which combines micrognathia with other craniofacial and distal limb anomalies.

The association of HSCR with craniofacial anomalies is of interest for a number of reasons. Morphogenesis of the craniofacial region is linked to neural crest development and involves common factors such as growth factor signaling [47, 197]. The neural crest supplies the membranous bones of the face during development and appears to exert some measure of control over craniofacial development. In our series, a number were related to the development of the eye which occurred in 9 of 408 (2.2%), and included 3 patients with micro-/anophthalmos and two with congenital ptosis [126]. In addition, certain of the craniofacial anomalies display characteristics of the DiGeorge syndrome [87], the most frequently encountered microdeletion syndrome in humans characterized by cardiovascular, thymic, parathyroid, and craniofacial anomalies.

We now know that the SMAD binding protein 1 gene (SMADIP1, MIM 605802), previously linked to HSCR [187], has been recently identified as a disease-causing gene in a polytopic embryonic defect (MIM 235730) including midline anomalies, facial dysmorphic features and ENS malformation (HSCR) [46]. Other craniofacial syndromes such as the Aarskog syndrome-faciogenital dysplasia have been linked to FDG1 gene [137]. One patient with Jeune asphyxiating frontonasal dysplasia has been reported [7].

Ophthalmic Anomalies

Variable expression of ophthalmological findings have been reported in association with HSCR and are probably the result of maldevelopment of neural crest cells from adjacent areas on the prosencephalon [120]. In our series, they occurred in 9 out of 408 patients studied (2.2%), and included three patients with micro-/anophthalmos (Fig. 9.3) and two with congenital ptosis [130]. Other ophthalmic anomalies include a number of reports of coloboma [64, 66, 77, 134].

Abnormalities of the eye and autonomic nervous system are also frequent in CCHS especially when associated with HSCR [36]. Ophthalmic anomalies may also be found in those with auriculovertebral syndromes (e.g. Goldenhar syndrome) [94]. Ptosis has also been previously reported [196].

This association is not surprising as the development of the eye precedes migration of nerve cell precursors from the craniocervical portion of the embryonic neural tube, and sympathoblasts from somites of the developing hindbrain [78, 121] contribute to both the superior cervi-

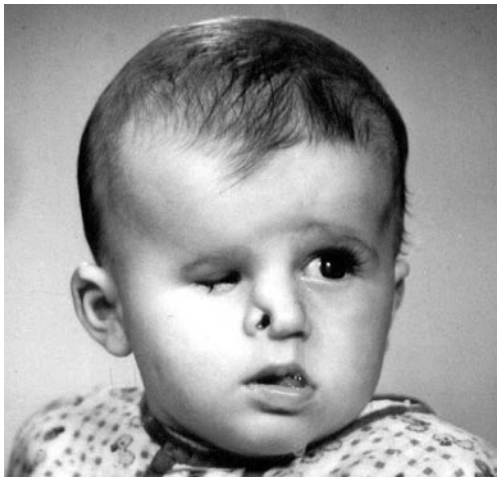

Fig. 9.3 Anophthalmos, craniofacial anomalies and HSCR

cal ganglion as well as ganglion cells of the distal colon [120, 121].

Coloboma of the iris has been reported with HSCR a number of times [64, 66, 77, 99, 134, 162, 164]. Coloboma and the possibly related aniridia trait is often genetically linked, having been mapped to the short arm of chromosome 2 [48]. It arises as a result of the failure of the optic fissure during the 6th week of embryonic life. It is mostly transmitted in an autosomal dominant fashion [99] especially in the renal-coloboma syndrome associated with Pax 2 [162].

In addition to the known association with chromosome 13 deletions [95, 164], the iris is involved in the Waardenburg-Shah association [43, 165]. More recent reports linking frame-shift and missense SOX10 mutations have been shown to result in the premature termination of the translation process of SOX10 protein generation (a key regulator of peripheral glial cell differentiation) [19, 31, 79]. This then gives rise to truncated SOX10-derived proteins which create the phenotype (PCWH in severe cases and Waardenburg-Shah in a milder form) [79]. Other reported genetic links of colobomas in HSCR include a 2p22 genetic variation [190] and the ZFHX1B gene [64].

Reported HSCR-coloboma associated clinical syndromes include the Goldberg-Shprintzen syndrome (HSCR, coloboma iris and microcephaly) [22, 54, 166, 196], and the Rubenstein-Taybi syndrome (callosal agenesis, iris coloboma and megacolon) [66]. Reports of "cat eye" syndrome with HSCR suggests a link with chromosome 22 as the chances of the two conditions occurring simultaneously has been estimated at 1 in 25 million [109].

9.5.3.7 Skin and Integumentary System

Pigmentary disturbances are anticipated in association with HSCR due to the established critical role of the endothelin system and its association with the Waardenburg's syndrome [71] and the development of melanocytes in EDNRB knockout mice [75]. The hypopigmentation and white forelock of WS4 is also encountered in the ABCD syndrome (albinism, black lock, cell migration disorder) [65], being ascribed to a homozygous mutation in exon 3 of the EDNRB gene [186]. Other possible links of pigmentary disturbances such as the Yemenite deaf-blind hypopigmentation syndrome have been shown to be an associated SOX10 mutation [65]. Other pigmentary disturbances with similar associations include the black locks albinism [108], and familial piebaldism [192].

In approximately 10% of patients with cartilage hair hypoplasia syndrome there is an association with HSCR [111, 112]. The cartilage hair hypoplasia syndrome has in turn been attributed to an RMRP gene mutation [18] which has been mapped to chromosome 9p13 [175], once again underlining the possible importance of this region.

A number of other skin-related syndromes have been associated with HSCR [113]. In keeping with other reports of the KID (keratitis, ichthyosis and deafness) syndrome [113] we have reported one patient with familial ichthyosis associated with HSCR [130]. Although the exact genetic links with ichthyosis are unknown, two recent publications indicate Xp22.3 [100] and 2q35 [86], both of which are close to areas with known HSCR connections.

9.6 Other Less Common Associations with HSCR

9.6.1 Syndromes Related to Cholesterol and Fat Metabolism

HSCR has been reported in severely affected patients with the Smith-Lemli-Opitz syndrome [27, 142, 150], a disturbance in cholesterol metabolism due to the 7-dehydrogenase cholesterol reductase gene located at 11q12-q13 [188].

HSCR has been described in association with Bardet-Biedl non-syndromic obesity (BBS) [80, 104, 107]. The related McKusick-Kaufman syndrome [74] has a 10% HSCR incidence [77]. Congenital heart defects are part of the syndrome and in one report HSCR was associated with situs inversus [104]. Although BBS is related to a number of chromosomal anomalies including 2q, 3p, 11q, 15q and 16q, mutational overlap between these two conditions has been described [170].

9.6.2 Tumors Associated with HSCR

Neurocristopathies are associated with a number of clinical phenotypes, which include a variety of tumors of neural crest origin resulting from oncogene upregulation and tumor suppressor gene inactivation. These include inherited familial predisposition to tumors which include

medullary carcinoma of the thyroid [132], pheochromocytoma [194] and other tumors related to the MEN2 phenotype. These are discussed in Chapter 18.

In addition, HSCR is associated with tumors of neural origin which include neuroblastomas (NB) [124], ganglioneuromas/ganglioneuroblastomas [68] and retinoblastoma [172, 191]. HSCR is also associated with neurofibromatosis and other autonomic nervous system disturbances [33] which may be related to neural tumors.

NBs, along with HSCR, are considered abnormalities of cell development and control. Genetic linkage is suggested because of the uncommon autosomally dominant familial inheritance in NB [152] as well as associations with other genetically linked conditions such as HSCR and CCHS [182]. The major HSCR susceptibility genes (RET and EDNRB) do not appear to be involved in familial NB [115]. Having said this, there are suggestions that certain retinoblastomas may be related to chromosome 13 [164, 172, 191]. Reported cases include ganglioneuroma/ganglioneuroblastoma occurring in the mother with NB being identified in the child [68].

The PHOX2B gene is a candidate for this association as it appears to be the major susceptibility gene in CCHS, as well as being associated with familial NB [182] and HSCR-NB associations [33]. PHOX2B has subsequently been suggested to predict tumor risk in familial NB [183]. Further study of non-familial cases has not confirmed PHOX2B mutations as being a major locus for NB but have been suggested as representing second-site modifications responsible for a specific phenotype [143].

In addition to these known tumor associations, chromosome 10q loss of heterozygosity (including the RET site) has been described in early-stage chondrosarcomas [152] and T-cell lymphoma [92]. Other tumor associations include certain carcinomas which occur years later [50]. These may, however, be unrelated to HSCR per se but represent the normal population incidence.

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Enterocolitis Complicating Hirschsprung's Disease

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10

10.1 Introduction

Enterocolitis is a clinical condition with symptoms including diarrhea, abdominal distension, pyrexia, colicky abdominal pain, lethargy and the passage of blood-stained stools [1]. Enterocolitis is a significant complication of Hirschsprung's disease (HD) both in the pre- and postoperative period [2]. Hirschsprung's-associated enterocolitis (HAEC) can occur at any time from the neonatal period onwards into adulthood and can be independent of the medical management and surgical procedure performed. Recurrent enterocolitis can occur even in the presence of a diverting colostomy which is termed "diversion enterocolitis" [3–5].

The incidence of enterocolitis ranges from 20% to 58% (Table 10.1) [6–13]. The incidence in the preoperative period is 16% and in the postoperative period is 18% [1]. Fortunately, the mortality rate has declined over the last 30 years from 30% to 1% [11, 13–15]. Results from Japan demonstrate a decline in mortality from 1978 to 1998 from 6.5% to 0.7% [13]. This decrease in mortality is related to earlier diagnosis of HD and enterocolitis, rectal decompression, appropriate vigorous resuscitation and antibiotic therapy.

Although the concept of HAEC was alluded to in the literature in 1950 by Burnard [16], Fisher and Swenson [17] in 1956 and Dorman [18] in 1957, it was not until 1962 that Bill and Chapman [19] presented the first definitive description of the condition.

10.2 Pathogenesis

Despite multiple investigations and studies, a complete understanding of the etiology of HAEC is still unavailable. Numerous theories have been put forward to explain its occurrence including a physical dilatation of the proximal bowel, variations in the mucin components and production, rotavirus, *Clostridium difficile*, increased prostaglandin E1 activity, mucosal immunity defects, a Schwartzman-type reaction, disordered motility associated with protein sensitization and sucrase-isomaltase deficiency. Other histological and immunological studies indicate that some patients are prone to recurrent HAEC

Table 10.1 Incidence of HAEC

Reference	Incidence (%)	Mortality (%)
6	38	0
7	35	3
8	34	0
1	32	4
9	30	10
10	27	4
3	25	3
12	23	1
13	17	0.7

due to persistent inflammation within the bowel, or an immune deficiency either local or systemic with defective white cell function [20–22].

10.3 Theories of Pathogenesis

10.3.1 Mechanical Obstruction

Bill and Chapman [19] argued in 1962 that partial mechanical obstruction was involved in the pathogenesis of HAEC causing mechanical dilatation of the proximal bowel leading to fecal loading and stasis resulting in further dilatation and thus mucosal ischemia and bacterial invasion which was cured by colostomy [19]. This suggests that enterocolitis only occurs in dilated ganglionic proximal bowel. However, this theory does not explain the enterocolitis that occurs in distal colon with a defunctioning proximal stoma, the occurrence of enterocolitis in postoperative patients or histological evidence of enterocolitis in aganglionic bowel [12, 13]. In discussing the theory of Bill and Chapman it is important to note that the length of the aganglionic segment has been identified as a possible risk factor for HAEC. Studies including our own have shown that longer segments of aganglionosis have a higher risk of HAEC [8, 9, 23]. It is postulated that the increased length of aganglionic bowel implies a greater proximal obstruction with greater intraluminal pressure, increased bacterial stasis and proximal dilation. However, other studies on this condition have shown no difference as regards length of the aganglionic bowel [11, 19, 24].

10.3.2 Sucrase Deficiency

In 1973 Ament and Bill [25] presented the case of a 6-year-old boy with chronic enterocolitis following surgery for HD. Clinical investigations revealed the presence of a sucrase-isomaltase deficiency, and the child recovered on a low sucrose diet. This led to the postulation that nonobstructed HAEC is caused by an inborn error of metabolism. Its is important to note that this has not been replicated and that Ament and Bill acknowledged that the boy was an Eskimo, and that 10% of Greenland Eskimos are sucrose-intolerant [25].

10.3.3 Shwartzman Reaction

Berry and Frazer [26] in 1968 suggested that HAEC is initiated by a sensitivity reaction similar to a Shwartzman reaction caused by intraluminal organisms invading the submucosa [26]. They injected endotoxin directly into the exteriorized rabbit bowel proximal to an obstruction and produced enterocolitis in six of nine animals.

10.3.4 Prostaglandins

A single case was reported by Lloyd-Still and Demers [27] of HAEC with fulminant unresponsive diarrhea which revealed high PgE1 levels [27]. In response to cholestyramine a 12-fold decrease in prostaglandin E (PgE) levels in the colostomy fluid was detected. It was postulated that increased PgE activity, enterotoxin, and bile acid malabsorption may be involved in HAEC [27].

10.3.5 Defective White Cell Function

In 1988 Wilson-Storey et al. [22] postulated that defective white cell function may be a predisposing factor for HAEC [22]. White cell counts were analyzed in nine patients with HD of whom five developed HAEC and ten age-matched controls. Their data showed a statistically significant difference between the neutrophil count (2.0, 3.6, 8.6×10^9/l) in those with HAEC, HD and controls, respectively. This relative neutropenia worsened in three patients during and after an episode of HAEC. Wilson-Storey et al. also postulated that white cells in HAEC patients are "sluggish" in response to the inflammation.

10.3.6 Immature Mucosa

Blood group-associated antigen Le^b is normally present in fetal colon and absent in a normal ganglionated bowel [28]. Fujimoto and Miyano demonstrated strong expression of Le^b which was uniformly present along the entire length of the crypts of the aganglionic bowel [20]. This expression in aganglionic bowel could indicate a proliferation of the immature crypt cells, or that the colonic mucosa has not matured and hence the mucosa persists in a fetal stage. Thus it is postulated that there is an underlying abnormality of the epithelium lining found in HAEC which may be causative rather than related to the effect.

10.3.7 Mucin

Other theories focus further on the role of increased and altered intestinal mucin/mucus. Clinically the voluminous amount of mucus produced during HAEC is quite obvious and dramatic. Needless to say this has led to speculation that the mucus is a pathogenetic factor in this condition. The preepithelial mucus or mucin consists of glycoproteins and secretory immunoglobulins (IgA) and acts as the first line of defense by binding and inactivating organisms. In the normal bowel most of the mucin is silated or sulfated, and thus there is relatively little neutral mucin present. The neutral mucin is present in the upper half of the crypts and the acid mucin in the

lower [20]. The colonic mucin is kept in a stable ratio by the rapid removal of epithelial cells in the crypts and the routine desulfation of the mucin by the bacteria [29].

In 1981 Akkary et al. performed rectal biopsies in ten patients with HD after formation of a colostomy and in six controls with normally ganglionated bowel, and reported abnormal mucin composition in the patients with HD [30]. They found a "marked increase" in the volume of sulfated mucin and that most of the goblet cells contained less mucin especially in patients with severe diarrhea [30]. They postulated that increased bacterial stimulation leads to both decreased mucosal cell renewal and increased sulfatization of the mucin causing abnormalities of the mucin ratio. This alteration of the ratio leads to increased adherence of enteropathogenic organisms to enterocytes. Changes in the mucin may lead to altered susceptibility to bacterial degradation [20]. Increased amounts of neutral mucin and a decrease in the acidic sulfated mucins were also detected in the resected enterocolitic bowel using PAS-AB staining [20, 31, 32].

Teitelbaum et al. proposed in 1989 that the presence of HD implies an alteration in the mucins of the large bowel with associated mucin retention and crypt dilatation [32]. Teitelbaum et al. proposed a histological grading system ranging from normal to gross abnormality using both histological features and the feature of mucin retention which is unique to HD and cystic fibrosis (Table 10.2) [32]. They demonstrated that 88% of patients with HAEC had grade III or higher while 83% of those without HAEC had grade II or lower. Despite the high incidence of HAEC in infants with trisomy 21, their histology findings were frequently inconsistent with their clinical features. This supports the theory that their decreased immunity allows increased susceptibility to HAEC with a less severe immune response. If patients with trisomy 21 were excluded from the study, 100% of patients with HAEC had grade III or higher. Teitelbaum et al. have used this grading system to predict the development of enterocolitis in patients with HD of grade III or higher. However, they admit that the uneven distribution of HAEC histological changes in resected specimens makes clinical correlation difficult. These histological changes demonstrate how the mucosa has become susceptible to enterocyte-adherent organisms which release toxins. The toxins cause both local (crypt abscesses, ulceration and perforation) and systemic (sepsis and coagulopathy) inflammatory responses.

Aslam et al. demonstrated that total mucin turnover is significantly reduced in patients with HD compared with to age-matched normal controls. Although ganglionated colon demonstrated similar mucin turnover alterations the changes were more significant in the aganglionic bowel [33]. This signifies an abnormal mucus defensive barrier in the colon of patients with HD, even in the histologically normal bowel. The same team also studied the colonic mucins of the proximal ganglionated bowel in nine patients with HD at the time of pull-through [34]. Radioactive precursors ^{35}S-sulfate and ^{3}H-glucosamine were added to the mucins of the intact remaining mucosa and the patients were followed for a mean of 30 months. They found that four patients without enterocolitis had a turnover rate six times higher than those with HAEC [34]. This reduced turnover of mucins will give rise to a defective mucus-defensive barrier allowing enterocyte adhesion and toxin release [34]. In 1999 Aslam et al. demonstrated that the mucin glycoproteins in children with HD, although quantitatively deficient, show no qualitative histological or immunological differences from those of normal controls [35]. The mucin gene expression and the quality of mucins was also similar to those of normal controls [35]. Yet those patients who developed HAEC had mucin turnover rates that were seven times lower than those without HAEC [35]. Gork et al. showed that mucin inhibits bacterial translocation in vitro across both fetal and adult cultured intact enterocyte monolayers [36]. Also in this study, they demonstrated that the inhibitory effect on translocation is lower in the fetal cells than in the adult cells.

Table 10.2 Histological grading system for HD

Grade	Histopathology
0	No abnormalities
I	Crypt dilatation and mucin retention
II	Cryptitis or two crypt abscesses
III	Multiple crypt abscesses
IV	Fibrinopurulent debris and mucosal ulceration
V	Transluminal necrosis or perforation

MUC-2 has recently been shown to be the predominant mucin gene expressed in human bowel [37]. Mattar et al. have shown that MUC-2 protein expression is significantly lower in patients with HD than in controls (19.8±15 vs 121±47) and not detectable during active enterocolitis [38]. The decline in MUC-2 expression in patients with no inflammatory response implies an intrinsic problem which could allow bacterial adherence and translocation. The authors suggest the use of probiotics prophylactically, such as *Lactobacillus casei* strain *GG*, in order to increase the epithelial expression of MUC-2 and possibly decrease bacterial translocation [38].

Overall the evidence has not proven whether mucin alteration is due to the underlying aganglionic condition or a result of the enterocolitis. However, the balance of data supports the concept that the mucin variations are an expression of an altered mucosal barrier and the underlying aganglionic process itself [38].

10.3.8 Intestinal Wall Defenses

Secretory IgA immunoglobulin provides a major immunological barrier in the gastrointestinal tract. IgA is the predominant immunoglobulin at all levels in the intestinal tract both in the lumen and within the wall. Albanese et al. have shown that secreted IgA binds to bacteria and prevents bacterial translocation across an intact segment of viable intestinal tissue [39].

Piebald mice have a congenital megacolon with absent distal ganglion cells, and hence are an excellent model of HD [40–42]. A number of studies have been performed in our center with a breeding colony of piebald mice to investigate the model and establish mucosal secretory function in HAEC [43, 44]. Two distinct patterns of mortality occur with the majority of mice (64%) characterized by becoming unwell acutely with evidence of acute enterocolitis at 3 to 4 weeks and then dying quickly or dying between 9 and 11 weeks due to ileus with massive abdominal distension and megacolon [43]. Interestingly two different immunological responses were evident. Those with a more acute history had acute splenitis and a severe diffuse lymphocytic response in the intestinal submucosa and lamina propria with a significantly raised level of IgA in contrast to controls and the late death group. The late death group had increased plasma cell distribution within the deep layer of the lamina propria only. This increased level of plasma cell infiltration in the ganglionic segment of the colon in the early death group implies that the local antigenic stimulation is the principle pathological event [43].

Wilson-Storey et al. postulated that there is a marked deficiency in the transfer of IgA across the intestinal mucosal membrane in patients with HAEC. They based this on the absence of secretory IgA in the buccal mucosa in patients with HAEC [17]. Five out of six patients with HD had no detectable secretory IgA in their saliva. These patients also had an increased amount of IgA in their buccal mucosal tissue. Imamura et al. demonstrated similar results in colonic resection specimens including elevated levels of IgM and J chain plasma cells in the bowel of those with enterocolitis [45]. Multiple factors including elevation of CD68-positive monocytes/macrophages and CD45RO-positive and CD57-positive natural killer (NK) cells were present in those with HAEC. Marked increases in IgA plasma cells in the lamina propria were found, yet there were a distinct reductions in the luminal IgA in four of the five patients with HAEC. Normal luminal and epithelial IgA was present in the ganglionated bowel.

Since 1976 the question has been asked as to whether the decrease of luminal IgA reflects a primary deficiency in transfer of IgA out of the cells onto the luminal surface or whether it is due to inflammatory change [46]. Turnock et al. attempted to answer the question as to whether or not there is a premorbid deficiency of the intestinal immune response in patients who develop HAEC [47]. They examined rectal suction biopsies of 20 patients with HD of whom eight developed HAEC. They found no evidence of a significant deficiency or difference in population in the IgA, IgM or IgG plasma cells in the lamina propria in patients with HD, HAEC or normal controls [47]. Overall there is evidence that IgA function and formation are normal in the cells but that there is a deficiency in the transfer of the immunoglobulin into the lumen to assist the mucin in its role in the front line of immunological response; however, this hypothesis has not been proven conclusively.

Mucosal neuroendocrine cells (NE) mediate intestinal function through synthesis and storage of neuroendocrine neuropeptides and biogenic amines which act as chemical messengers [48, 49]. Soeda et al. demonstrated in 1992 that NE cells are increased in the aganglionic segment of bowel in HD as opposed to the ganglionated bowel and normal controls [50]. In 1993 they noted a marked reduction in NE cells in ganglionated bowel in HAEC compared to those without. These diminished NE cells may represent an impaired immune response or a deficiency which may facilitate the initialization of inflammation [51]. This impaired immune response theory is echoed in trisomy 21. The combination of HD and trisomy 21 is associated with a higher incidence of enterocolitis with 50% of patients with trisomy 21 and HD developing HAEC in contrast to 29% among the normal population [1]. Infants with trisomy 21 have an intrinsic immune deficiency due to both decreased cytotoxic T-lymphocytes and derangement in humoral function which may explain their increased risk of HAEC [52–54].

Histological evidence of enterocolitis consists of a number of features including crypt abscesses, leukocyte aggregates, ulceration and Paneth cell metaplasia [31]. Paneth cells are normally present in the small bowel and secrete lysozymes which digest the bacterial wall membranes. Their presence in HAEC colon suggests an attempt at reinforcement of the mucosal immunity [31]. ICAM-1 is a cell surface intercellular adhesion glycoprotein which is involved in leukocyte recruitment when inflammation occurs. Kobayashi et al. have demonstrated that ICAM-1 shows increased expression in the endothelium of both the ganglionated and aganglionic bowel in patients with HAEC [55]. This emphasizes the importance of endothelial cell activation in HAEC pathogenesis. Elhalaby et al. postulated that the occurrence of a single episode of HAEC can alter intrinsic intestinal immunity by causing a chronic change to the mucosa to an increased the risk of further episodes [8]. This would help to explain the lower but real recurrence rate of HAEC following a "diversion" colostomy or a successful pull-through [8, 9].

10.3.9 Abnormal Motility and Macrophages

Suzuki et al. in 2004 used endothelin receptor null rats as a model for long-segment HD as they have a megaileum proximal to a constricted aganglionic region [56]. They

showed that the number of macrophages is increased in the tunica muscularis suggesting that macrophages play an important role in the inflammation of tunica muscularis in rats [56]. They postulated that the increased numbers and activation of macrophages may result in damage to networks of interstitial cells of Cajal leading to disordered intestinal rhythmicity in regions of the gut in which myenteric ganglia are intact. This disordered movement may encourage stasis, bacterial growth and, with the abnormal mucins, increased translocation.

10.4 Microbiology

Bacteria and viruses have been linked to enterocolitis by a number of studies. *Clostridium difficile* was first reported in 1982 by Thomas et al. when high titers of the toxin were detected in four of six patients with HAEC [57]. In 1986 Thomas et al. detected the cytopathic toxin in 7 of 13 (54%) and *C. difficile* was isolated in 77% of children with HAEC [58]. In the control groups *C. difficile* was isolated in 18% of those with HD and in 30% of children without. Thomas et al. postulated that the toxin was pathogenetic due to the incidence of toxin in the feces, the magnitude of the toxin levels and the isolation rates for *C. difficile* which were significantly higher in HAEC patients than in those without HAEC or even HD [58]. The possibility that HAEC could prevent the development of a "benign" colonic bacterial flora and aggressively treating *C. difficile* could improve this made this a very exciting theory. However, this has not been proven on subsequent investigations: 50% of all patients with HD have *C. difficile* and there is no variation in incidence between before and after surgery [59]. Wilson-Storey et al. in 1990, demonstrated a broad spectrum of organisms present in the stools with no significant difference in the Clostridium carriage rate between those with HAEC and those without HAEC or normal controls [60]. Stool samples in our center reveal a wide range of colonic flora present during episodes of HAEC. However, after an episode of enterocolitis, 70% of patients with HAEC have *C. difficile* present as opposed to 42% of those without HAEC [61]. It is postulated that after the initiation of the enterocolitis episode alteration in mucosal immunity allows *C. difficile* to flourish. Although it may not be causative, it can significantly complicate the colitis. Pseudomembranous colitis with stools positive for *C. difficile* is rare and has been reported in four patients with a 50% mortality despite vancomycin therapy [62].

Bacterial adherence has been viewed as an important factor for the last 15 years being demonstrated histologically in up to 40% of pull-through specimens in patients with prior HAEC. When in the mouse model intestinal mucus was removed there was an increased adherence of *Escherichia coli* colonic mucosal layers [63]. *Escherichia coli*, *C. difficile* and *Cryptosporidium* were the adherent organisms found, suggesting that the adherent nature of the organism is an important factor. Suzuki et al. observed abnormal intestinal flora with a marked increase in gram-negative aerobes (Enterobacteriaceae) and anaerobes (Bacteroidaceae) in the distended region of the small intestine of their endothelin receptor-null rats [56].

Imamura et al. hypothesized that the diversity of the altered local response in HAEC is due to a multifactorial microbiology etiology [45]. They examined the entire resected colon from 12 patients with HD. CD57-positive NK cells which act as antiviral agents were found to be significantly increased in the ganglionic segment of the HAEC patients while no difference was found in those without enterocolitis or the normal controls. This has led to the postulation that the increase in these antiviral cells implies a viral etiology [45].

Wilson-Storey agrees that HAEC has a multifactorial infective etiology [61]. Rotavirus was identified in seven of nine patients with enterocolitis [60]. Of note, there were no symptoms of vomiting in these patients which is pathognomonic for rotavirus gastroenteritis. Also there was no evidence of contact before, during or after admission to hospital [60]. However, these results have not been replicated.

10.5 Pathology

Historically in 1886 Harold Hirschsprung described "deep ulcerations that penetrate to the serosa … an abscess under the mucosa … mottled spaces that can be seen in the submucosa containing pus" in his first report of the condition [64]. Thus he became the first to describe a number of key pathological features of HAEC. Histological evidence of enterocolitis consists of a number of features including crypt abscesses, leukocyte aggregates, ulceration and Paneth cell metaplasia [31]. Paneth cells are normally present in the small bowel and secrete lysozymes which digest the bacterial wall membranes. Their presence in the colon of those with HAEC suggests an attempt at reinforcement of the mucosal immunity [31].

10.6 Risk Factors for Enterocolitis

A number of factors have been proposed as important in the etiology of HAEC. These factors include delay in the initial diagnosis of HD, gender, a family history of HD, and the presence of trisomy 21. Delays in the diagnosis of HD leads to a higher incidence of enterocolitis as the presenting condition [65]. Our own series [9] in 1994 revealed that the incidence of enterocolitis in neonates increased from 11% in the first week of life to 24% after. In Ann Arbor a decrease in the incidence of preoperative enterocolitis has been explained by a protocol of early diagnosis of HD and washouts. The incidence of preoperative enterocolitis has also significantly fallen in Japan. In a nationwide study of 3852 patients over 30 years the

incidence fell from 29% in 1978–1982 to 17% in 1998–2002 [13]. Historically early decompression enterostomy was recommended but now commencement of an early washout program and prompt surgery are viewed as key features in prevention of HAEC [66]. The length of the aganglionic segment has been identified as a risk factor. Studies have shown that HAEC is significantly more common in patients with aganglionic segments longer than the sigmoid [8, 23]. Our own experience reflects this result [9]. A neonate with total colonic aganglionosis can present with perforation of the ganglionic bowel. However, some studies on this condition have found no difference as regards length of the aganglionic bowel [11, 19, 24].

Some studies have shown a higher incidence since the introduction of the pull-through procedure ranging from 2% to 27% [67]. The high HAEC incidence of 21% has been reported after Swenson's pull-through operation by Swenson himself in a 40-year follow-up [12]. However, Wildhaber et al. [68] demonstrated no correlation between the incidence of HAEC and the type of pull-through performed. Higher HAEC incidences of up to 55% have been noted in Ann Arbor, but the center acknowledges a very low threshold for diagnosis and treatment [69, 70]. Our study, similar to that of Polley et al. [71], found no difference in the incidence of HAEC following different types of pull-through [9]. No increase in HAEC has been found in the postoperative period after a primary pull-through without stoma formation [6]. Down's syndrome is associated with an incidence of 3–16% of HD of all causes [72, 73]. The combination of HD and trisomy 21 is associated with a higher incidence of postoperative morbidity, prolonged hospitalization and poor long-term bowel function. Infants with trisomy 21 have an intrinsic immune deficiency due to both decreased cytotoxic T-lymphocytes and derangement in humoral function which may explain their increased risk of HAEC [74–76]. Of patients with trisomy 21, 50% develop HAEC as opposed to 29% in the normal population [1]. In our experience 47% of patients with trisomy 21 and HD develop one or more episodes of HAEC [72, 73]. HAEC occurs in 54% of patients with trisomy 21 [24].

Some postulate that the occurrence of a single episode of HAEC can alter intrinsic intestinal immunity leading to an increased risk of further episodes [8]. Carneiro et al. [1] reported that HAEC occurs predominantly in females (50% vs 29%); however, although this has been noted by others, it has not been found to be statistically significant [8, 77].

The presence of associated anomalies is also associated with an increased incidence of HAEC. Klein et al. [78] initially reported associated anomalies in 35% of patients with HAEC in 1984. Carneiro et al. and Elhalaby et al. reported HAEC in 53% and 47% of those with anomalies, respectively [1, 8]. A lower incidence of 15% was noted in South Korea [3].

In 1977 we reported a case of intestinal neuronal dysplasia (IND) in association with HD [79]. In 1995 10 of 31 patients following a definitive pull-through procedure were demonstrated to have IND in the proximal margin of the resected bowel [80]. All ten patients with IND had persistent bowel problems after the definitive operation for HD, including enterocolitis (n=5), soiling, and constipation. Only 4 of the other 21 patients had persistent bowel symptoms. This suggests that IND is commonly associated with HD, and emphasizes the importance of histochemical examination of the resected segment to predict postoperative bowel function in patients with HD.

Our experience demonstrates that although HAEC does occur with a defunctioning colostomy, its incidence is substantially lower [9]. Hackam et al. [77] evaluated 62 cases of HAEC in 33 patients at a mean of 8 months from definitive surgery. They found no significant difference in gender, age at pull-through and weight at surgery, the type of operation, or the number of stages. The presence of an anastomotic leak and bowel obstruction requiring release of adhesions were significant risks for HAEC with a relative risk of 2.8 and 3.0, respectively [77].

10.7 Clinical Presentation and Diagnosis

As stated at the start of this chapter enterocolitis is a clinical condition with diarrhea, abdominal distension, pyrexia, colicky abdominal pain, lethargy and the passage of blood stained stools [1]. A grading system for the clinical features of HAEC is presented in Table 10.3. In the neonate the classical presentation consists of a history of constipation from birth associated with occasional loose foul-smelling stools and progressive abdominal distension. Among neonates with HD, 16–33% present with diarrhea [2, 8, 9, 15]. The presence of diarrhea is pathognomonic of enterocolitis which occurs in 93% of patients with HAEC [1, 2, 8, 9, 12, 23]. Vomiting rarely occurs in HAEC. A markedly distended hyperresonant

Table 10.3 Clinical grading system for HAEC

Grade	Clinical symptoms		
	Explosive diarrhea	Abdominal distension	Systemic manifestations
I	Mild	Mild to moderate	None significant
II	Moderate	Moderate to severe	Mild
III	Severe	Marked	Shock or impending shock

abdomen occurs in 32–83%, vomiting in 9–76%, pyrexia in 12–54%, and less commonly rectal bleeding in 5–9% of patients with HAEC [8]. Rectal examination either by digit or soft catheter which is both diagnostic and therapeutic results in a characteristically explosive foul smelly stool and gaseous decompression which once witnessed is never forgotten. Patients after a pull-through operation or those with a defunctioning stoma will present in the same fashion.

The significant morbidity associated with HAEC occurs with the toxic megacolon which is characterized by bilious vomiting, fever, dehydration, marked abdominal distension, and signs of shock [81]. Fortunately, bowel perforation is a rare complication occurring in only 2–3% of patients [1, 8].

Although in the majority of patients the diagnosis can be made easily on clinical evaluation, plain abdominal radiographs are the most useful investigation. Simple anterior-posterior and lateral decubitus abdominal radiographs can show thickening of the bowel wall, mucosal irregularity, dilated bowel loops, pneumoperitoneum and evidence of toxic megacolon (grossly dilated colonic loop) (Fig. 10.1). A large 40-year study of 880 patients following Swenson's procedure revealed a 3% incidence of spontaneous perforation [12].

A barium enema in a patient with HAEC can demonstrate mucosal nodularity, ulceration and edema, speculation, narrowing of the anorectal junction and colonic dilatation (Fig. 10.2). However, most of the radiological findings can persist after the cessation of the active enterocolitis and have no specificity. Elhalaby et al. [8] assessed 150 plain radiographs acquired during and between episodes of HAEC. Colon dilatation was the most radiologically sensitive sign (90%), but it had a sensitivity of only 24%. "Intestinal cut-off" sign which appears when the gaseous intestinal dilatation is abruptly cut off at the pelvic brim was both sensitive (74%) and specific (86%) for HAEC.

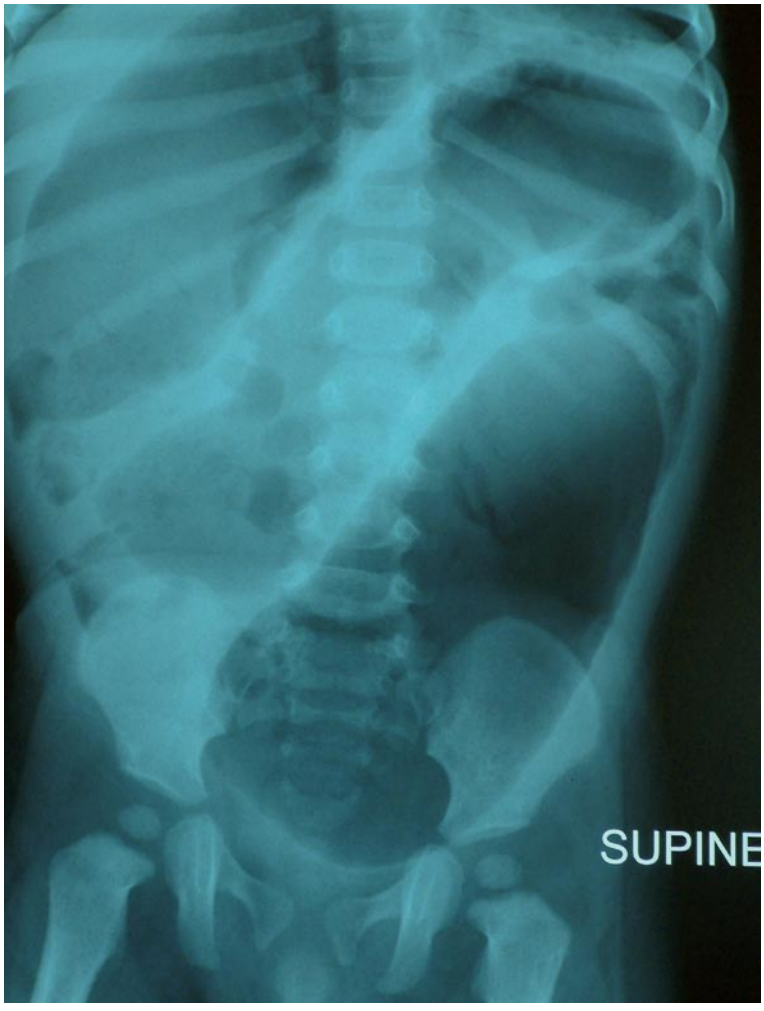

Fig. 10.1 Plain abdominal radiograph demonstrating thickened bowel wall, gross distension and the "pelvic cut-off" sign

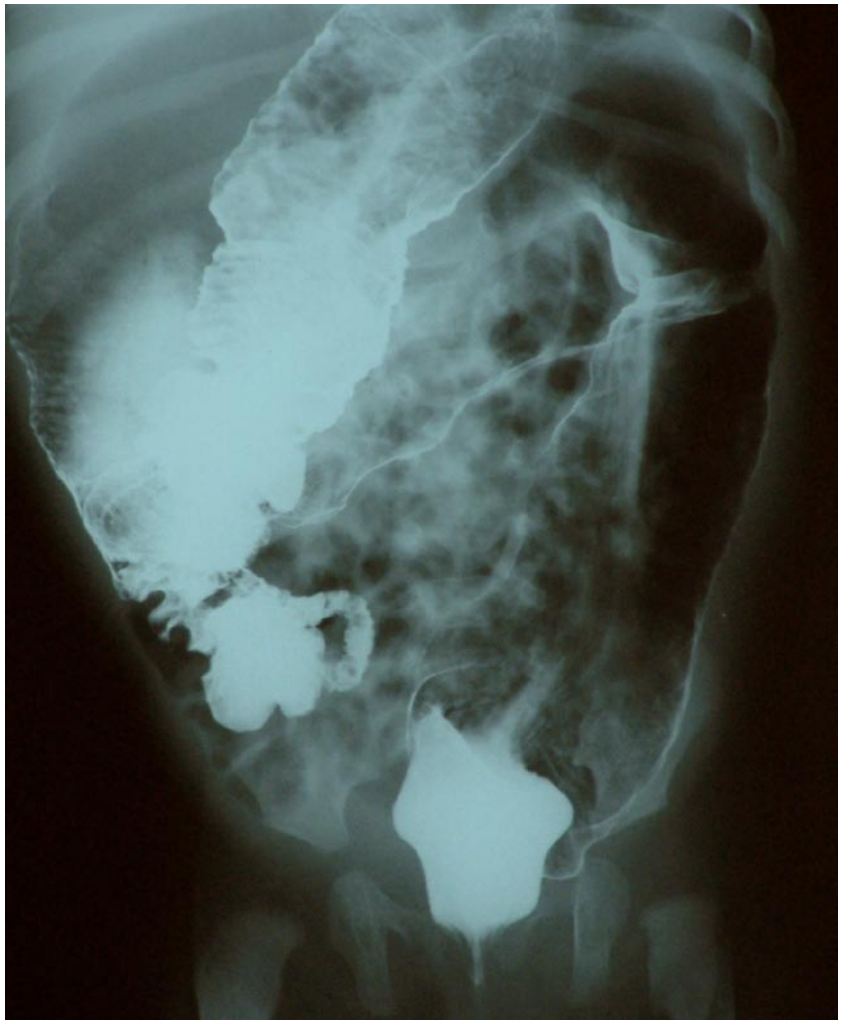

Fig. 10.2 Barium enema demonstrating colonic distension, speculation, edema and mucosal nodularity

10.8 Treatment

The key step in the initial management of a patient with HAEC is urgent resuscitation and correction of electrolytes. Shim and Swenson [82] recommended the use of a flatus or rectal tube to enable colonic decompression. Rectal washouts should be performed as soon as possible using a large-bore soft catheter with multiple side holes. The tube is well lubricated and advanced into the colon. In preoperative HAEC the tube should be passed into the transient zone if technically possible. Chest tubes with extra side holes have been used with some success in our institution to treat patients with HAEC who do not decompress via smaller catheters. Repeated tube decompression and gentle rectal washouts with 30–50 ml of normal saline make a significant clinical impact on these patients.

Vancomycin can be given either orally or via enema if *C. difficile* is found on stool culture. It has been reported by Carneiro et al. [1] to be successful in 14 of 15 "stool-positive" patients with episodic enterocolitis. Oral metronidazole has also been used with some success. Clinical deterioration in the neonate particularly those with long-segment disease in which washouts have a high failure rate may require an emergency decompression colostomy.

Concerns over the mortality rate due to fulminant enterocolitis in the postoperative period led Marty et al. [10] to suggest routine postoperative rectal washout to decrease both the incidence and the severity of episodes of enterocolitis following definitive surgery. They recommend a policy of rectal irrigation performed by the parents commencing 2 weeks following surgery twice daily for 3 months followed by once daily for 3 months. This

policy reduced their incidence of HAEC from 36% (34 of 95 patients) to 10% (4 of 40 patients).

In episodes of recurrent enterocolitis which can develop in up to 56% of patients, anal dilatation has been recommended [8]. However, prior to commencing a treatment regimen a repeat contrast enema should be performed to rule out a mechanical obstruction. In our center rectal biopsies are also taken to ensure the presence of ganglionated bowel. Patients with a normal rectal biopsy may require a sphincterotomy [8, 12]. Wildhaber et al. [69] found that 59% of patients had recurrent enterocolitis of whom 75% were symptom-free following a posterior myotomy/myectomy. Similar results have been reported by Menezes and Puri [83]. Redo pull-through operations when appropriate appear to be as effective as primary procedures in terms of continence and stooling frequency, and can decrease episodes of HAEC [84]. Rintala and Lindahl [85] treated eight patients with recurrent HAEC with sodium cromoglycate, a mast cell stabilizer that is used successfully in patients with inflammatory bowel disease. Significant clinical improvements were noted in six of the eight patients, four of whom had trisomy 21. No side effects of sodium cromoglycate were noted. Sodium cromoglycate may be a useful adjunct in the therapy of recurrent HAEC, especially in the difficult management of trisomy 21 combined with HD.

10.9 Prognosis

The medical management of those with HAEC is 2.5 times more costly than of those with just HD. Mortality rates in enterocolitis have fortunately fallen from 30% to 1% [13, 32]. Results from Japan demonstrate a decline in mortality from 6.5% in 1978 to 0.7% in 1998 [32]. This decrease in mortality is related to earlier diagnosis of HD and HAEC, rectal decompression, appropriate vigorous resuscitation and antibiotic therapy [1, 65, 86].

However, despite the improvement in mortality rates in HAEC, the morbidity has a profound impact with prolonged hospitalization with a mean of 13 days ranging from 6 to 29 days [1]. Teitelbaum et al. found that neonates with HAEC have a mortality rate of 5% and a morbidity rate of 30%, and their hospitalization is twice as long as neonates without HAEC [32].

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Diagnosis of Hirschsprung's Disease and Allied Disorders

J. Kelleher and N. Blake

11

11.1 Radiological Diagnosis

Major advances have taken place in the histochemical diagnosis of Hirschsprung's disease in recent years. While rectal manometry remains a reliable screening procedure, radiology still has an important role to play. The diagnosis of Hirschsprung's disease may be suggested on plain films, which may also demonstrate the serious complication of enterocolitis. Barium enema is usually diagnostic and should show both the transition zone and the length of the distal aganglionic segment of bowel.

11.2 Initial Radiographs

Most cases of Hirschsprung's disease present in the newborn period with abdominal distension and delayed passage of meconium. Approximately 90% of patients fail to pass meconium in the first 24 hours of life [1]. Supine and lateral decubitus plain films are performed routinely. The supine film will show gaseous distension of bowel loops with distribution of loops sometimes suggesting large-bowel involvement. The level of obstruction may be indicated by the presence of undilated colon or rectum (Fig. 11.1). The horizontal beam film may show multiple fluid levels in the distended bowel loops and also serves to exclude perforation, which is a rare complication. We strongly recommend a lateral decubitus view with right side raised instead of the erect view. This is less traumatic to the baby than the erect view and provides more diagnostic information.

If the diagnosis is still uncertain from the initial plain films, a prone lateral view with buttocks elevated may be helpful (Fig. 11.2). The infant will be comfortable in this position for 10 minutes or longer, allowing gas to rise from the distended colon into the undilated rectum. A cone-shaped or funnel-like appearance of the transitional zone between the distended proximal bowel and the narrowed aganglionic distal segment may be shown (Fig. 11.3). All three plain films in suspected Hirschsprung's disease can be taken without removing the baby from the warm protective environment of the incubator. We hope that the practice of dangling babies upside down for inverted views has been abandoned.

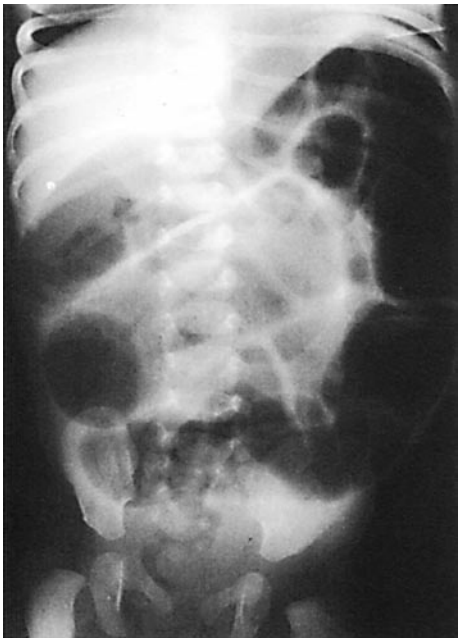

Fig. 11.1 Supine abdominal radiograph at 30 hours with abdominal distension and failure to pass meconium. Marked gaseous distension of colon to sigmoid level with undilated rectum consistent with Hirschsprung's disease. This was confirmed with barium enema and rectal biopsy

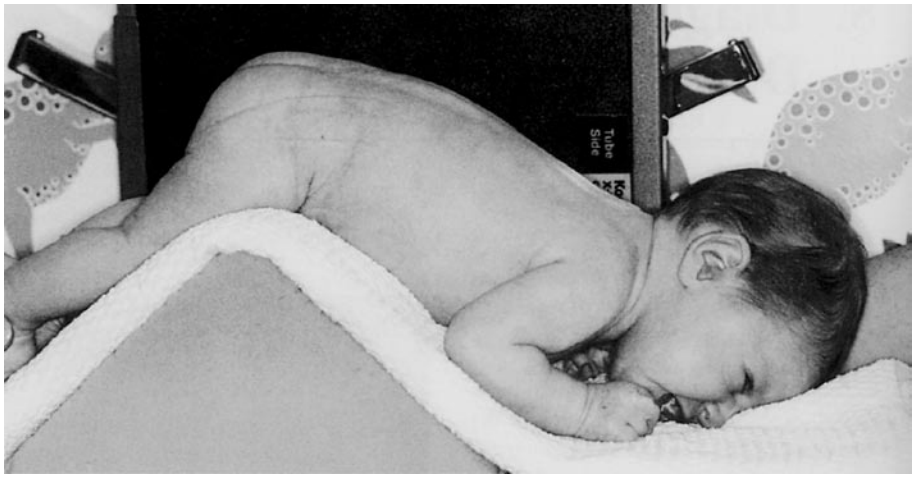

Fig. 11.2 Baby comfortably in position for prone lateral view with horizontal beam and buttocks elevated

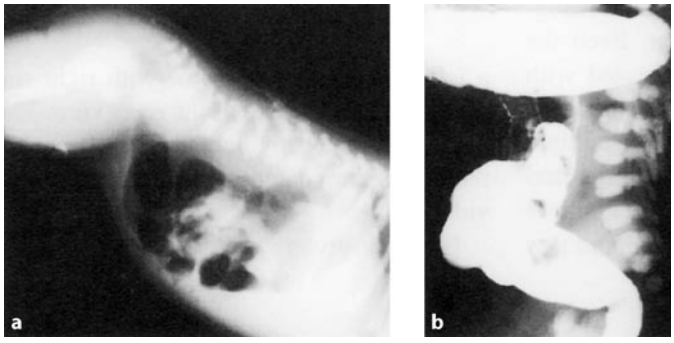

Fig. 11.3a,b Rectosigmoid Hirschsprung's disease. **a** Prone lateral view with buttocks elevated showing relative narrowing of rectum and sigmoid with marked proximal bowel distension and fluid level. **b** Barium enema confirms diagnosis with cone-shaped transitional zone at rectosigmoid junction

11.3 Differential Diagnosis

Colonic atresia may give similar plain film findings to Hirschsprung's disease but is readily excluded with contrast enema, which will show complete mechanical obstruction. In cases of distal small-bowel atresia, there is often marked dilatation of multiple loops of small bowel, with many abnormal air-fluid levels. The loop immediately proximal to the obstruction often demonstrates the widest fluid level.

In meconium ileus the typical mottled granular appearance of gas trapped in the thick meconium may be seen. Furthermore, clear sharp air-fluid levels are usually not a feature in the lateral decubitus views in meconium ileus. Hirschsprung's disease can sometimes simulate meconium ileus on plain films, but the correct diagnosis is usually obvious on Gastrografin or barium enema (Fig. 11.4).

Both meconium plug syndrome and neonatal small left colon syndrome probably represent part of a spectrum of similar functional disorders related to delayed "maturity" of the colon in the newborn [8]. Associated factors include prematurity, maternal diabetes, preeclampsia and maternal drug ingestion. In both of these conditions the clinical presentation and plain film findings may suggest a diagnosis of Hirschsprung's disease. However, it is notable that in both conditions the rectum is normally distensible in contrast to true Hirschsprung's disease where the rectum remains abnormally narrow. The functional obstruction in both meconium plug syndrome and in the small left colon syndrome will usually resolve with Gastrografin enema. However, as a minority of these infants will actually have Hirschsprung's disease, all should have a rectal suction biopsy performed.

11.4 Enema Technique

A carefully performed contrast enema is usually very reliable in either confirming or excluding the diagnosis

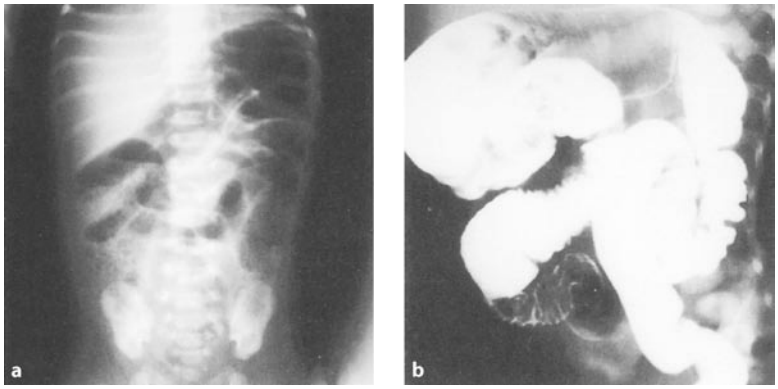

Fig. 11.4a,b Long segment Hirschsprung's disease simulating meconium ileus. **a** Supine film showing mottled appearance of air trapped in meconium with bowel distension. Fluid levels were not a prominent feature in the erect film. **b** Barium enema reveals typical transitional zone at the splenic flexure in the oblique view

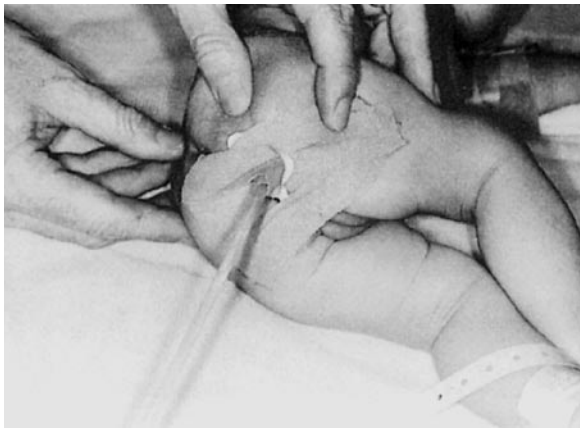

Fig. 11.5 Buttocks tightly strapped with adhesive tape. Tube is secured with a loop of tape

of Hirschsprung's disease and, if positive, in identifying the transition zone. Rectal wash-outs are contraindicated and even digital examination should be avoided or kept to a minimum prior to the barium enema. Otherwise the distended proximal bowel may be decompressed, with distortion of the transition zone leading to a false-negative diagnosis.

The fluoroscopy room should be warm and an intravenous line should be in place. A recent horizontal beam radiograph should be reviewed to exclude perforation.

A soft rubber catheter of appropriate size is inserted into the rectum and secured in position with firm strapping drawn tightly across the buttocks (Fig. 11.5). If a balloon catheter is used, the balloon should not be inflated due to the risk of perforation and distortion of the transition zone by the distended balloon.

The choice of contrast medium is somewhat controversial. In the neonate with suspected intestinal obstruction of uncertain etiology, we often begin with a diagnostic enema using an iso-osmolar, non-ionic water-soluble medium before changing to either Gastrografin or barium solution as appropriate. If Hirschsprung's disease has already been confirmed by prior rectal biopsy, we use barium to identify the transition zone. We allow a period of 24–48 hours to elapse after suction biopsy before such an enema is performed.

Barium sulfate suspension is diluted 50% with warm saline and injected slowly under fluoroscopic control using a 50-ml syringe with the baby in the lateral position. Slow injection of the contrast agent avoids over-distension and obliteration of a potential cone-shaped transition zone. This zone may be observed at the classical rectosigmoid level in the supine and lateral positions (Fig. 11.3b). However, rotation of the baby into oblique positions is usually required for a longer segment lesion involving the sigmoid or descending colon (Fig. 11.4b).

When the injected barium clearly outlines the distended proximal normal ganglionic colon, no more is injected. Appropriate views are taken to show the transition zone.

11.5 Enema Findings

Many patients will show an abrupt transition from the narrow distal aganglionic segment to the dilated proximal bowel, thus confirming a diagnosis of Hirschsprung's disease. However, we usually take supine and lateral films approximately 4 hours after the enema and sometimes at 24 hours. This allows us to assess the barium residue, and a transition zone may be accentuated and more easily identified (Fig. 11.6). Occasionally repeat films at 48 hours are performed in equivocal cases. Frequently, abnormal irregular contractions may be observed on fluoroscopy (Fig. 11.7) [3]. With the use of manometry and modern histochemical methods for diagnosis, most problem cases should be more readily clarified.

A modification of the barium enema to incorporate the study of rectosphincteric reflex during balloon inflation of the rectum has been described by Nagasaki et al. [4]. This might be useful in screening older children with severe constipation, but seems unlikely to replace rectal manometry.

Total colonic aganglionosis is rare and often difficult to diagnose as the colon may not be significantly narrowed. If reflux of barium into a grossly dilated ileum is observed, the diagnosis should be strongly considered. However, the ileocaecal valve may be competent preventing ileal filling (Fig. 11.8). In this situation, Hirschsprung's disease cannot be confidently differentiated from ileal atresia or meconium ileus, and a definitive diagnosis of total colonic Hirschsprung's disease may only be made histologically from frozen sections of surgical specimens.

If meconium ileus or meconium plug syndrome is diagnosed on contrast enema (Fig. 11.9), Gastrografin may be introduced to reduce the obstruction.

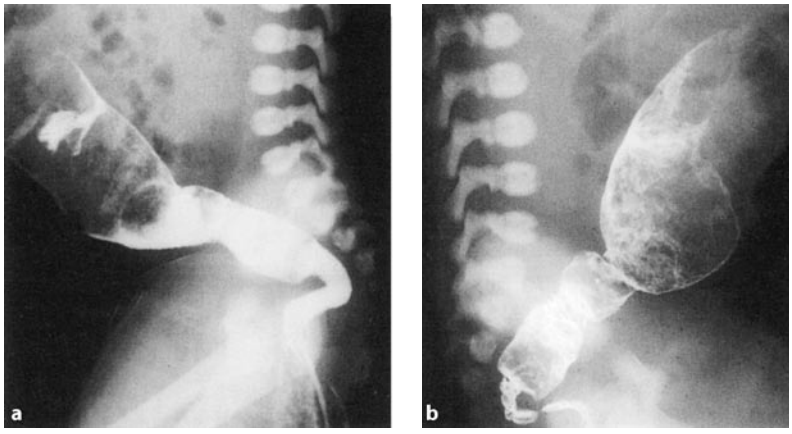

Fig. 11.6 **a** Barium enema shows narrow rectum with sigmoid colon gradually widening proximally to give the "tunnel funnel" appearance of Cremin. **b** Routine film at 24 hours showing a sharp transitional zone at the distal sigmoid level

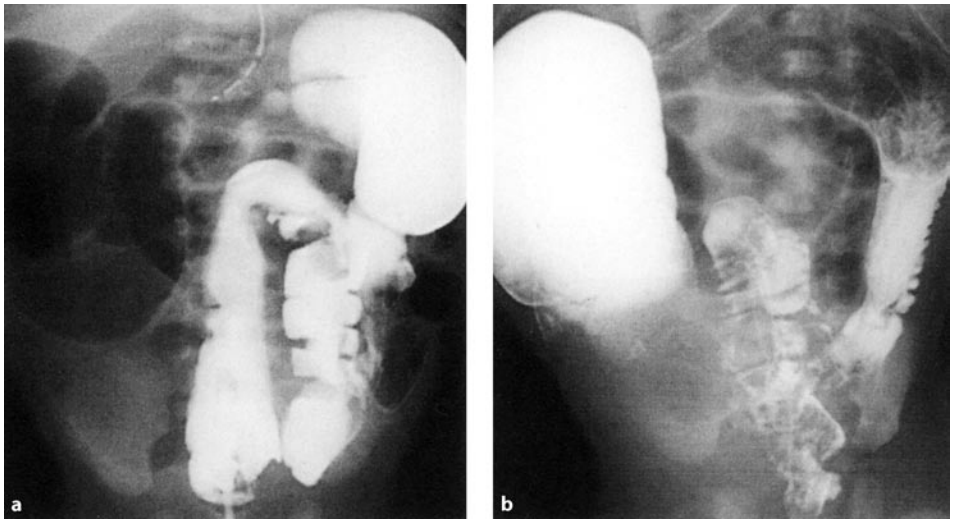

Fig. 11.7a,b Abnormal contractions in long-segment Hirschsprung's disease. **a** Deep contractions are shown in the sigmoid colon. The transitional zone is at the proximal descending colon. **b** Spicular contractions in the descending colon

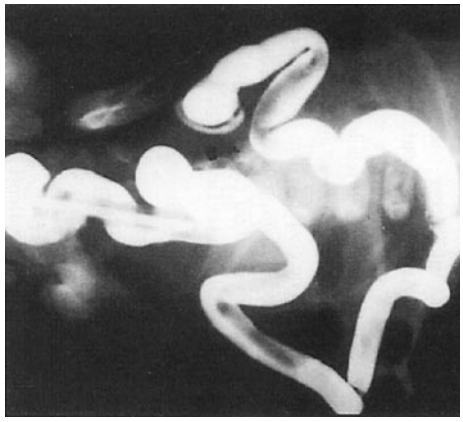

Fig. 11.8 Total colonic Hirschsprung's disease. Gastrografin enema shows generalized narrowing of the colon without specific features. No contrast could pass through the competent ileocecal valve. The diagnosis was made at surgery

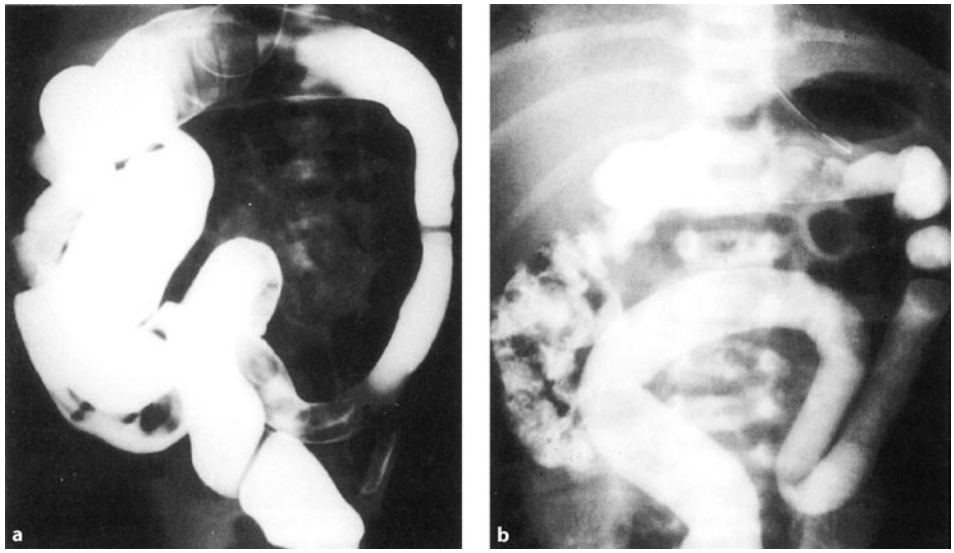

Fig. 11.9a,b Meconium plug. **a** Barium enema reveals a normal-caliber rectum and a narrowed left colon with marked distension from the splenic flexure. Note extensive filling defects due to plugs of meconium. **b** Gastrografin enema 2 days later shows complete reduction. Subsequent rectal biopsy was normal

11.6 Enterocolitis

This is the most feared and serious complication of Hirschsprung's disease and is potentially fatal [5]. Often referred to as Hirschsprung's enterocolitis (HEC), it is a particular risk in children with Hirschsprung's disease and trisomy 21, with Carneiro et al. [9] reporting a 50% incidence in these children compared to 29% in all other children. The risk seems greatest before the diagnosis of Hirschsprung's disease is established. However, Murthi and Raine have found that the highest incidence (22%) of HEC occurs following pull-through surgery [11]. Radiological findings include the presence of distended loops of bowel (Fig. 11.10), abnormal air-fluid levels, bowel wall thickening, mucosal edema, pneumatosis intestinalis or signs of perforation [10].

The presence of necrotizing enterocolitis (NEC) in a full-term infant should raise the possibility of Hirschsprung's disease, and rectal biopsy should be performed at an appropriate time. A contrast enema should not be performed on a baby with suspected HEC (Fig. 11.11, films from an outside hospital).

11.7 Postoperative Examinations

Most children will require little postoperative radiological imaging. Any suspected complications relating to the anastomosis, e.g. leak, fistula or abscess, can usually be safely diagnosed by a combination of sonography, water-soluble contrast enemas (Fig. 11.12) and, if necessary MR imaging. Recently there has been much interest in

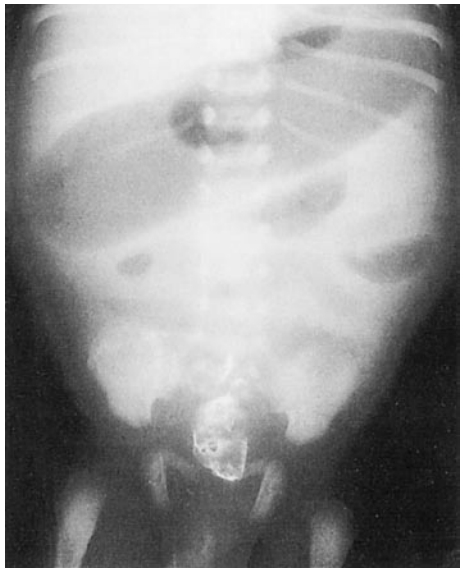

Fig. 11.10 Toxic megacolon. Gross distension of the transverse colon at 5 days with generalized abdominal distension and opacity due to ascites. Note retained barium in the rectosigmoid following enema 2 days earlier

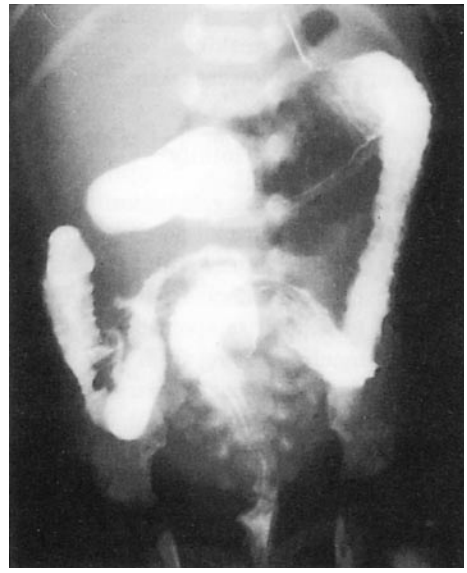

Fig. 11.11 Enterocolitis complicating Hirschsprung's disease. Barium enema shows mucosal swelling with fine ulceration in the descending and sigmoid colon. The transverse colon remains dilated with areas of spasm and mucosal irregularity in the proximal colon and terminal ileum

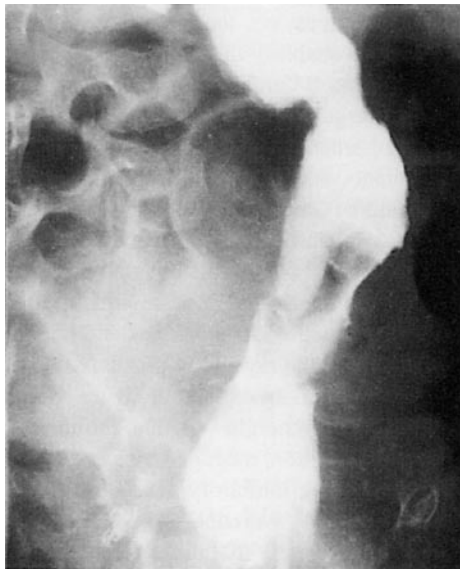

Fig. 11.12 Postoperative water-soluble contrast enema reveals a minimal leak into a widened rectosacral space. With extension of parenteral feeding and antibiotic therapy satisfactory healing occurred

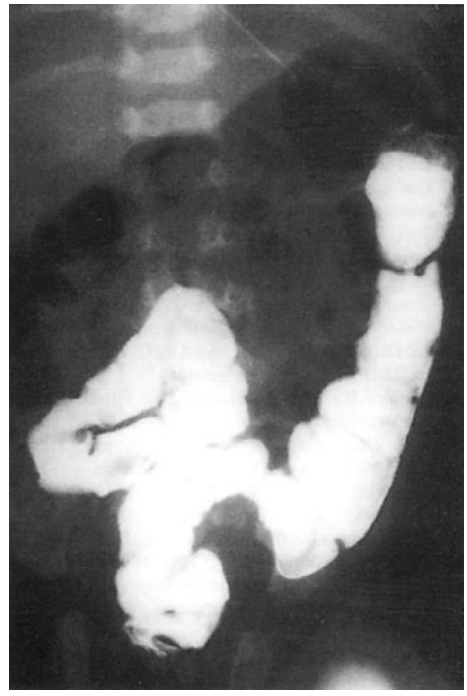

Fig. 11.13 Intestinal neuronal dysplasia. Delayed film in 3-day-old boy at 24 hours shows no specific features but barium residue is marked. Histology was classical with giant, ectopic ganglia and hyperganglionosis on rectal biopsy

the use of endoanal sonography in evaluating the anal sphincters in children after surgery for Hirschsprung's disease. It seems to hold out significant promise for the future but, as yet, experience is limited [12, 13, 14].

11.8 Intestinal Neuronal Dysplasia

In most patients, intestinal neuronal dysplasia (IND) is clinically indistinguishable from Hirschsprung's disease at presentation [6, 7]. Our experience of barium enema in these patients suggests that the findings are often equivocal and could delay diagnosis (Fig. 11.13). It should be born in mind that IND combined with Hirschsprung's disease occurs in 25–35% of patients with Hirschsprung's disease [15]. The diagnosis of IND is essentially histological and histochemical but the pediatric radiologist should always keep this condition in mind when presented with a patient who does not easily fit the criteria of Hirschsprung's disease.

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Functional Diagnosis

12

A.M. Holschneider and I. Steinwegs

Besides aganglionosis, there is no clear correlation between histomorphology and function of the bowel. Therefore functional diagnosis of chronic constipation is of great importance for the diagnosis and treatment of Hirschsprung's disease (HD) and allied disorders.

12.1 Anorectal Motility

The musculature of the gastrointestinal tract and the draining urinary tract is composed primarily of smooth muscle cells. Because of their low resting membrane potential, these cells tend to depolarize spontaneously. The electrical impulses arising from the spontaneous depolarization and repolarization, the basal electrical activity (basal electrical rhythm, BER), are responsible for the muscular tone. Any major shift of the membrane potential fluctuations in the direction of depolarization leads to the occurrence of stronger electrical impulses in the form of volleys of action potentials, which present as spikes in the slow basal rhythms. These rapid action potentials are responsible for the segmental and peristaltic contractions of the smooth musculature (Fig. 12.1). Both the origin and the propagation of the progressive contractions, that is the propulsive waves, and in all probability the segmental contractions, that is those confined to one bowel segment, of the gastrointestinal tract are regulated via the intramural bowel wall plexus. Distension of the bowel wall by a stool bolus produces an excitatory impulse which, after traversing the submucous plexus and being transmuted by the myenteric plexus, leads to a cholinergic contraction oral to the bolus and to nonadrenergic–noncholinergic (NANC) relaxation, mediated by inhibitory neurons containing nitric oxide (NO), aboral to the bolus [1–3]. Adrenalin modulates the acetylcholine release at cholinergic synapses. NO has recently been recognized as a neurotransmitter that mediates relaxation of the smooth muscles of the gastrointestinal tract [4]. It is identical to nicotinamide-adenine-dinucleotide-phosphate-diaphorase (NAPDH-diaphorase) [5] which can therefore be used as a diagnostic marker for HD. It is suggested too, that a lack of NO synthase in pyloric tissue is responsible for pylorospasm in infantile hypertrophic pyloric stenosis [6] (Fig. 12.2).

At the time of circular muscle relaxation, contraction of the longitudinal muscles, and thereby shifting of the bowel contents, occurs. Beside NO-containing inhibitory neurons, many other peptidergic neurons, storing vasoactive intestinal peptide (VIP), substance P (SP), enkephalin, neurokinin A (NA), histidine isoleucine, gastrin-releasing peptide (GRP) and many other factors are involved in the peristaltic reflex. They are lacking or abnormal in HD and allied disorders [3, 7]. Also a decreased expression of nerve growth factors (NGFs), fibroblast growth factors (FGFs), extracellular matrix (ECM) such as laminin, and cell-adhesion molecules (CAMs)

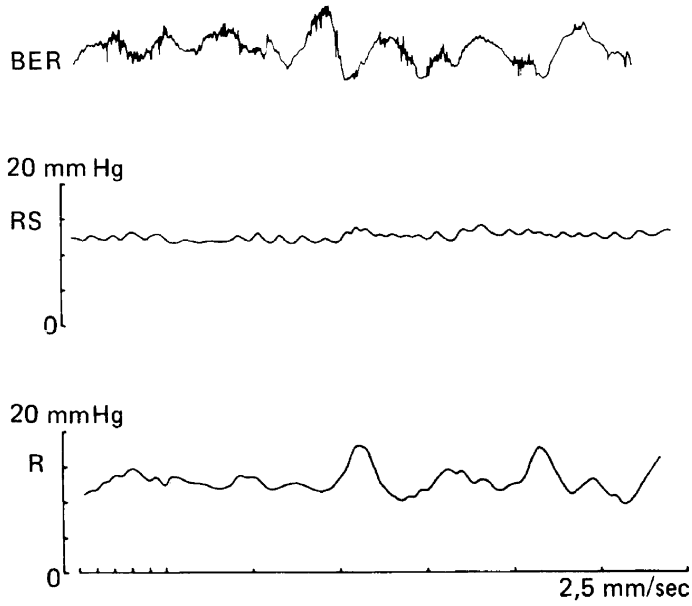


Fig. 12.1 Spike potentials on the top of the slow waves in the colon of a healthy child (*BER* basal electrical rhythm, *R* rectum, *RS* rectosigmoid)

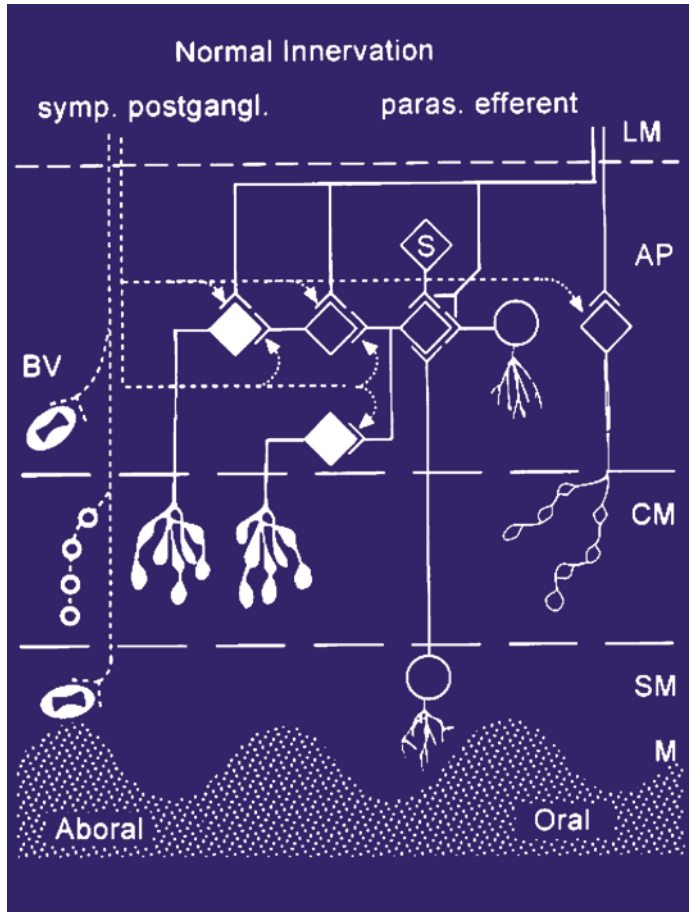


Fig. 12.2 Schematic diagram of the peristaltic reflex, showing the intramural plexus and the efferent postganglionic adrenergic and preganglionic cholinergic axons entering the bowel. The impulses from the mechanoreceptor cells are transmitted via interneurons (*white squares*) over cholinergic synapses to the NANC inhibitory neurons (*dark squares*). The finely drawn neuron with white circles in its terminal axons represents a postganglionic adrenergic axon. Stimulation of the NANC neurons leads to a neurogenically produced and peptidergically transmitted relaxation aboral to the bolus. Oral to the bolus, a myogenically produced contraction of the circular muscle occurs (rebound excitation). The sympathetic system acts as a modulator of the acetylcholine release at the cholinergic synapses. *AP* Auerbach's plexus, *asterisks* peptidergic transmitters, *BV* blood vessels, *circles* sensory neurons, *CM* circular muscle, *S* pacemaker neuron with spontaneous activity situated in the ICCs of Stach's plexus (plexus submucosus extremus), *SM* submucosa

such as neural cell adhesion molecule (NCAM), NCAM L1 (L1CAM), and N-cadherin lead to neuronal abnormality in HD. CAMs play an important role in cell–cell interactions, which regulate the development and maintenance of multicellular organisms. FGFs can induce diverse cellular responses in multiple biological systems including neurite outgrowth. Yoneda et al. [8] showed that there is an altered interaction between FGF-CAM and FGF receptors (FGFR) in aganglionic segments of HD. The number of CAM-positive fibers in aganglionic segments is markedly decreased compared to ganglionic segments, whereas there is not such a difference in FGF and FGFR expression. This suggests that CAM-FGF signaling is altered in HD and may be responsible for the failure of neural cell migration in the intestinal tract.

Beside the submucous and myenteric plexus, interstitial cells of Cajal (ICCs) have important regulatory functions in human gut musculature [9]. These cells are pacemaker cells which generate slow waves and facilitate active propagation of electric events and neurotransmission of the gastrointestinal tract. The ICCs can also be recognized immunohistochemically by the demonstration of their surface-receptor-tyrosine-kinase-kit (c-kit). Mesenchymal ICC precursors which carry the c-kit receptor require the kit ligand provided by neuronal or smooth muscle cells. The ICCs develop as either myenteric ICCs (ICC-MY) or intramuscular ICCs (ICC-IM). Solari et al. [10] demonstrated in c-kit immunoreactive ICC-IM in the circular and longitudinal muscle layers in normal sigmoid colon and ganglionic bowel in patients with HD and in those with total colonic aganglionosis (TCA). These long, thin, bipolar cells are connected to each other via one or two processes, whereas ICCs-MY create a dense mesh-like network surrounding the myenteric plexus. This characteristic 3D network can only be visualized with the whole-mount technique where the mucosa is separated in one layer from the muscularis mucosa attached to the submucosal layer. In aganglionic bowel only sparse and single ICC-MY were observed between the circular and longitudinal muscle layers. Similarly, in whole-mount preparations ICC-IM were markedly reduced. Wu et al. [11] reported that neurons might be necessary for development of highly differentiated ICC-MY and a mature ICC network. This could explain the absence or reduction of c-kit-immunopositive cells in HD. Nemeth and Puri [12] found that the characteristic 3D network observed in normal colonic mucosa is replaced in HD by thick nerve trunks that do not form any network and run up and down in a serpentine manner.

12.2 Physiology of the Internal Anal Sphincter

The internal anal sphincter is influenced by five nervous mechanisms:

1. Alpha adrenergic excitatory nerves
2. Beta-adrenergic inhibitory receptors
3. Cholinergic neurons
4. Nonadrenergic noncholinergic neurons
5. Peptidergic nerves

Alpha Adrenergic Excitatory Nerves

Alpha adrenergic excitatory nerves travel in the hypogastric nerves and maintain sphincter tone via alpha excitatory receptors [13]. In the basal state hypogastric nerves do not play a significant role in the resting internal anal sphincter pressure and rectoanal reflex-induced relaxation. However, there is a significant sympathoexcitation in response to higher volumes of rectal balloon distension [14]. Yamato and Rattan [15] conclude that alpha-2 adrenoreceptors exert important neuromodulatory influences on the rectoanal inhibitory reflex, while alpha-1 adrenoreceptors may exert modulatory effects on the resting internal anal sphincter tone. Adrenergic influences via the hypogastric nerves contribute to the tone of the internal anal sphincter.

Injury to the hypogastric nerves leads to reduced sphincter contraction to 70% of baseline and increased activity in the rectum, whereas after lumbar colonic nerve resection internal sphincter tone decreases to 32% of baseline [16]. Spinal anesthesia or blocking of the pudendal nerves is followed by a decrease of the sphincter tone as is known from patients with myelomeningocele.

Beta-adrenergic Inhibitory Receptors

The pharmacological stimulation of beta-adrenergic inhibitory receptors leads to relaxation of the muscle [17–20].

Cholinergic Neurons

The influence of cholinergic neurons on the sphincter is not yet adequately understood because parasympathetic drugs act differently in different animal species and in the upper and lower segments of the sphincter.

Nonadrenergic Noncholinergic Neurons

NANC inhibitory neurons are situated in the myenteric plexus, and contact neurons of the cholinergic system. They are of great importance both in the peristaltic reflex and in internal sphincter relaxation. This is evidenced by the fact that the rectoanal inhibitory reflex can still be elicited after both sacral nerves and both hypogastric nerves have been severed and the blood supply to the anorectum has been isolated [21]. Their absence causes pathophysiology of the narrow segment and anal sphincter achalasia [5, 22–25]. The NANC innervation of the

internal anal sphincter involves an inhibitory substance generated from the l-arginine-nitric oxide pathway [26–30].

Peptidergic Nerves

Peptidergic nerves seem to play an additional role in internal sphincter relaxation by modifying adrenergic and cholinergic transmission. Neuropeptide Y (NPY), VIP, SP and metenkaphalin lead to relaxation of the colonic smooth muscle [31]. VIP and nitric oxide synthase are present and frequently coexist in neurons of the internal sphincter of the opossum. These neurons may be important too in mediating rectoanal reflex-induced relaxation [26, 32, 33]. According to Fujimoto et al. [34], there are only a moderate number of these fibers in normal internal anal sphincter tissue, whereas these peptide-containing nerves are abundant in the sphincters of patients with neurogenic internal sphincter achalasia. NPY causes an increase in internal anal sphincter pressure and inhibits internal anal sphincter relaxation [31].

The relaxing phase of the peristaltic reflex, mediated by the ganglion cells, manifests itself at the caudal end of the gastrointestinal tract as relaxation of the internal anal sphincter. Manometric evidence of internal sphincter relaxation can therefore be considered as proof of the presence of intramural ganglion cells and a normal neurotransmission in the most caudal segments of the terminal anorectum, and places the diagnosis of aganglionic HD out of the question.

12.3 Comparison of the Internal Anal Sphincter and the Rectum

The internal anal sphincter cannot be regarded as a simple terminal convolution of the circular muscle of the rectum. O'Kelly et al. [35] demonstrated that the response in vitro of the human anal canal longitudinal muscle layer to cholinergic and adrenergic stimulation also shows evidence of sphincter specification of the longitudinal muscle coat. Comparing the histological and pharmacological properties of the rectum and the internal anal sphincter, the following are particular to the anal constrictor. The internal anal sphincter has no or far fewer ganglion cells at least in its lower two-thirds than the adjacent rectum. Tafazzoli et al. [36] provided reference data about the quantitative distribution of nerve cells and ganglia within the submucosal plexus of the

Table 12.1 Summary of the most important anatomical, physiological and pharmacological data on the internal anal sphincter, rectum and aganglionic segment in HD. Inhibitory influence: NANC neurons, peptidergic neurons, inhibitory β-receptors. Excitatory influence: cholinergic neurons (?), α1 and α2 receptors, high norepinephrine content, no spike potentials (*BER* basal electrical rhythm, *GFA* glial fibrillary acid protein, *GRP* gastrin-releasing peptide, *MetEnk* metenkephalin, *NANC* nonadrenergic-noncholinergic neurons, *NCAM* neuronal cell adhesion molecule, *NO* nitric oxide, *NPY* neuropeptide Y, *SP* substance P, *VIP* vasoactive intestinal peptide)

Internal anal sphincter	Rectum	Hirschsprung's disease
Only a few ganglion cells in the uppermost part of the muscle [113]	Normal distribution of ganglion cells (18,000/cm²) [117]	Ganglion cells absent [119]
Dense sympathetic nerve fibers [114]	Normal nerve fiber distribution [113]	No inhibitory NANC neurons [5, 22–24]
No NANC-inhibitory neurons [5, 22, 23]	Low norepinephrine content (0.23 μg/g fresh weight) [114, 116]	No peptidergic neurons storing Enk, GRP [120]
Peptidergic inhibitory neurons storing NPY, VIP, SP, MetEnk, and other peptides	Inhibitory NO storing NANC-neurons [1–4]	Fewer VIP and SP-containing nerve fibers [7, 20]
Inhibitory β-receptors [20, 115]	Peptidergic neurons storing VIP, SP, MetEnk, GRP, histidine, isoleucine, neurokinin A [31–34]	Hypertrophied sympathetic nerve fibers [121]
High norepinephrine content (0.46 μg/g fresh weight) [116]	Few α-excitatory receptors [118]	Hypertrophied parasympathetic nerve fibers [122]
Many α1- and α2-excitatory receptors [13–15]		High norepinephrine content (0.4 μg/g fresh weight) [115, 123]
Cholinergic neurons [21, 115]		Lack of GFA [124]
No spike potentials on the BER [37]		Abnormal expression of NCAM [125]

human anorectum from healthy subjects showing that there is no uniform distribution pattern of ganglia or nerve cells, but a continuous decrease towards the anus. Morphometric analysis has demonstrated the presence of nerve cells and ganglia even in the most distally located segments of the rectum, the region of the anal canal. Although the number of nerve cells and ganglia in these segments is less than 50% of the numbers found in the remaining segments, the distal anorectum is characterized by hypoganglionic conditions and not by aganglionic conditions. It has a denser adrenergic innervation than the neighboring rectal segment and double the norepinephrine content. It is rich in alpha-excitatory receptors, and the NANC inhibitory neurons are absent from its lower two-thirds. In humans the tonic sustained contraction of the muscle is not induced by bursts of spike potentials, in contrast to the findings in many animal species and to the proximal rectum [37–39]. Therefore, the internal anal sphincter cannot be characterized as a megacolon-like narrow segment [40] (Table 12.1).

12.4 Electromanometry

Electromanometry allows the recording of pressure changes around hollow muscular organs of the gastrointestinal tract and the bladder. The intraluminal pressures of the rectosigmoid, rectum and anorectum are transformed into electrical impulses and after appropriate amplification registered by a recorder. This permits the function of the anorectal continence mechanism to be assessed. Of foremost interest are the propulsive motility, and the contractile and opening abilities of the internal anal sphincter. Electromanometry is a screening investigation for chronic obstipation and anal incontinence and should be performed before other examinations such as radiography or transit-time studies are performed, or suction biopsies are taken [41].

12.4.1 Technique of Anorectal Manometry

Electromanometry is a combination of pull-through and three-point measuring procedures. Polyvinyl feeding tubes closed at the tip and with a lateral opening 3 to 5 cm from the tip are usually used as pressure receivers. These are connected to a recording system via pressure transducers and amplifiers. Another type of manometric device is the anal pressure vectography. The advantage of this system, in contrast to other methods, is the three-dimensional reconstruction of the intrasphincteric pressure. This may be useful for measuring the anorectal pressure profile in patients with anorectal malformations after sphincter reconstruction operations and in children with stool incontinence after sphincteromyotomy. However, for the recording of internal sphincter relaxations or propulsive waves one has to switch to other probes. Besides, the pressure values recorded by six circularly arranged single tubes inside one probe differ enormously (Fig. 12.3). Therefore, the calculated mean values are not representative.

Other measuring procedures utilizing large-volume single-balloon systems and small miniballoons connected in series [42, 43] have also proven useful. They only allow the recording of internal sphincter relaxation, not the other manometric parameters, and cause, particularly in small children, marked irritation due to their foreign body effect [44]. For this reason, measurements made with feeding tubes have prevailed. Measuring catheters are introduced into the rectosigmoid, rectum and anorectum, respectively. In addition a stimulating catheter is placed in the rectosigmoid and a Foley catheter with a balloon volume of 30 to 50 cm^3 in the rectum to simulate a bolus effect. Introduction and reliable placement of the rectosigmoid catheters is achieved using an intestinal tube which is withdrawn over the carefully positioned catheters back to the anus. The anorectal catheter is then placed in the rectum and in the sense of a pull-through procedure is slowly withdrawn toward the anus. By this means, the height of the anorectal pressure plateau can be determined and hence accurate positioning of the catheter in the area of the internal anal sphincter achieved. The appearance of typical anorectal fluctuations, that is, slow fluctuating waves expressing the basal electrical activity of the sphincteric smooth muscle cells, confirms the accuracy of the positioning.

12.4.2 Anorectal Pressure Profile

An irrigation catheter withdrawn from the rectum down through the anal canal at a constant rate reveals a constant rise in pressure with the maximum pressure plateau in the caudal portions of the sphincter. At a standardized speed of withdrawal, the length of the pressure profile corresponds to the length of the sphincteric high-pressure zone and its height to the anal constrictive pressure produced under resting conditions mainly (85%) by the internal anal sphincter [41, 45]. The length of the high-pressure zone ranges in normal individuals from 3 to 7 cm (Fig. 12.4).

12.4.3 Internal Sphincter Relaxation

The muscle tone of the internal anal sphincter is myogenic in nature and maintained by the basal electrical rhythm. In contrast to the colon, the slow waves give rise to phasic contractions which are not accompanied by spike potentials (Fig. 12.5). The amplitude of the slow waves in vitro are about 10 mV, and in vivo 200–500 μV. However, there is interindividual variation and most pa-

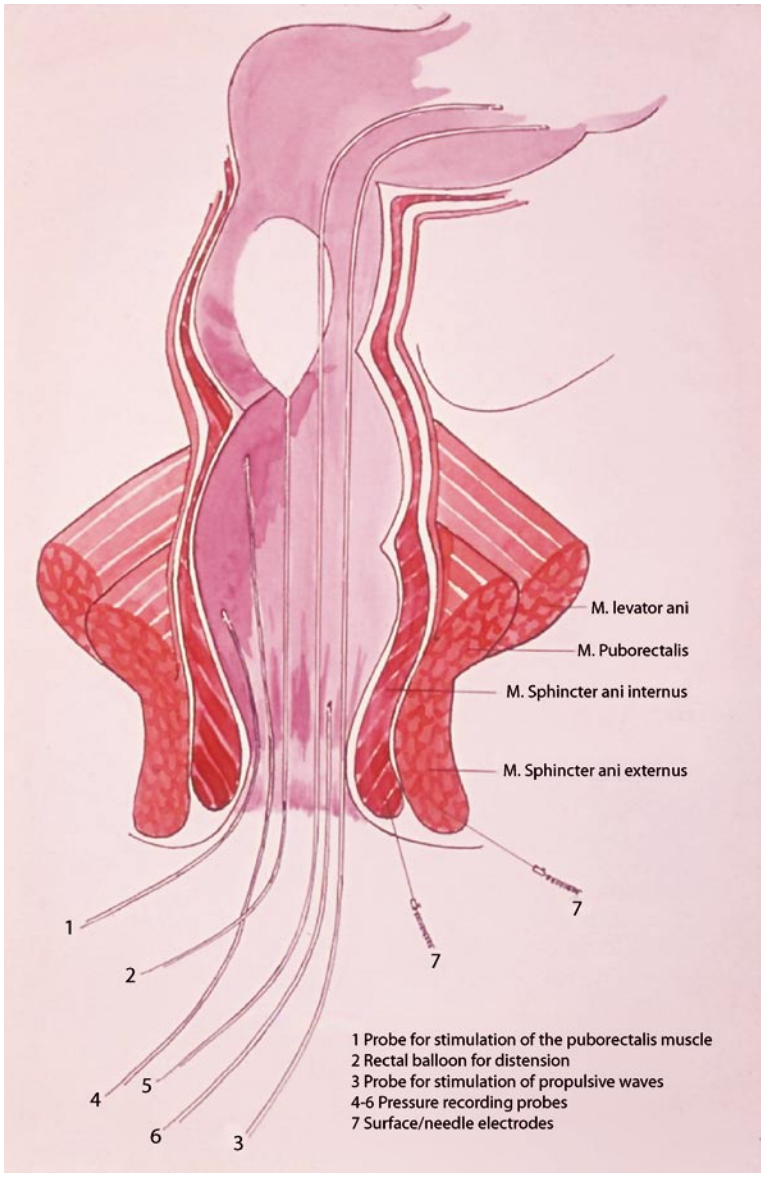


Fig. 12.3 Schematic diagram of the position of the manometric catheters in the rectum and anorectum

tients show a rhythmic increasing and decreasing pattern. According to Wienbeck and Altaparmakov [46], however, the frequency of these pacemaker activity waves remains constant in each individual. The slow wave potentials and mechanical contractions migrate in the oral direction. The frequencies of the electrical control activity are 20 cycles/min at the dentate line, 13.5 cycles/min at the anorectal junction, and 5 cycles/min in the rectum, indicating pacemakers in the smooth sphincter muscle.

The relaxation reflex, a physiological criterion for sphincters, corresponds to the relaxing portion of the most caudal peristaltic reflex, and can be manometrically demonstrated by showing relaxation of the muscle when a balloon is simultaneously distended in the rectum (Fig. 12.6).

The basal electrical activity of the sphincteric smooth muscle becomes desynchronized, and at the same time the mechanical pressure drops. The summation effect is lost, and electrical activity is no longer demonstrable via extracellular leads (Fig. 12.7). As soon as the lowest pressure drop occurs, the electrical and mechanical rhythms reappear, the pressure rises, and sphincter tone is restored. The mean intrasphincteric pressure profile in children is 47±18 mmHg, and the resting pressure in the rectum is 7.5±2.5 mmHg. A rectal distension of a 10-ml balloon should evoke a relaxation of 6 seconds duration.

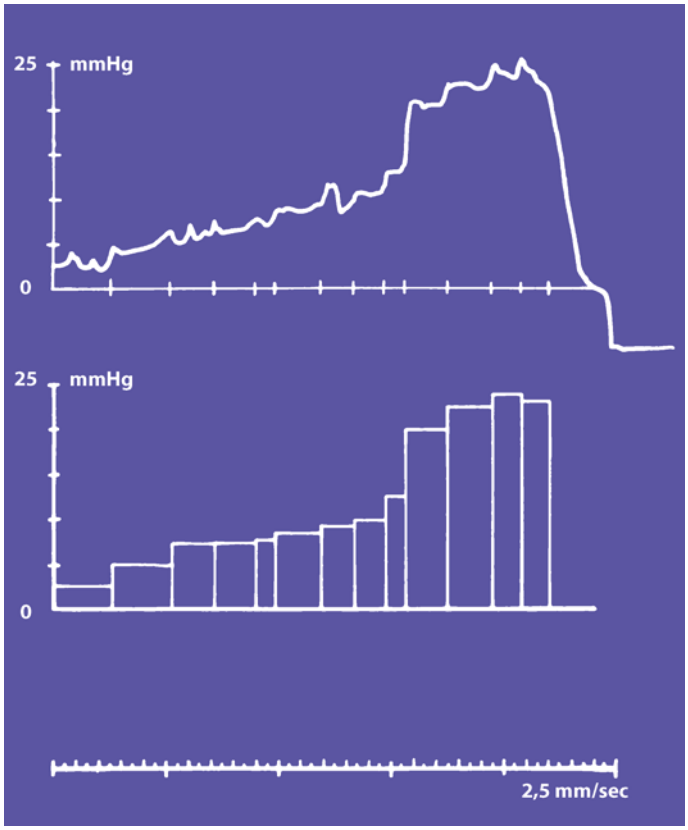


Fig. 12.4 Schematic diagram of the anorectal pressure profile. One horizontal notch represents 1 cm

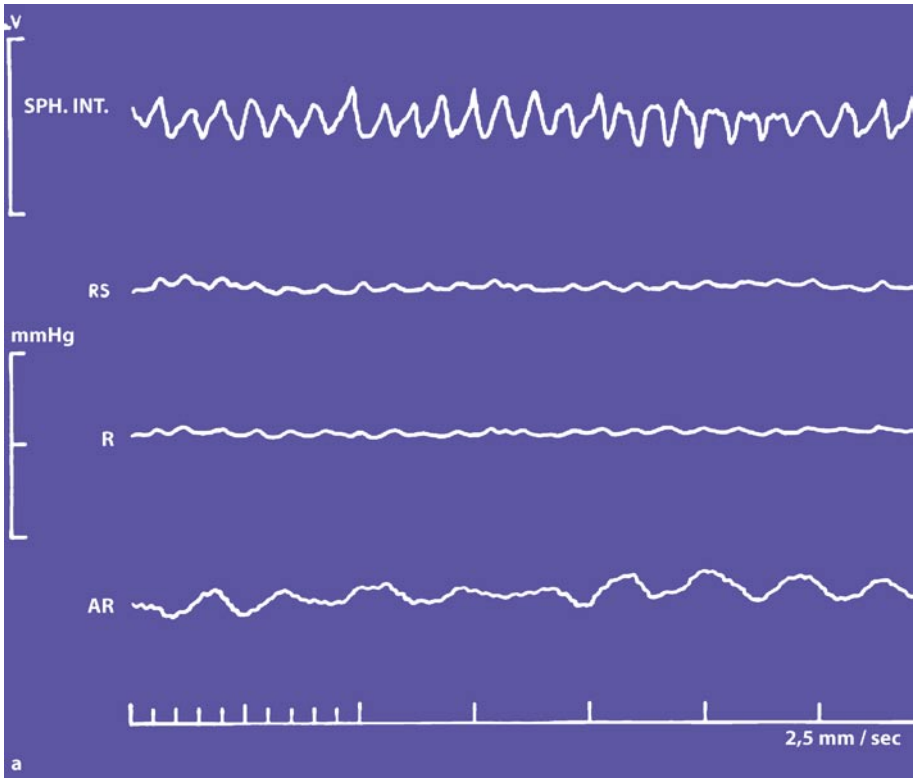


Fig. 12.5a,b Basal electrical rhythm of the internal anal sphincter (SPH.INT) : **a** constant pattern of the slow waves; **b** *see next page* (*RS* rectosigmoid, *R* rectum, *AR* anorectum)

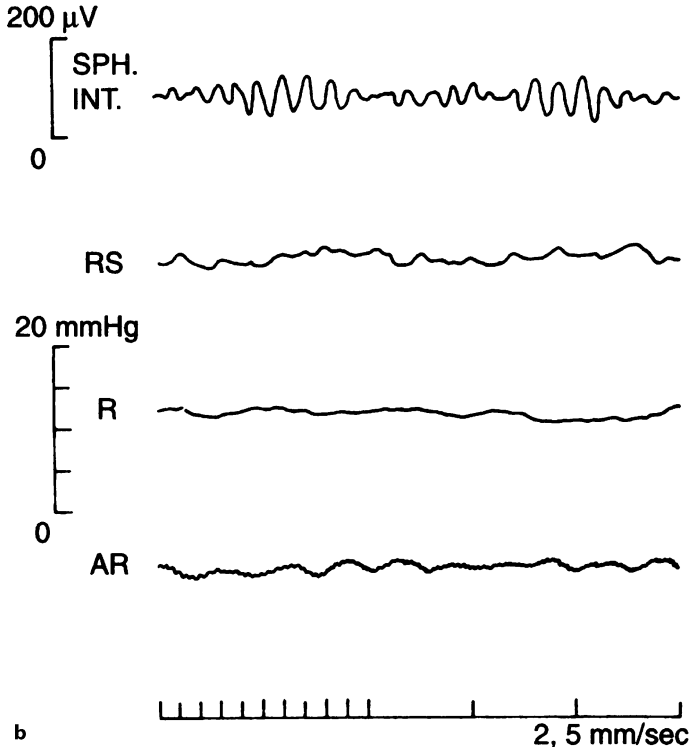


Fig. 12.5a,b *(continued)* Basal electrical rhythm of the internal anal sphincter: **b** wave-type pattern (*AR* anorectum, *R* rectum, *RS* rectosigmoid)

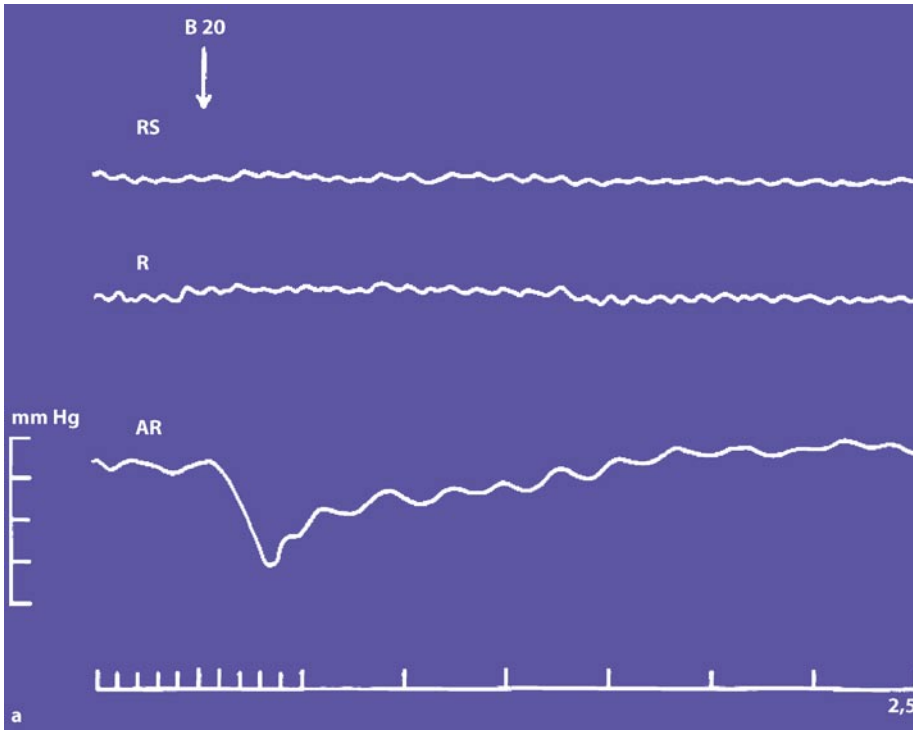


Fig. 12.6 Normal internal anal sphincter relaxation after distension of a rectal balloon (B) with 20 ml of air (*RS* rectosigmoid, *R* rectum, *AR* anorectum). Note: the fluctuating waves of the anorectum resume at the deepest point of the relaxation!

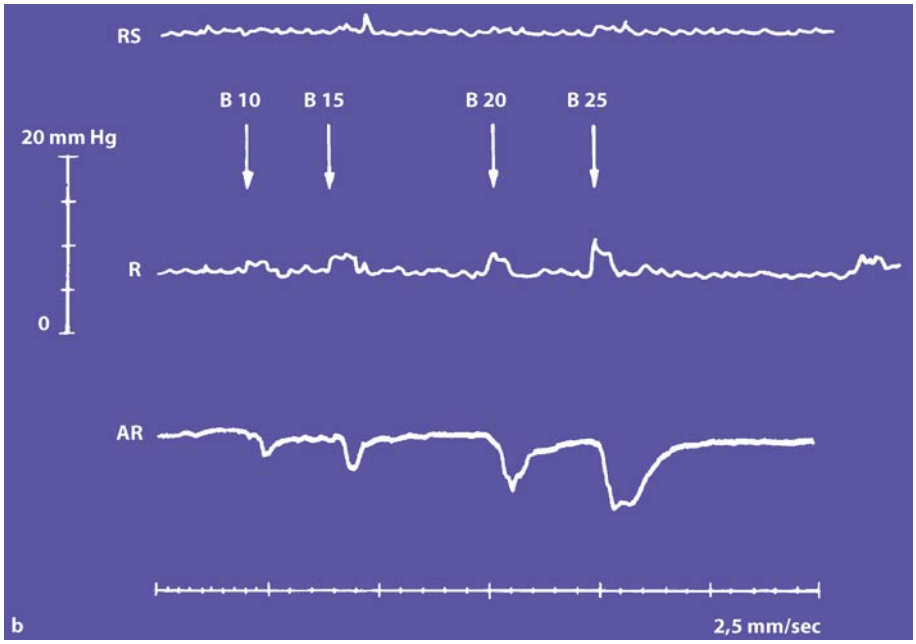


Fig. 12.6 (*continued*) **b** Direct proportionality between the rectal distension volume and the depth and length of the internal sphincter relaxations (*AR* anorectum, *B* balloon, *B10* distension volume 10, 15, 20, 25 ml, *R* rectum, *RS* rectosigmoid)

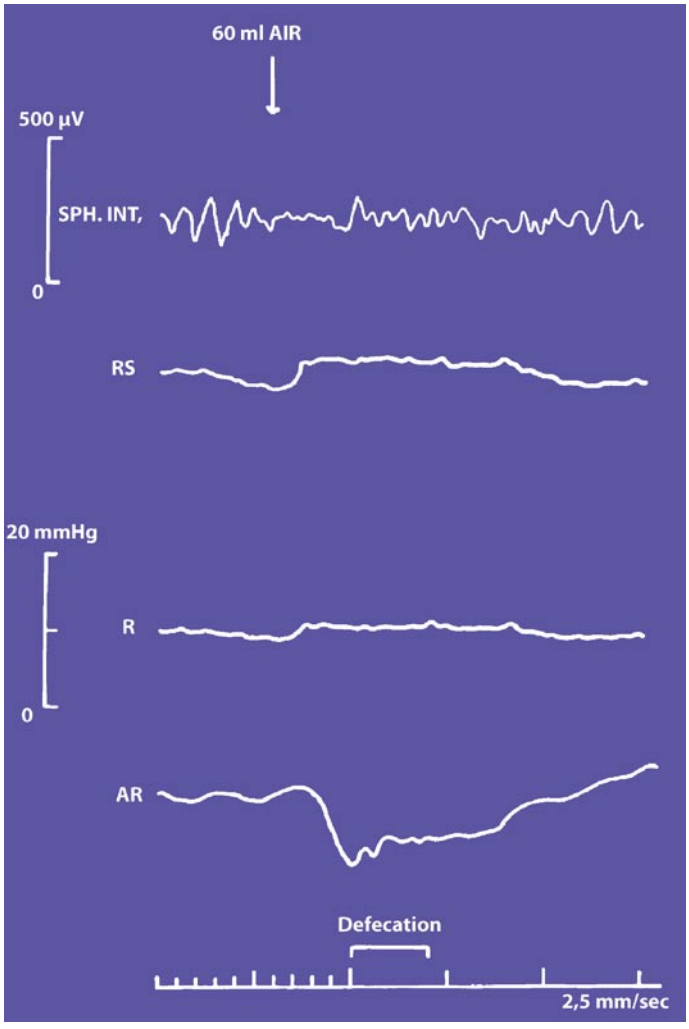


Fig. 12.7 Simultaneous inhibition of electrical and mechanical activity of the internal anal sphincter (SPH.INT) after injection of 60 ml air into the rectosigmoid (*RS*). Slight adaptation reaction in the rectosigmoid and rectum (*R*). Once the lowest point of relaxation is reached, electrical and mechanical activity resumes (*R* rectum)

12.4.4 Continence Reaction

Simultaneous recording of electrical activity in the striated sphincter muscles reveals a substantial increase in activity during internal sphincter relaxation. This is the rectosphincteric reflex (Fig. 12.8).

This reflex serves to constrict the anal canal opened by the relaxation in order to prevent stool soiling. Transient opening of the anal canal for a few stool particles is necessary so that discrimination of gaseous, liquid or solid bowel contents in the upper anal canal is possible. Manometrically, this reflex contraction of the external anal sphincter is expressed as a contraction spike during or at the end of internal sphincter relaxation, which is termed the continence reaction. The simultaneous contraction of the puborectalis muscle is also included in this continence reaction (Fig. 12.9). The simultaneous contraction of the puborectalis muscle, however, can be tested more exactly by direct stimulation of the muscle with 0.1 ml of physiological saline solution [47]. Under resting conditions the external anal sphincter contributes only up to 15% to the anorectal pressure barrier, but up to 60% during sudden rectal distension. The tone of the sphincter is reflected by the intensity of its spike potential discharge and increases even with breathing. The only exception to this very sensitive increase in spike potential is during defecation which is induced by contraction of the abdominal wall muscles followed by simultaneous interruption of the electrical and mechanical activities in both the internal and external anal sphincters (the defecation reflex; Fig. 12.10). The striated muscle contains two types of muscle fibers: type I fibers for tonic contraction and type II fibers for phasic contraction. The proportions and distribution vary from fetal life to adulthood [48] (Figs. 12.11 and 12.12).

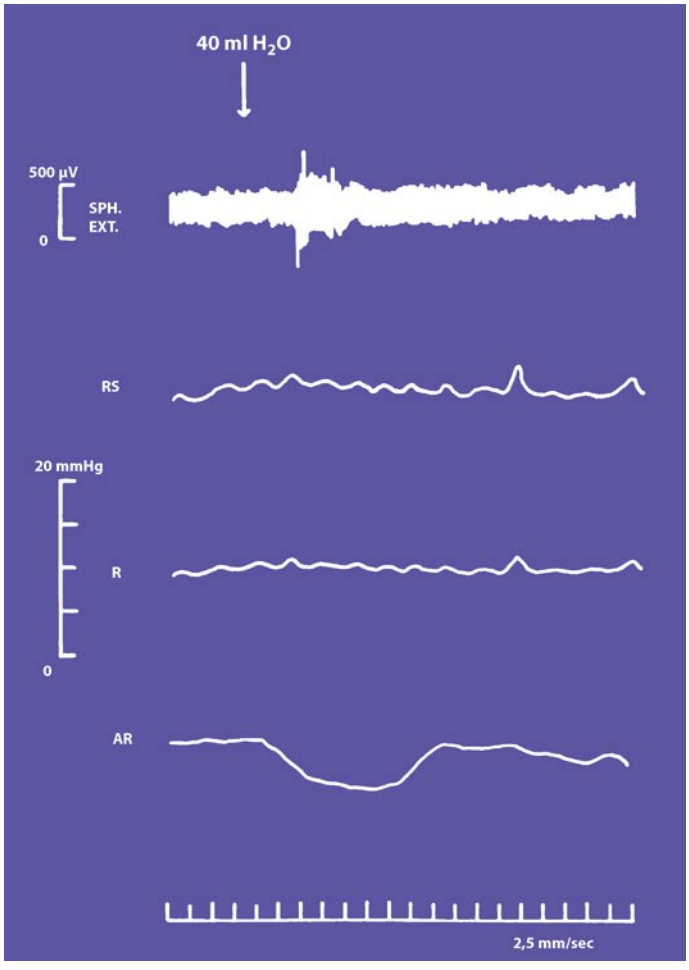


Fig. 12.8 Increased activity in the external anal sphincter during internal sphincter relaxation produced by injection of 40 ml water into the rectosigmoid (rectosphincteric reflex) (*AR* anorectum, *R* rectum, *RS* rectosigmoid)

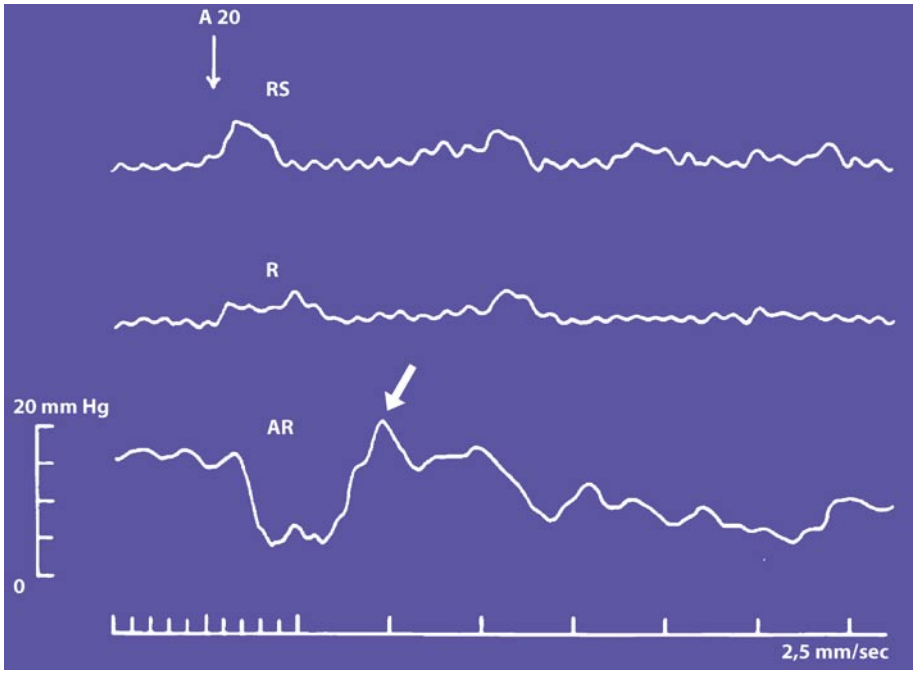


Fig. 12.9 Continence reaction (*CR*): the injection of 20 ml air (*L20*) into the rectosigmoid (*RS*) is followed by deep relaxation in the anorectum (*AR*). There is a subsequent distinct contraction spike in the anorectum (*arrow*), expressing the contraction of the striated sphincter and puborectalis muscles (*R* rectum)

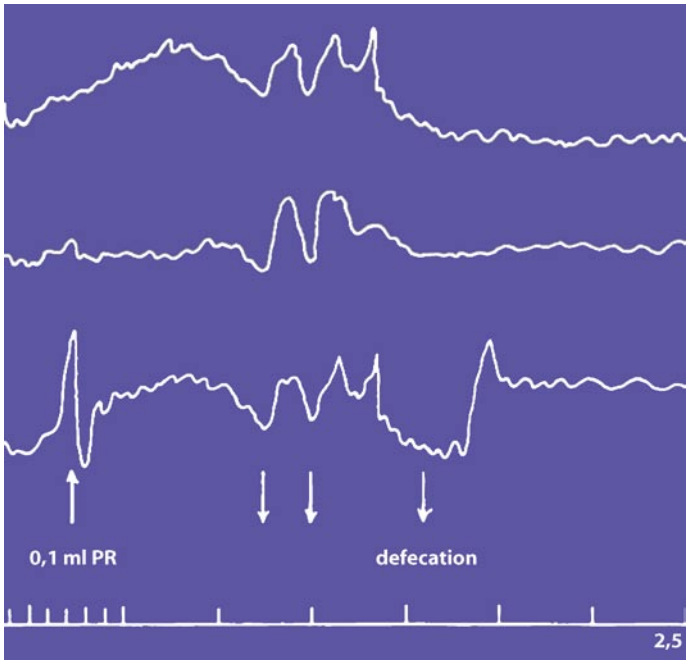
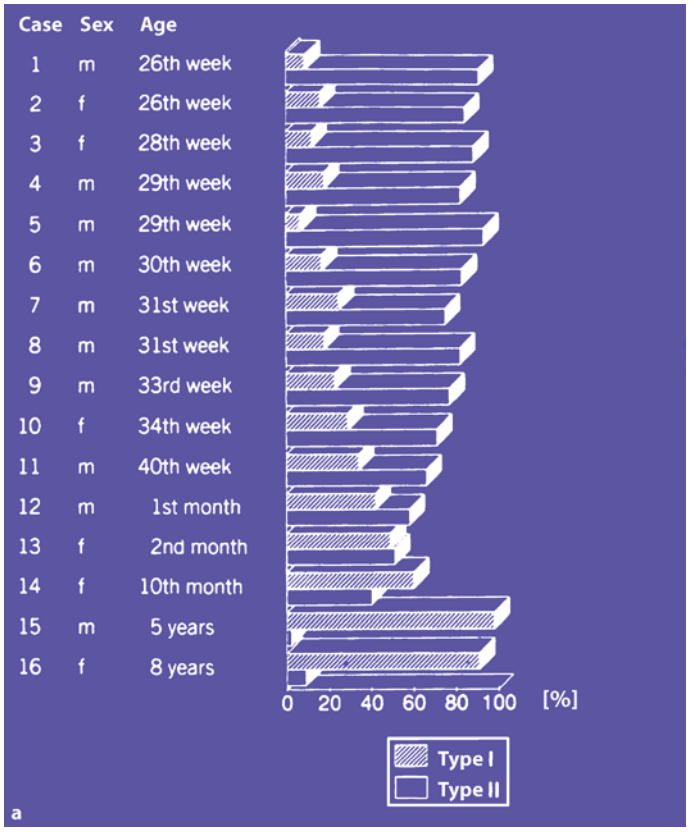


Fig. 12.10 Defecation reflex: simultaneous interruption of the electrical and mechanical activities in both the internal and external anal sphincters stimulated by injection of 0.1 ml of physiologic saline solution at the puborectalis sling (*PR*), lead to the propagation of a propulsive wave which is followed by a drop in the anorectal pressure and recurrent defecation of small amounts of liquids



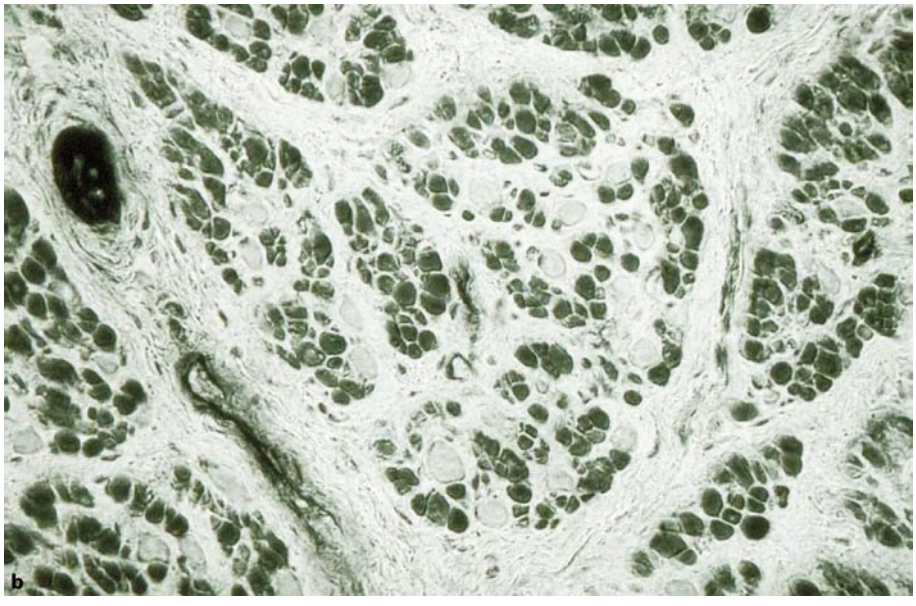
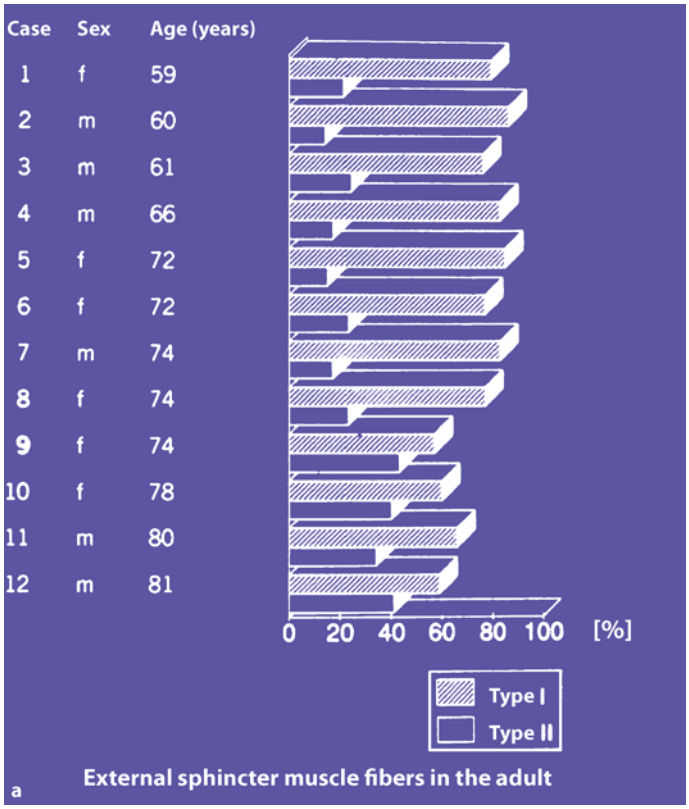


Fig. 12.11 **a** Relative Distribution of type I (tonic) and type II (phasic, rapid) fibers in infants, fetuses and children. **b** Cross-section through external anal sphincter of 29-week fetus. Large type I fibers (clear) surrounded by small type II fibers (dark). ATPase reaction at pH 10.4, enlarged 100× (taken from 48). In Fetuses rapif Type II muscle fibers predominate. With increasing age infants Type I fibers increased and showed a predominace in adults



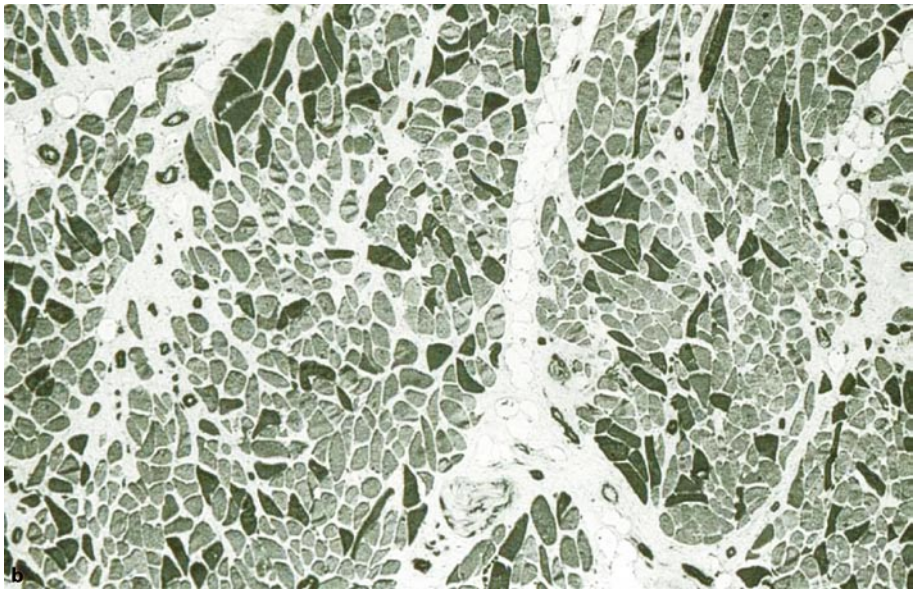


Fig. 12.12 **a** Relative distrubution of Type I and Type II muscle fibers in adults; **b** Predominace of Type I fibers (clear);

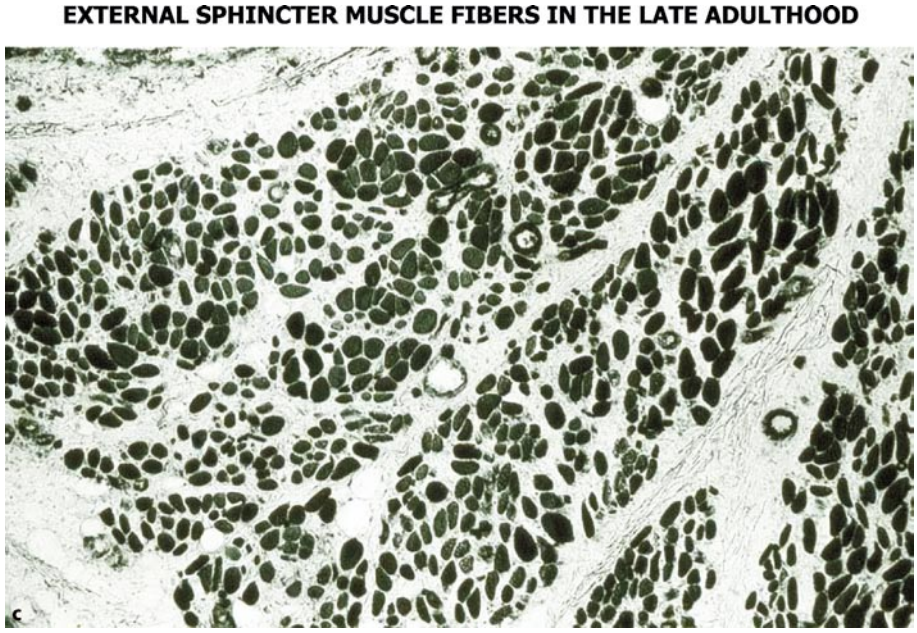


Fig. 12.12 *(continued)* **c** Increasing proportion of Type II (rapid, dark) muscle fibers. The pattern of late adulthood corresponds to the findings in newborns

12.4.5 Rectal Motility

Insertion of one measuring catheter into the rectum and another about 5 to 10 cm above the first into the rectosigmoid, or insertion of a probe with at least three sideholes placed at a distance of 3 to 5 cm according to the age of the patient permits observation of the segmental and propulsive contractions of the colon. When the rectosigmoid is stimulated with water or air, a rapid pressure increase followed by a slow pressure decrease occurs in the rectum, an expression of the plastic adaptive ability of this organ to sudden changes in pressure. Manometrically, this reaction is called the adaptation reaction (Fig. 12.13). The quotient of volume difference and pressure difference is designated as rectal compliance, and can to some extent indicate the elasticity of the rectum (Fig. 12.14). Thus, the compliance is distinctly elevated in a greatly dilated secondary megacolon, while it is below 1 ml/mmHg in a "rectal colon" after abdominoperineal pull-through.

12.5 Pathological Electromanometric Criteria

Indications for anorectal manometry are the differential diagnosis of HD and chronic constipation and for the functional analysis of fecal incontinence.

12.5.1 Habitual Chronic Constipation

In habitual chronic constipation, marked segmental contractions are found in the rectum which lead to spontaneous internal sphincter relaxations. The amplitude of the relaxations is directly proportional to that of the contractions in the rectal waves (Fig. 12.15).

12.5.2 Neurovegetative-Psychogenic (Functional) Anal Sphincter Achalasia

In neurovegetative-psychogenic or functional anal sphincter achalasia, the opening ability of the internal anal sphincter is interrupted or prevented by a simultaneous voluntary contraction of the striated sphincters and pelvic floor muscles. Even with large stimulating volumes, only rudimentary relaxations accompanied by voluntary contraction spikes before, during or after internal sphincter relaxation can be observed. Their appearance is a sign that the child is not willing to defecate, but is retaining stools. If the patient's attention is diverted, however, normal internal relaxation patterns can be observed during the same session (Fig. 12.16). Decisive in the diagnosis of functional anal sphincter achalasia is thus the simultaneous appearance of both rudimentary and normal relaxation patterns. About 90%

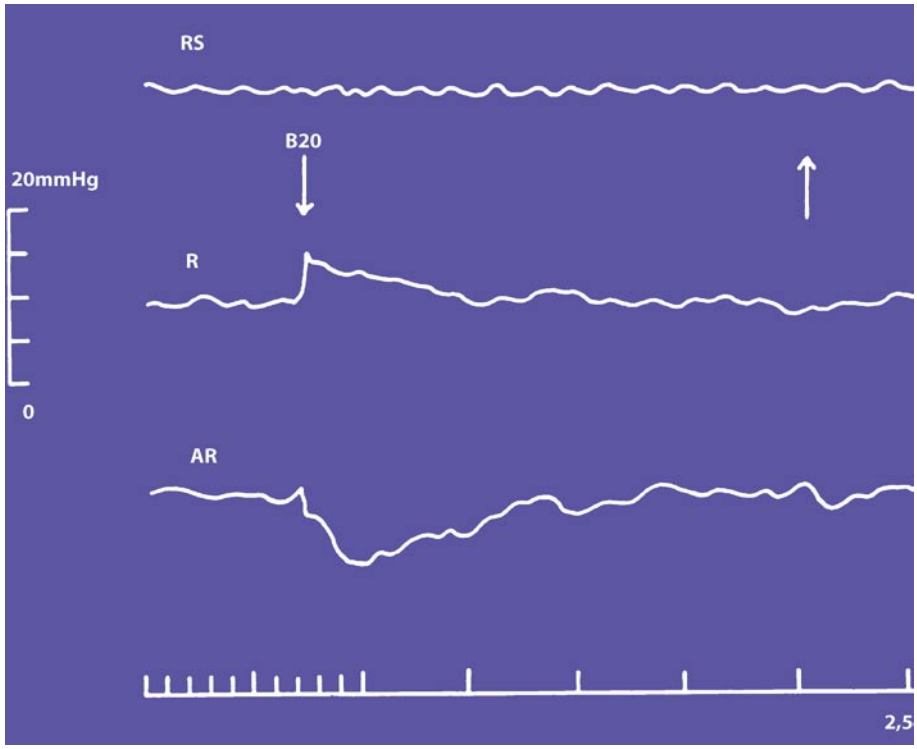


Fig. 12.13 Adaptation reaction: after distending a rectal balloon (B) with 20 ml of air. Definite adaptation reaction is seen in the rectum (*R*), with a rapid pressure increase and slow decline to resting values. In addition, there is relaxation in the anorectum (*AR*), (*RS* rectosigmoid)

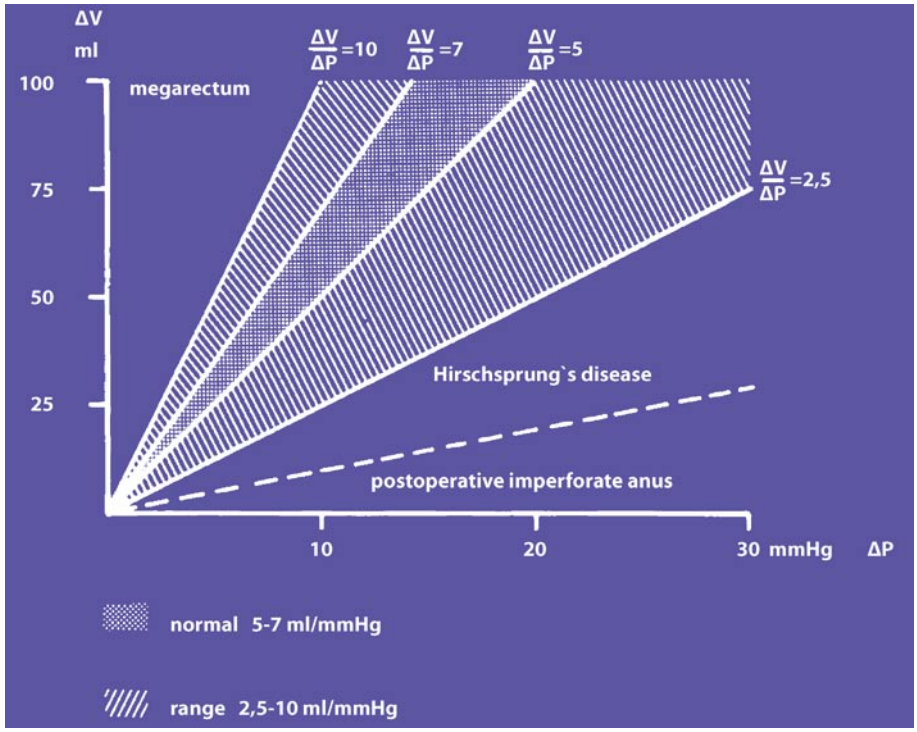


Fig. 12.14 Schematic diagram of rectal compliance: >10 ml/mmHg in the distended rectum in HD, <2.5 ml/mmHg after surgical correction of high anal atresia, 2.5–10 ml/mmHg normal range

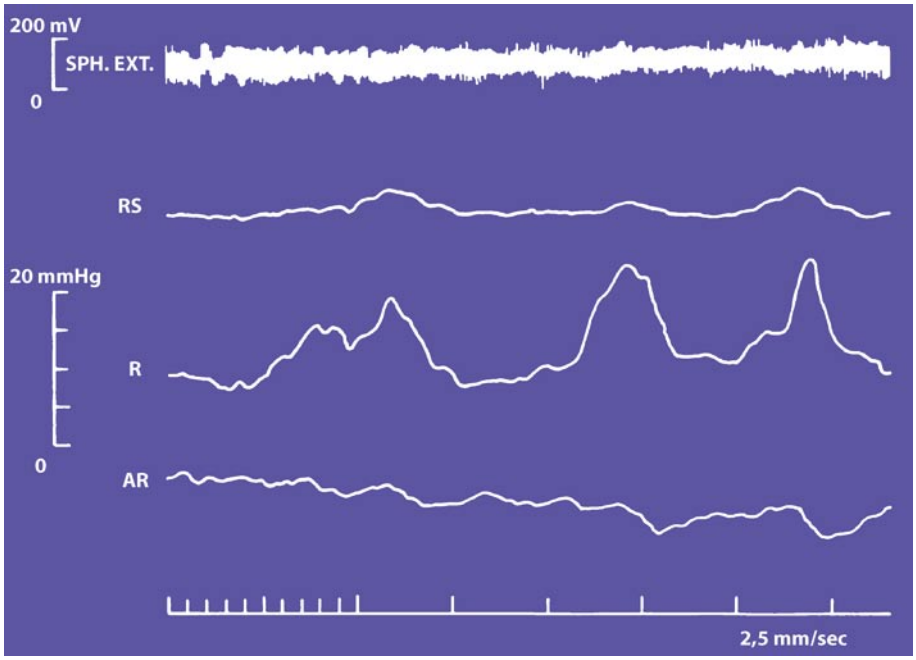


Fig. 12.15 Habitual constipation: high segmental and propulsive contractions in the rectum (*R*) lead spontaneously to internal sphincter relaxations in the anorectum (*AR*), whose amplitude is proportional to the intensity of the rectal contractions, (*RS* = rectosigmoid, SPH.EXT. = external anal sphincter EMG)

of all constipated children suffer from functional anal sphincter achalasia.

12.5.3 Myogenic Anal Sphincter Achalasia

In organic-myogenic anal sphincter achalasia, the sphincter muscles have fibrosed due to previous inflammations, abscesses, fissures, fistulas, chronic diarrhea, lacerations etc. The sphincter can no longer open wide enough to allow adequate defecation to occur. The inflammation spreads to the internal anal sphincter, causing progressive fibrosis of the smooth muscle [49] which can proceed to total sclerosis. Electromanometrically, rudimentary sphincter relaxations with reduced amplitude and shorter duration are found in this form of anal sphincter achalasia. The direct proportionality between the amplitude and duration of relaxation and the rectal distension volume is abolished (Fig. 12.17). Normal patterns of relaxation are no longer observed, although the relaxation reflex is still demonstrable. Myogenic sphincter achalasia is however very rare in children. Since our report in 1973, we have operated on no further patients with this diagnosis [50].

12.5.4 Neurogenic Anal Sphincter Achalasia and Hirschsprung's Disease

Neurogenic anal sphincter achalasia occurs to a different degree in all patients with neuronal intestinal malformations such as aganglionosis, intestinal neuronal dysplasia (IND), hypoganglionosis, immaturity of ganglion cells, hypogenesis and others. In children with aganglio nosis restricted to the sphincter and lowermost parts of the anal channel it corresponds to a megacolon with an ultrashort segment, and behaves manometrically like a congenital Hirschsprung's megacolon. Since the basic pathophysiology in aganglionic megacolon is the absence of intestinal nervous plexus and thus neither ganglion cells nor inhibitory NANC neurons are present, the intestinal inhibitory reflex of the internal anal sphincter cannot be elicited [41, 45] (Fig. 12.18). Normal peristalsis is not possible. Whereas the demonstration of internal sphincter relaxations excludes the presence of HD, the absence of the relaxation reflex is only pathognomonic when the anorectal fluctuations typical of the smooth muscle cells of the internal anal sphincter are observed prior to and after relaxation and when the patient is not a newborn less than 14 weeks of age [51]. In newborns the internal sphincter relaxation reflex may be absent or rudimentary due an immaturity of its nerve supply. According to Wood [52], the discharging frequency of the action potentials in the aganglionic segment increases from its proximal to its distal parts. This leads to an increased contractile tendency in the aganglionic bowel segment and to manometrically demonstrable multisegmental mass contractions (Fig. 12.19).

12.5.5 Intestinal Neuronal Dysplasia

In patients with IND no pathognomonic morphology of the relaxation reflex exists. The reflex mechanism may be

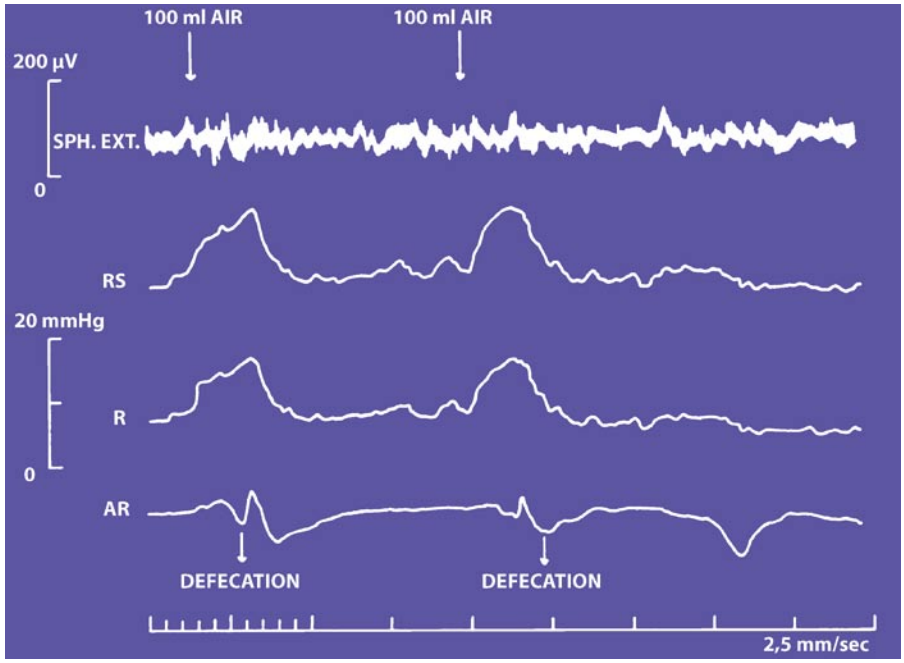


Fig. 12.16 Neurovegetative-psychogenic (functional) anal sphincter achalasia: simultaneous occurrence of spontaneous normal internal sphincter relaxation and rudimentary relaxations take patterns in the anorectum (*AR*) after injection of 100 ml air into the rectosigmoid. The rudimentary relaxation takes place due to simultaneous voluntary contraction of the puborectalis muscle and external anal sphincter, meaning an increased continence reaction, during internal sphincter relaxation, (SPH.EXT. external anal sphincter EMG, *RS* rectosigmoid, *R* rectum, *AR* anorectum)

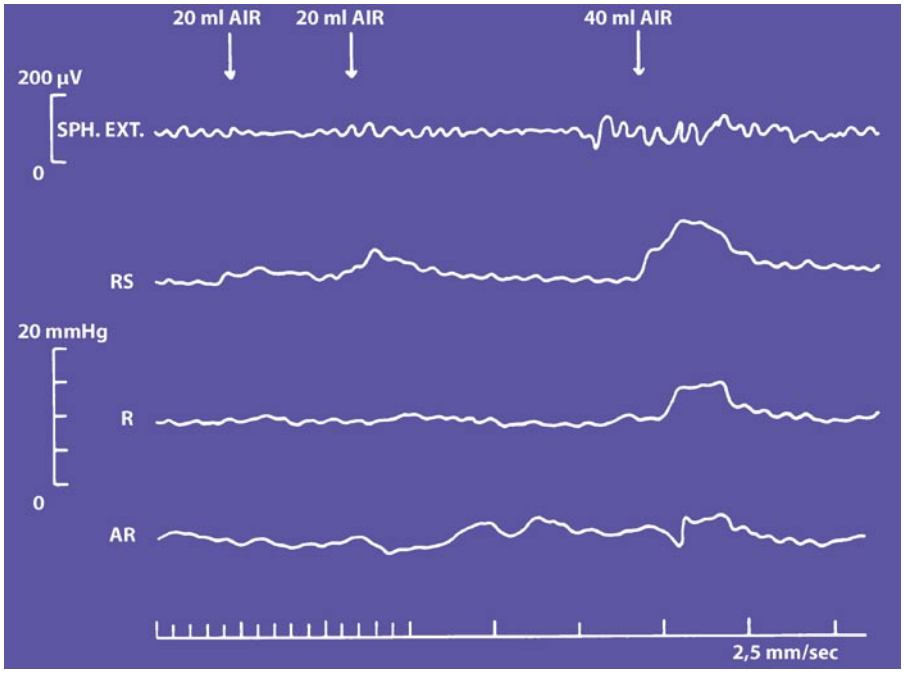


Fig. 12.17 Myogenic anal sphincter achalasia: rudimentary internal anal sphincter relaxations after injection of 20–40 ml air into the rectosigmoid (*RS*). The direct proportionality between the distending volume and the amplitude of the relaxation is abolished. Unobtrusive relaxations, such as those seen in functional achalasia, are not observed, (SPH.EXT. external anal sphincter EMG, *RS* rectosigmoid, *R* rectum, *AR* anorectum)

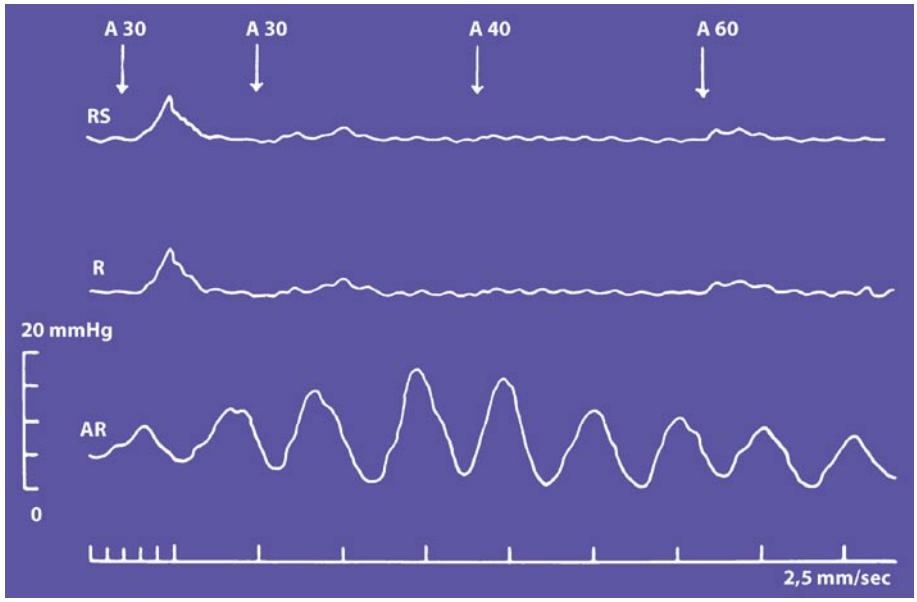


Fig. 12.18 Congenital Hirschsprung's megacolon: absence of internal sphincter relaxation during the injection of increasing volumes of air into the rectosigmoid (*RS*). Strikingly high anorectal fluctuations are apparent in the anorectum (*AR*), (*R rectum*)

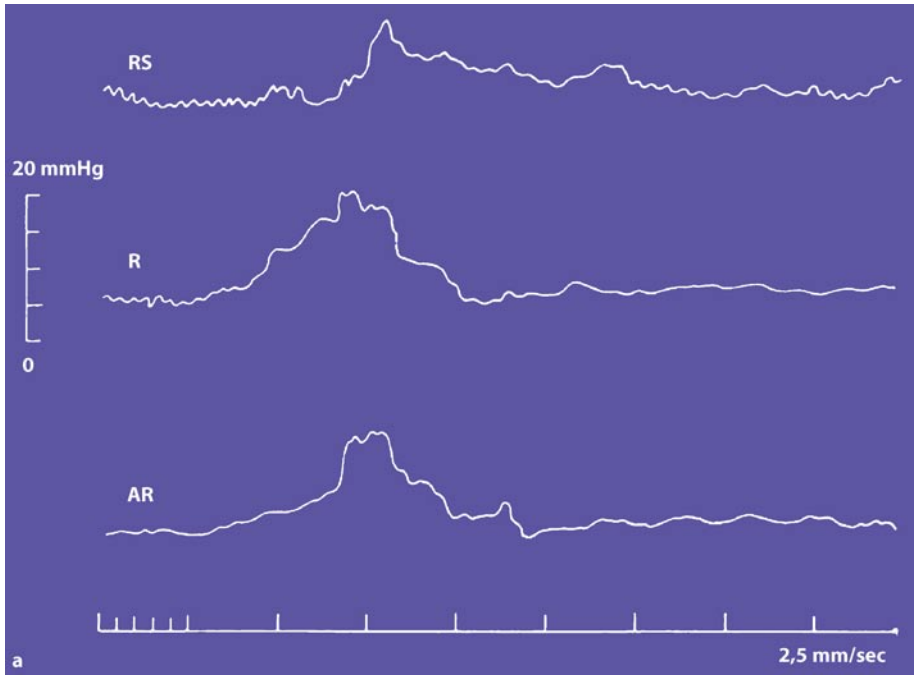


Fig. 12.19 **a** Spontaneous multisegmental mass contractions in the rectosigmoid, rectum and anorectum in congenital Hirschsprung's megacolon, **b** *see next page*

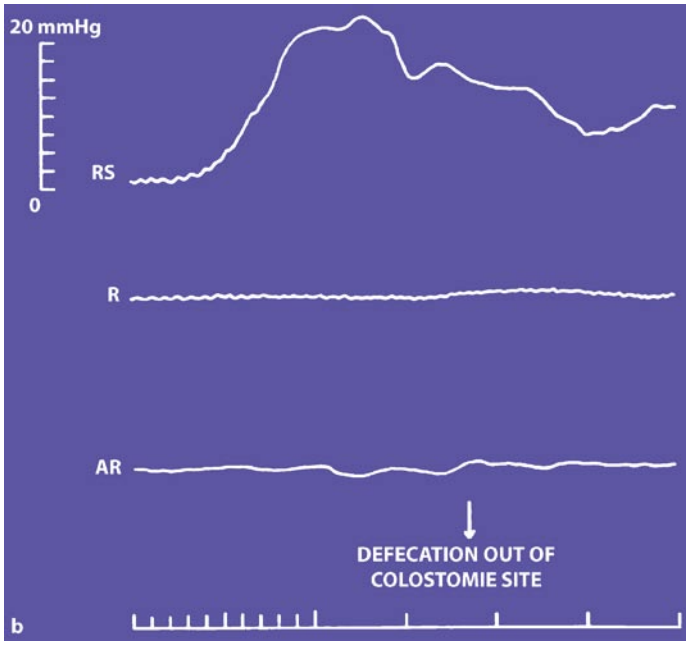


Fig. 12.19 *(continued)* **b** Spontaneous multisegmental mass contractions in the rectosigmopid (*RS*); no propagation of the wave in the rectum (*R*) and anorectum (*AR*); Defecation out of the colostomie site.

normal, rudimentary or absent (Fig. 12.20). The same is true for hypoganglionosis and immaturity of ganglion cells.

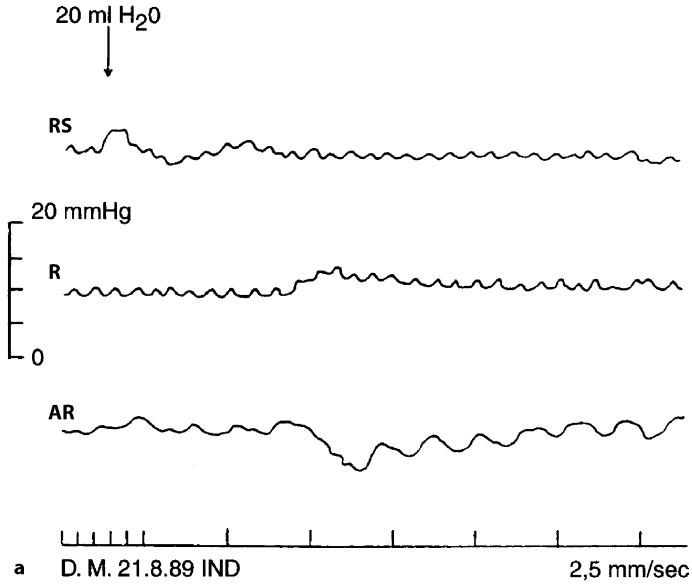
The internal anal sphincter sometimes also has an elevated tone with an increased anorectal pressure profile (Fig. 12.21). This can be true in HD as well as in hypoganglionosis and IND [53–55].

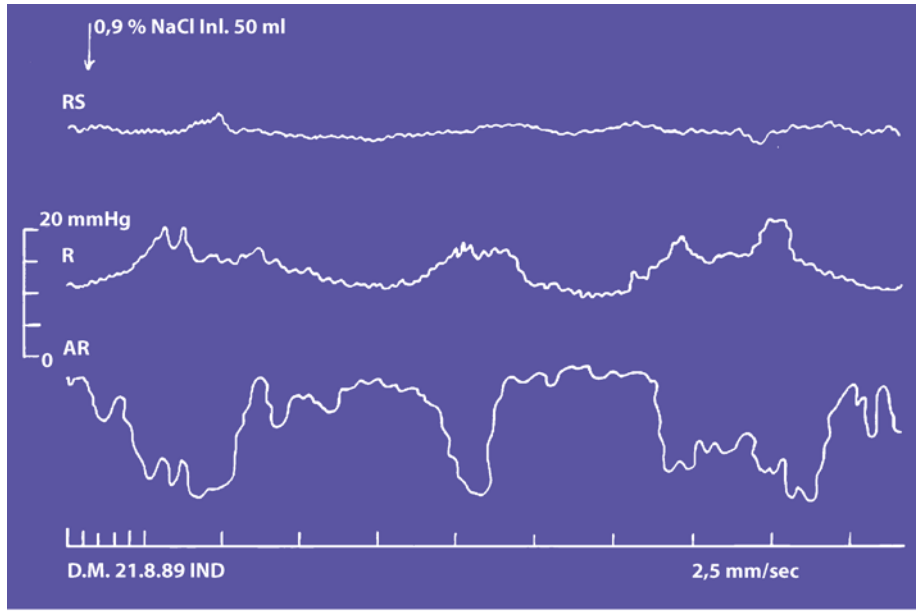
12.6 Potential Electromanometric Errors

The absence of internal sphincter relaxation is, then, crucial for the diagnosis of aganglionic megacolon. Demonstration of genuine internal sphincter relaxation with anorectal fluctuations at the beginning of the relaxation reflex and after return to the resting pressure level excludes the diagnosis of HD. The absence of internal sphincter relaxations cannot, however, be assumed to be reliable evidence of HD. One should keep in mind that in newborns, internal sphincter relaxation can physiologically be absent up to the 14th day of life or longer. It has been shown by measurements in premature infants weighing 1400 to 1900 g at gestational and composite ages of from 35 to 51 weeks that there are great variations in the rate of maturing of anorectal reflex activity which is dependent on both individual and environmental factors [51]. In that study, we found some premature infants who had internal sphincter relaxations as early as 35 weeks of age and others who lacked it after the 41st week. Traumatic skull fractures at delivery [56] and respiratory diseases [57], by way of example, can lead to delays in the maturing of the intramural plexus due to intrauterine hypoxia, causing signs of intestinal obstruction in the newborn.

In addition, the pre- and postnatal development of the rectal ganglion cells and nerve fibers varies enormously from one individual to another [58]. According to studies by Munakata [59] the acetylcholinesterase-positive nerve fibers first develop at 6 to 9 months, which likewise speaks for immaturity of the intestinal intramural plexus. For this reason, the authors recommend that the acetylcholinesterase preparation should be omitted and a silver stain done instead. According to Bughaighis and Emergy [60], at birth two-thirds of all intestinal neurons are immature, and maturation is finished not before the 5th year of life.

Electromanometrically, the type of obstruction due to delayed maturing of the nervous plexus cannot be distinguished from other neuronal intestinal disorders. Howard and Nixon [61] reported six newborns in whom laparotomy was performed with the diagnosis of acute abdomen. The configuration of the colon and rectosigmoid was similar to that found in HD: a zone of abrupt narrowing followed by proximal dilatation. Colostomy was performed and a biopsy taken which showed unremarkable bowel with ganglion cells present, so that the colostomy could subsequently be closed. Today we would suggest that these children were suffering from IND. We have observed the same clinical course in three children with histologically proven IND [62]. Besides, it seems possible that the small left colon syndrome may also be related to a similar maturational disorder, whereby in-





b **RS =Rectosigmoid; R = Rectum; AR = Anorectum**

Fig. 12.20a,b Reduced and enlarged internal sphincter relaxations in a patient with IND a normal internal sphincter relaxation, but reduced amplitude; **b** internal sphincter relaxations enlarged in amplitude and duration after injection of 20 ml (**a**) and 50 ml (**b**) of physiologic saline solution into the rectosigmois (RS). (*R* rectum, *AR* anorectum)

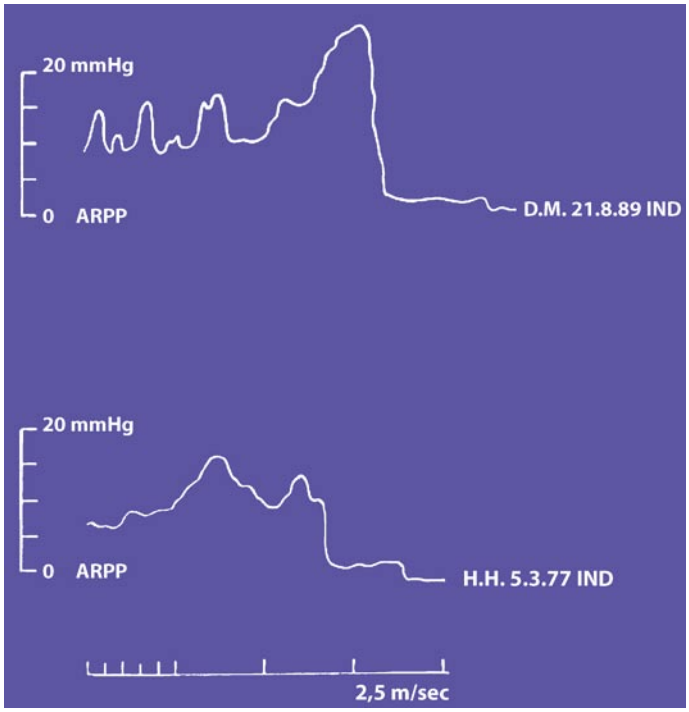


Fig. 12.21 Different values of the anorectal resting pressure profile in two patients with IND

creased condensation of stool due to maternal diabetes may play an additional role.

A further limitation is that catheters which are open at the tip occasionally become occluded. They can only be irrigated very cautiously in newborns, however, since even the small volumes used can induce defecation. For these reasons the value of manometry is limited in newborns whereas it is superior to all other diagnostic procedures except histochemistry in older children..

12.7 Accuracy of Electromanometry

Schnaufer [63] and Tobon et al. [64] consider anorectal manometry to be an absolutely reliable method which can be used with up to 100% accuracy for the diagnosis of aganglionosis. Arhan et al. [65] also reached similar conclusions. Internal sphincter relaxation was demonstrable in all their patients with so-called functional megacolon and was absent in all patients with HD. Frenckner and Euler [66] likewise reached the correct diagnosis of HD with manometry in all their patients with no false results, and similar findings were reported by Tamate et al. [67] and Verder et al. [68]. However, the findings of Meunier et al. [69] and von Issendorff [70] indicate that a certain degree of caution should be exercised in interpreting electromanometric findings in newborns and premature infants. Meunier et al. [69] found nine false-negative and six false-positive results in children from 3 to 31 days of age and seven premature infants. This would mean a diagnostic failure rate of 71.4% in premature infants and 26.4% in infants up to 31 days of age. Penninckx et al. [71] demonstrated 4% false results in 261 consecutive patients. In 11% the manometric result was equivocal. The value of anorectal manometry was most limited below the age of 1 month. According to Iwai et al. [72] a definitive diagnosis in patients with chronic obstruction was obtained in 95% of the patients, whereas in the neonatal period the diagnosis was obtained only in 81% of the children. Sumomito et al. [73] therefore recommend clarifying obscure rectoanal reflexes by the administration of prostaglandin F2 alpha. Since Holschneider et al. [51] have shown that internal anal sphincter relaxation is physiologically not demonstrable prior to the 14th day of life and the maturation of the relaxation reflex can also be delayed, these findings are not surprising.

Studies by Munakata [59] also indicate that acetylcholinesterase staining begins increasing in intensity, and is thus of diagnostic value, between the 6th and 9th month of life. Meunier and co-workers [69, 74] accordingly found only two false-positive and one false-negative result in the 1- to 6-month age group, a distinct failure rate of 7.7%. This rate decreased to 2% to 3% in older children. Von Issendorff [70] studied the value of different anorectal parameters. No normal or atypical internal anal sphincter relaxation could be demonstrated in any of his patients, although atypical or absent relaxations did occur in 5 of 19 patients with chronic constipation. These false-positive results were, in the authors opinion, attributable to technical errors. Mass contractions were never observed in the patients with chronic constipation, but occurred in 40% of those with aganglionosis. The

anorectal pressure profile, at an average of 31.9 mmHg, was definitely higher in the patients with HD than in those with chronic constipation, who showed a pressure of 22.4±7.3 mmHg. Propulsive waves could never be elicited in the patients with aganglionosis and very rarely in children with IND or hypoganglionosis. The adaptation reflex was sometimes normal and sometimes atypical, and the compliance, at an average of 5.1 mmHg, was markedly lower than the average 14.8 mmHg found in the patients with chronic constipation. In a more detailed analysis we came to the same result [41].

We have not managed to achieve the convincing 100% accuracy of Tobon and Schuster [75] in our studies, but have found an electromanometric accuracy of 96% in both comparative histological/manometric studies [49] and comparative roentgenological/manometric studies [76]. The roentgenological misdiagnosis rate, on the other hand, was 25%, and that for histology 4 to 6%. The accuracy of 232 electromanometric tracings in the same number of patients with HD, chronic constipation, anal atresia and myelomeningocele, was 87.2% with only 9.4 faulty classifications [77]. Boston and Scott [78] attained an accuracy of 92% in 63 newborn infants.

The differing results from anorectal manometry are undoubtedly due to different degrees of experience of the individual authors with the manometric technique, to technical difficulties, particularly in the newborn period, and to the physiological range of variation in the appearance of internal sphincter relaxation. Since manometry is an innocuous and simple examination, however, it should definitely be employed as a screening method for all types of defecation disorders. Martin et al. [79] prefer total colonic manometry to measure directly intraluminal pressures and contractile functions of the entire colon in patients with functional colonic obstruction. Manometric tracings were obtained while fasting, after feeding, and after pharmacological stimulation. They concluded that total colonic manometry can be valuable in deciding the need for and timing of diversion, the extent of resection required, and the suitability of the patient for restoration of bowel continuity in refractory functional obstruction. In all uncertain cases, suction biopsies should be taken. In addition radiographic and transit-time studies are essential in order to dry to determine the length of the aganglionic bowel segment and bowel motility.

Yang and Wexner [80] evaluated 50 consecutive patients with fecal incontinence by anal pressure vectography, electromyography and anal sonography during the same visit. Of the 50 patients, 34 (68%) showed global defects of the sphincters on cross-sectional vectograms. out of 46 patients, 36 had isolated decreased electromyographic activity in a single quadrant. However, only 5 of 38 patients (13.2%) had the same defect localized by anal pressure vectography. In addition, 33 of these patients had anal ultrasonography, and 27 of them showed anal sphincter defects. However, only 3 of these 27 patients (11.1%) had the same defect localized by anal pressure vectography. The authors concluded therefore that anal pressure vectography has a poor correlation with other physiological tests and is of no greater value than normal anorectal electromanometry as described above.

12.8 Anorectal Manovolumetry

Anorectal manovolumetry is a method for simultaneous recordings of anal pressures and rectal volumes in response to graded rectal distension pressure [81, 82]. The technique enables recording of rectal compliance and may therefore provide further insight into rectal wall elasticity. It can be especially helpful in incontinent patients. A further possibility for investigating rectal reservoir function is fecoflowmetry introduced in 1990 by Shafik and Khalid [83, 84]. The principle of this method is similar to that of uroflowmetry.

12.9 Electromyography

The reports of Marin et al. [85], Inon et al. [86], Holschneider [45, 87] and Vanasin et al. [88] generated some enthusiasm for anorectal myography in the diagnosis of HD. By means of intraluminal electrodes slow wave activity was recorded from the rectal wall. Strict contact to the mucosa is mandatory but difficult to achieve. Slow wave activity was recognized as regular with 12–20 oscillations per minute. If no spikes are seen on the top of the slow waves, the test is interpreted as being consistent with a diagnosis of HD (Fig. 12.22).

Whereas the basal electrical rhythm continues whether or not the muscles of the bowel wall are contracting, spike potentials can only be demonstrated when an additional contraction of the musculature occurs. Frequency, velocity, and the aboral–oral direction of spread of rhythmic peristalsis are controlled by electrical slow waves. Spike potentials, however, are responsible for additional phasic contractions, but can also be observed without any associated change in pressure [89]. On the other hand, mass movements are correlated to sinusoidal oscillations of several slow wave cycles. Pickard et al. [90] therefore found no spike potentials (what they called abnormal for healthy subjects) in 10 of 41 histologically proven healthy subjects and spike potentials (what they called atypical for HD) in 5 out of 15 patients with HD. Among 45 patients with chronic constipation and HD we found spike potentials on the top of the slow waves of the colon in 31 out of 35 constipated children. In 8 out of 9 patients with HD, in contrast, no spike potentials could be observed. Yanagihara et al. [91] and Shafik [92] also recommend electromyography as a suitable test for HD. However, for the above-mentioned reasons this has to be considered with reservation.

The main role of electromyography in constipation is to exclude anismus as a cause of obstructed defecation.

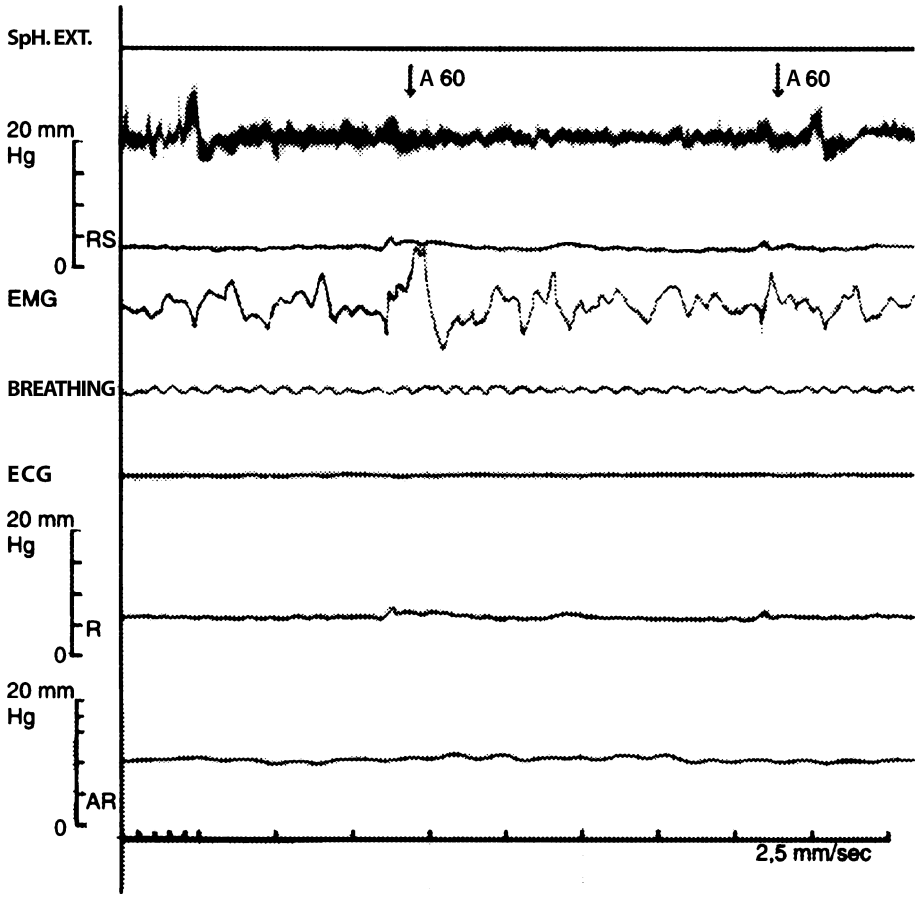


Fig. 12.22 No spike potentials on the top of the slow waves in a patient with HD (*RS* rectosigmoid, *R* rectum, ECG electrocardiography *AR* anorectum, SPH.EXT. external anal sphincter, EMG electromyography of smooth muscle layers)

Fink et al. [93] found anismus in 20% of patients studied. There was, however, a poor correlation between the finding of anismus on electromyography and failure of the anorectal angle to become widened during defecation.

12.10 Endosonography

Endoluminal ultrasonography of the anal canal is of no help in the diagnosis of HD or allied disorders. However, it can be a useful adjunct to physiological studies of anorectal function in patients with stool incontinence after surgical procedures particularly sphincteromyectomy [94–96] and for reproducible estimation of rectal compliance [97]. A combination of endoluminal sonography, electromyography of the external anal sphincter and manometric evaluation is favored by Tjandra et al. [95] and Gantke et al. [98]. Very promising is three-dimensional endorectal sonography which allows three-dimensional visualization of the pelvic floor and anorectal sphincters [99].

12.11 Transit-time studies

Transit-time studies are very helpful in the estimation of the length of the involved segment in patients with chronic intestinal obstruction. We never perform an extended colonic resection without having performed a transit-time examination [62]. Fink et al. [93] also recommend transit-time studies as a necessary requirement before performing a colectomy.

Gastrointestinal transit can be studied by means of indigestible metal particles followed on their way through the gastrointestinal tract by means of metal detectors [100], by transit scintigraphy with ^{111}In-DTPA [101], technetium Tc 99m sulfur colloid [102] or radiochromium (^{51}Cr) [103] or following ingestion of solid radiopaque markers [104–106].

We use a modification of the method of Hinton et al. [104]. A known number of commercially available pellets, usually 20, are swallowed by the patient and the disappearance of the markers from the gut or the appearance of the pellets in the stool is observed by serial radiographs at 24-hour intervals. The children receive normal food. Any laxatives or special diet is avoided. As transit-time studies are performed after X-ray enemas including defecography the bowel is clean at the beginning of the study. Six hours after ingestion the markers can be demonstrated in the ascending colon, where a physiological retroperistalsis can be demonstrated. In children usually, 80% of the pellets have passed after 48 hours. The markers can also be introduced in an enterostoma

to study the transit in the aboral segment of the bowel (Figs. 12.24–12.28). The pellets, usually 20, are inserted into the aboral segment and, according to the method of Hinton et al. [104], the disappearance and appearance of the pellets in the stool is also observed by several radiographs at 24-hour intervals. The ingestion of three sets of distinctive markers on three successive days as suggested by Metcalf et al. [107] has the disadvantage that the passage of the pellets through different segments of the gastrointestinal tract cannot be pursued and the radiograph taken on the 4th day gives just a global overview of bowel motility. According to Evans et al. [106], normal adults retain more than 20% of markers at 12 hours and less than 80% after 120 hours (Fig. 12.23).

Read et al. [102] studied the transit of a meal through the stomach, small intestine, and colon in 14 normal young adults and found that 50% of the markers were eliminated in just over 2 days, while it took just over 3 days to eliminate all the markers. Wagener et al. [108] measured the total and segmental colonic transit time in 22 healthy children without symptoms of constipation using the saturation method of Abrahamsson et al. [109]. The children swallowed ten radiopaque markers at a given time daily for 6 days, and a single abdominal radiograph was taken on the 7th day. The mean segmental transit times were 5.5 hours for the ascending colon, 10.9 hours for the transverse colon, 6.1 hours for the descending colon, and the longest period of 18.2 hours for the rectosigmoid colon. The mean total colonic transit time was 39.6 hours. A pathological transit was observed by Zenilman et al. [105] in 12 women with idiopathic colonic dysmotility and subsequent subtotal colectomy performed according to the results of the transit-time study and histological examinations of suction biopsy material. The use of ^{111}In-DTPA [101, 110] is more difficult due to the radioactivity of the markers with a half-time of 67.4 hours and the necessity to collect and eliminate radioactive stools. However, by investigating the patterns of colonic transit in 23 adults with chronic idiopathic constipation the authors were able to distinguish between two distinct patterns of colonic transit: colonic inertia and functional rectosigmoid obstruction, both of which had different pathogenetic and therapeutic implications.

As mentioned above, we used a modification of the method of Hinton et al. to assess the intestinal transit time in children with intestinal neuronal malformations [111]. In 53 patients with aganglionosis and in 37 out of 53 with other intestinal malformations, the intestinal transit time was prolonged. Of 16 children with IND type B, 8 had an abnormal transit time, 1 underwent anterior resection, and 2 had a temporary colostomy. Also 7 of 8 children with hypoganglionosis and 9 of 10 with a reduced parasympathetic tone showed a prolonged transit time. A resection was performed in 7 and 2 of these children, respectively. But only 11 of 17 children with heterotopia of the submucous plexus, dysganglionosis or immature ganglia had a prolonged intestinal transit time,

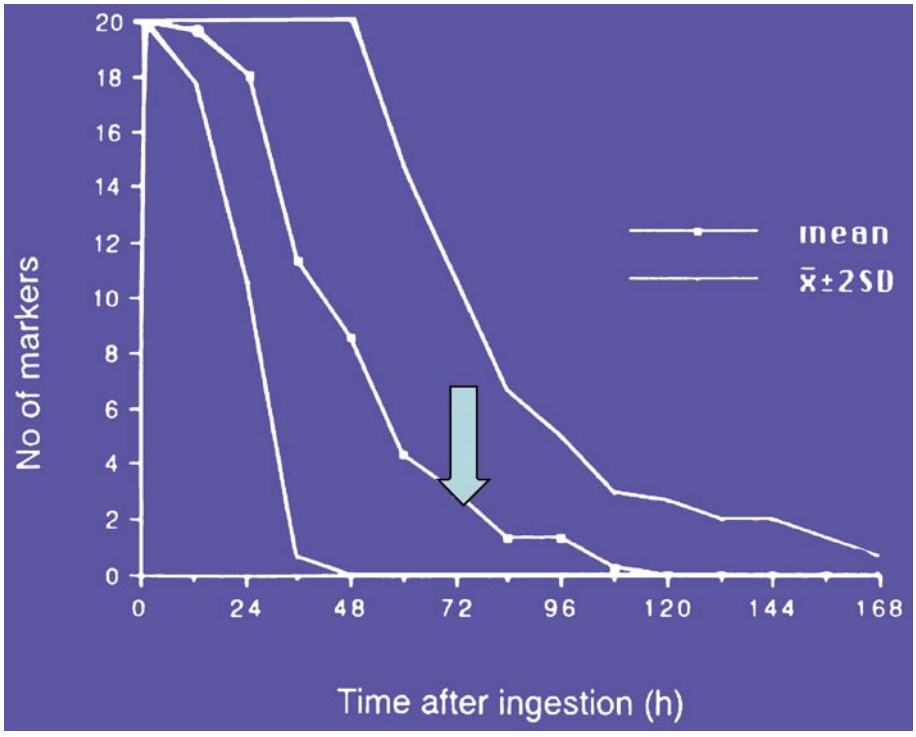


Fig. 12.23 Normal delivery of markers after oral ingestion in adults. The mean delivering time is marked by *arrow*

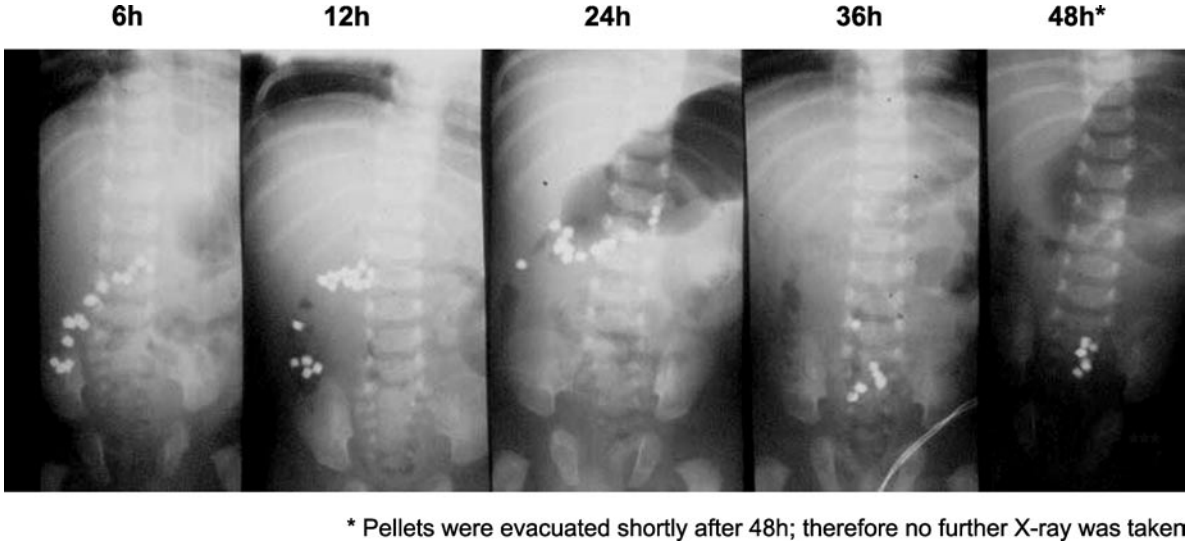


Fig. 12.24 Transit time study in a child with normal transit of markers to the rectum. In the radiograph taken 48 hours after swallowing 20 pellets, only 6 markers are visible in the rectum

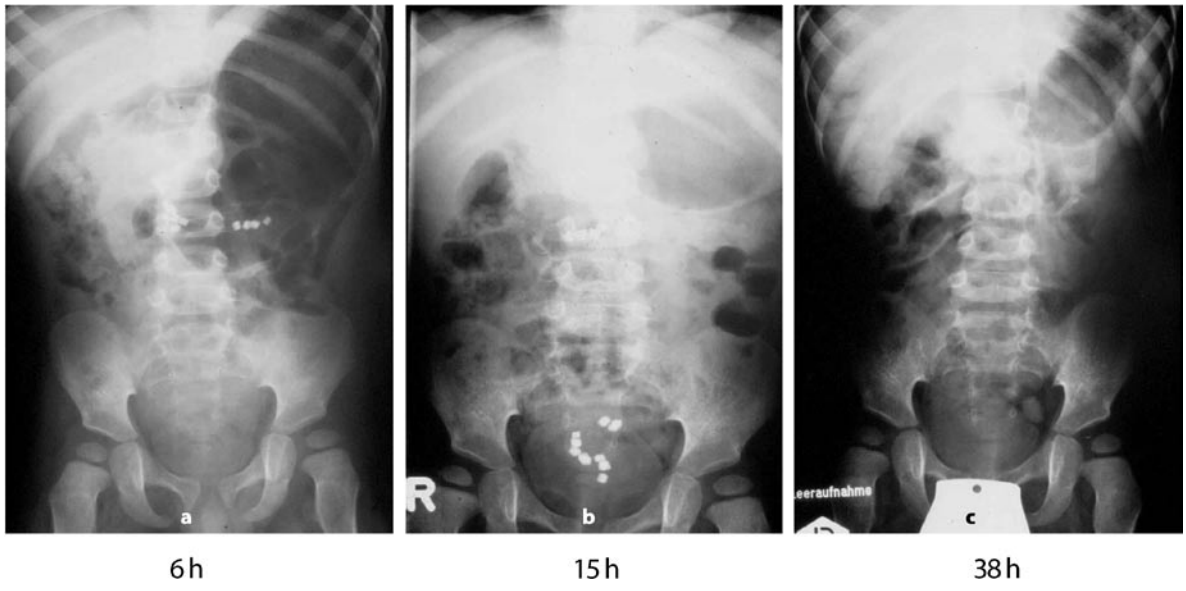


Fig. 12.25 Normal transit of pellets through the colon in a 12-year-old boy with a colostomy and IND after maturation. The radiographs were taken after 6 (*a*), 15 (*b*) and 38 (*c*) hours after ingestion into the aboral part of the colostomy

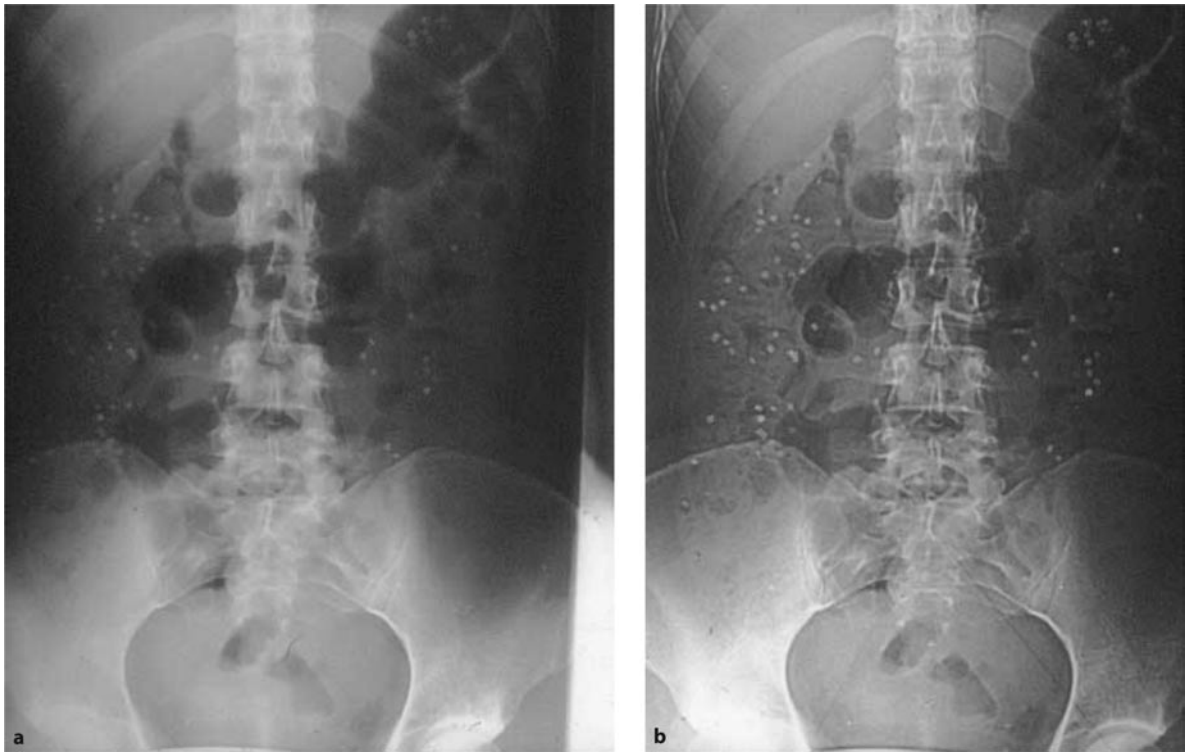


Fig. 12.26 IND in a 36-year-old woman. Twenty markers were ingested on three consecutive days. The radiographs taken at 5 days (*left*) and 11 days (*right*) show a severe transit delay

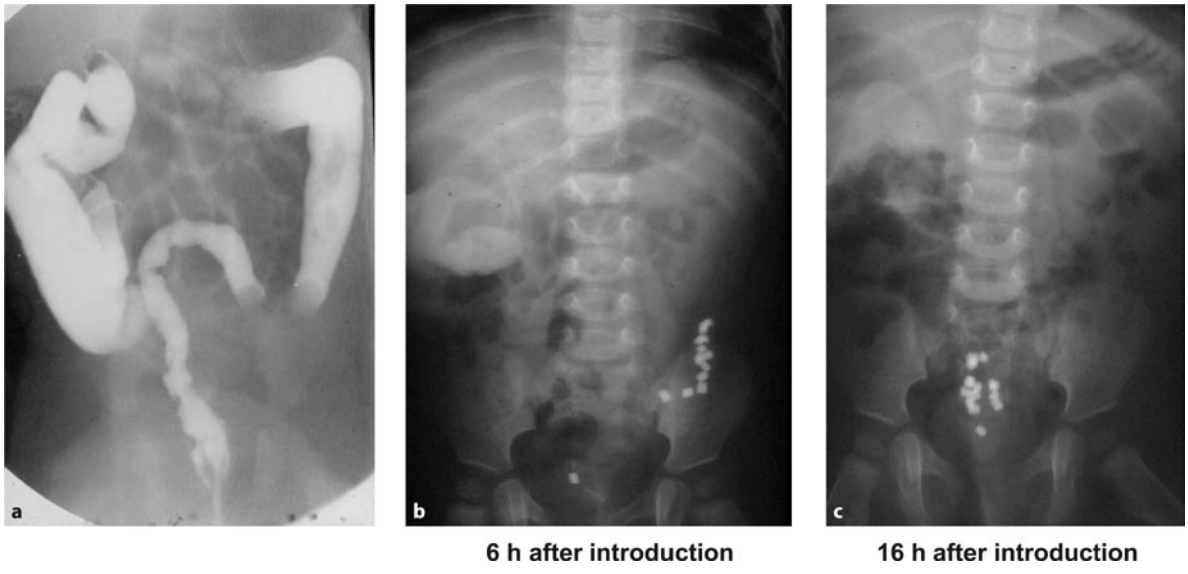


Fig. 12.27 HD and suspected IND in the transitory (unusually long transitory segment of a 10-year-old girl (*left a*). Contrast enema with gastrografin. Pellet study from colostomy site (*center b,* 6 hours after ingestion, *right c,* 16 hours after ingestion)

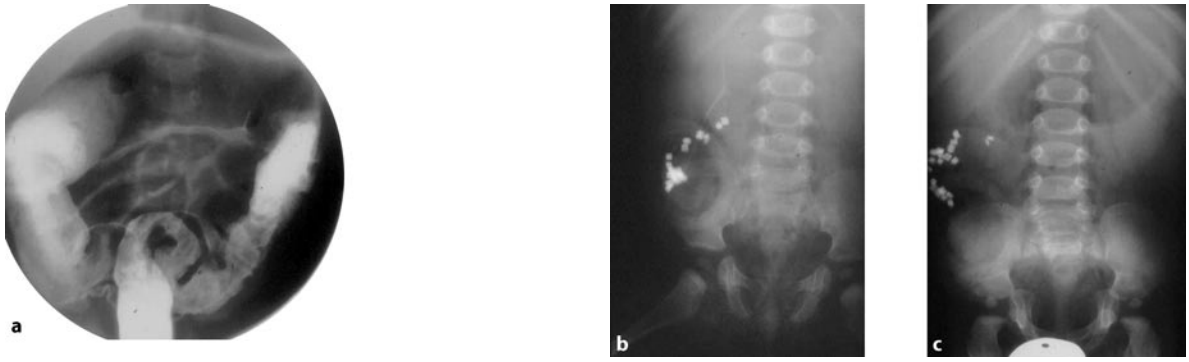


Fig. 12.28 Four month old boy with HD. *Left, a* Typical radiograph with narrow segment. *Top, b 24 hours* after ingestion of pellets into the colostomy, beginning transport through the transverse colon. *Bottom right c,* 48 hours later there is no further movement of the markers. The pellets were delivered by retrograde bowel movements in the colostomy bag

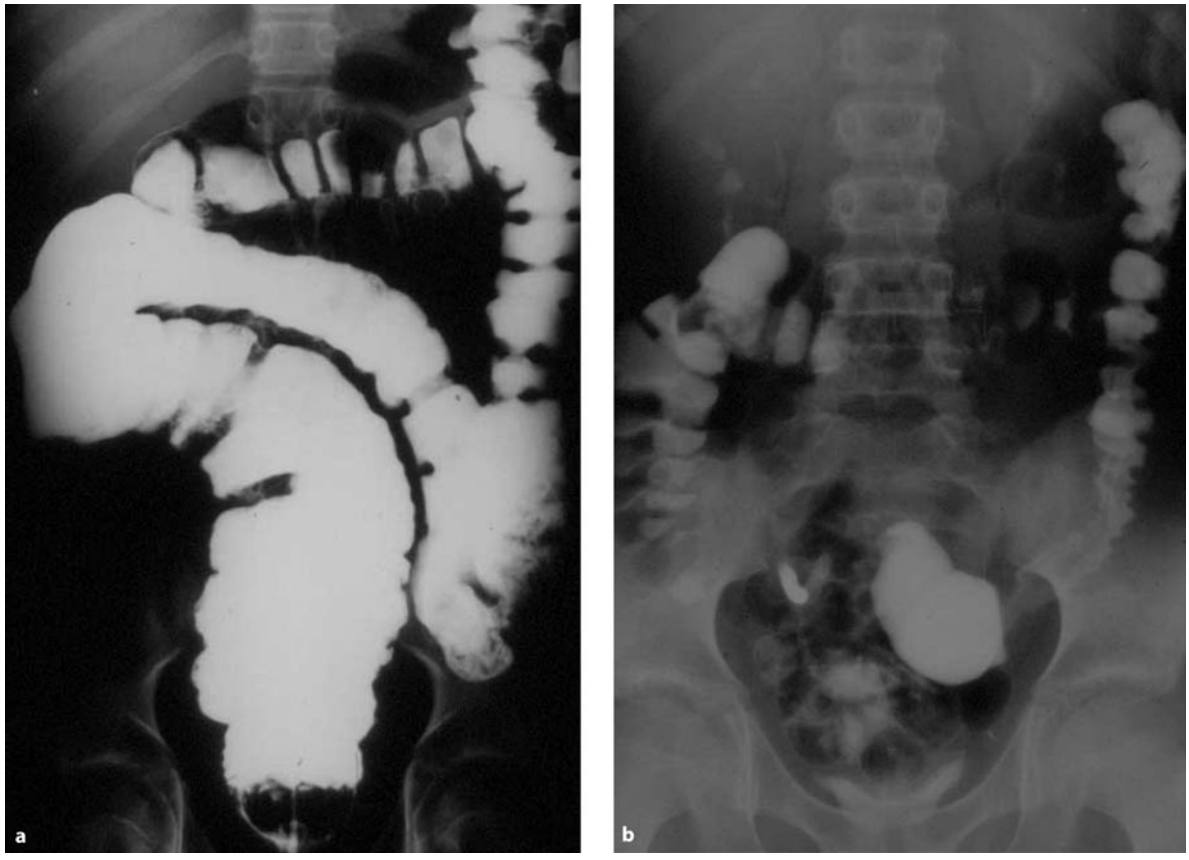


Fig. 12.29 Normal delivery of contrast material. Radiographs taken at the end of the procedure (*left*) and 20 minutes later (*right*)

and 2 underwent sphincteromyotomy. All children who required surgery had a prolonged intestinal transit time, but also 21 of 37 children who were successfully treated without surgery. Waldron et al. [112] used a technique of prolonged ambulant manometry and electromyography as well as transit-time studies in 8 patients with rectal inertia and 14 controls. External sphincter electromyography spike activity did not differ between the two groups. However, a reduced transit of feces to the rectum from the colon over a 24-hour period suggested the presence of a motor neuropathy in the rectum. Finally, late radiographs acquired 6, 12, 24 or 48 hours after contrast enema are very helpful in estimating the transit time of the contrast material (Figs. 12.29 and 12.30).

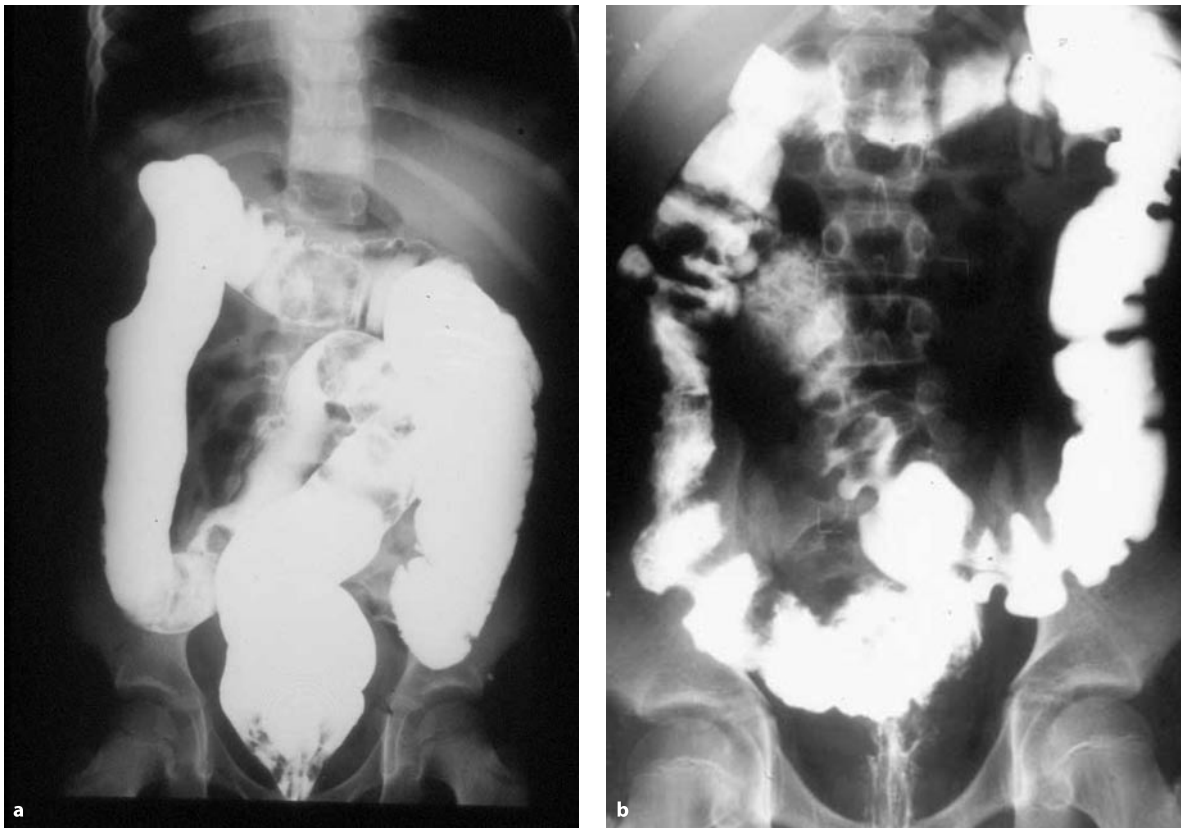


Fig. 12.30 Severe chronic constipation and IND. Radiographs taken at the end of the procedure (*left*) and 20 hours later (*right*)

12.12 Conclusions

Anorectal and colonic manometry are screening methods in patients with chronic constipation and are of high accuracy. Patients with normal internal sphincter relaxations can be treated conservatively. Vague patterns need further evaluation by radiography and transit-time studies. The most important diagnostic tools are histological and immunohistochemical evaluations of suction or full-thickness biopsies and whole-mount preparations of the bowel wall. However, the histological results should always be interpreted with special regard to the clinical symptoms and to the results of the electromanometric, transit-time and radiographic studies to avoid unnecessary surgery.

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Histopathological Diagnosis and Differential Diagnosis of Hirschsprung's Disease

W. Meier-Ruge and E. Bruder

13

13.1 Introduction

It has been well known since the reports of Dalla-Valle [6, 7] that the most characteristic feature of Hirschsprung's disease (HD) is the absence of ganglion cells in the narrowed segment. The aganglionic segment starts at the anal ring and extends proximally for a variable length. A reliable diagnosis of HD on a hematoxylin-eosin (H&E) staining of a mucosal biopsy requires considerable experience. There is the risk of rendering a false-positive diagnosis of HD in cases of hypoganglionosis. A diagnosis of an ultrashort Hirschsprung segment of less than 3–4 cm in length above the anal ring cannot be established by an H&E staining or immunohistochemical reaction. In contrast to immunohistochemistry or H&E staining, the introduction of the enzyme histochemical acetylcholinesterase reaction (AChE) has made the morphological diagnosis of HD easier and more reliable [12, 19, 20, 31].

In addition, the parallel use of lactate dehydrogenase (LDH) enzyme histochemistry allows confirmation of aganglionosis of the submucous plexus because nerve cells can be fairly electively stained with a LDH reaction. Therefore, without an appropriate dehydrogenase reaction there is a risk of incorrect positive or negative results. The routine use of LDH and succinic dehydrogenase (SDH) reactions [3, 24, 30, 36] which allow the visualization of ganglia and nerve cells in the submucous and myenteric plexus has made it possible to detect other abnormalities of colonic innervation with symptoms of HD [15, 20, 26, 36, 37].

With an NADH diaphorase or LDH reaction a rapid assessment of the myenteric plexus is possible and can be performed intraoperatively during colon resection. It is important for the surgeon to determine if the oral resection border consists of an abnormally innervated or normally innervated colon. In the course of such a quick intraoperative investigation, the intensity of the staining can be continuously monitored and the incubation time kept to the minimum necessary for a reliable diagnosis (8–10 minutes). In contrast to immunohistochemistry, enzyme histochemistry offers remarkable flexibility and a much faster result. Today, enzyme histochemical kits are commercially available (Districhem, Oberwil, Switzerland, and Bio-Optica, Milan, Italy).

The aim of this chapter is to demonstrate the enzyme histochemical characteristics of HD and its differential diagnosis from other functional anomalies of intestinal innervation showing symptoms of HD [26].

13.2 Hirschsprung's Disease

HD is characterized enzyme histochemically by the following easily identifiable features:

1. Absence of nerve cells in the submucous and myenteric plexus (ganglion cells are visualized by an LDH reaction (Fig. 13.1); and
2. Typical increase in AChE activity in the parasympathetic nerve fibers of the lamina propria mucosae (Fig.

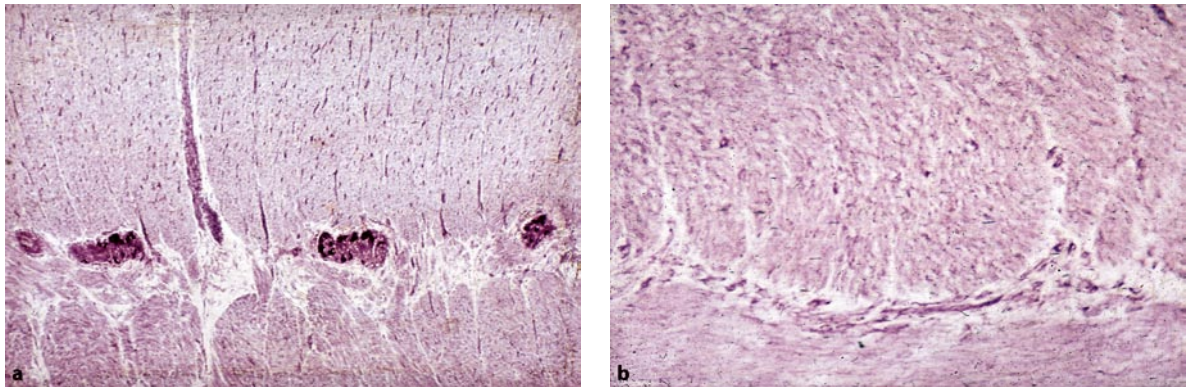


Fig. 13.1 **a** Normal myenteric plexus. **b** Aganglionosis with empty plexus cleft (LDH enzyme histochemistry, ×150)

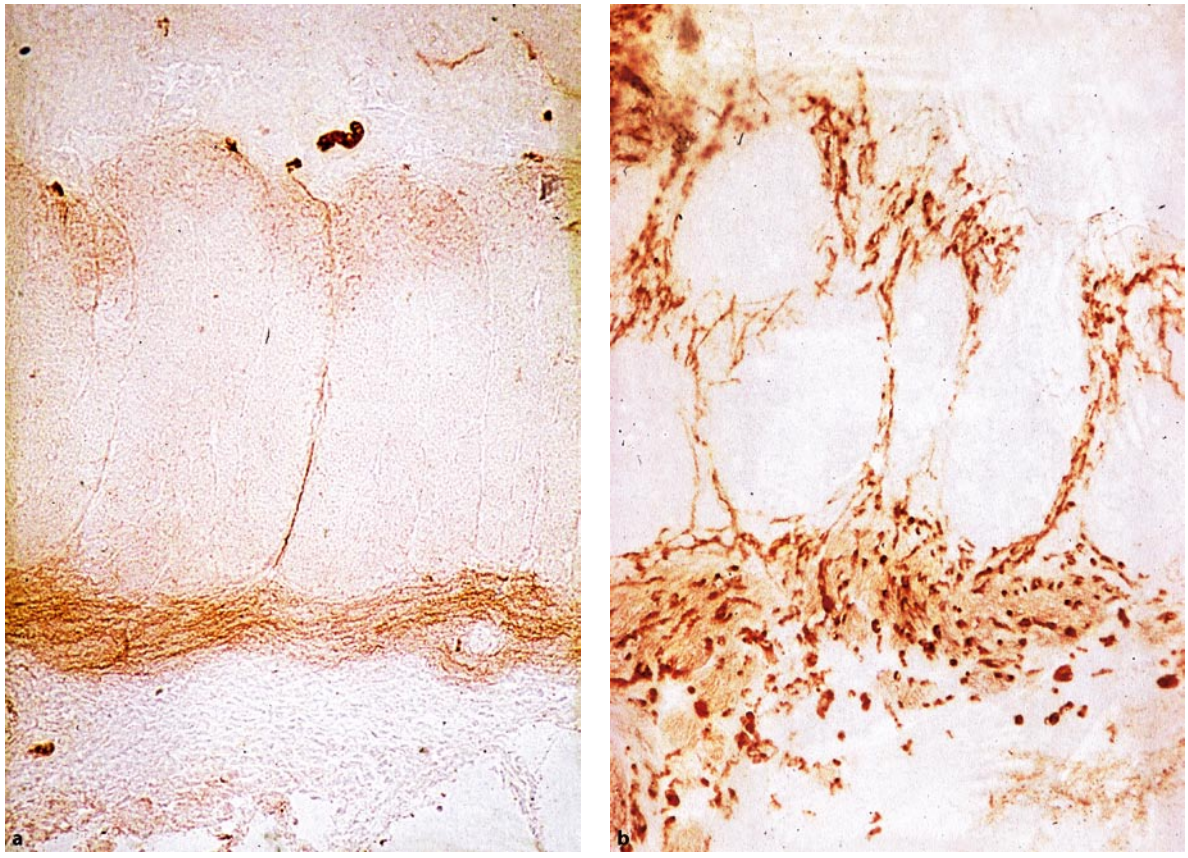


Fig. 13.2a,b Acetylcholinesterase (AChE) reaction in a suction biopsy of rectal mucosa. **a** Normally innervated rectum mucosa without AChE activity in parasympathetic nerve fibers. **b** HD. Aganglionic rectum mucosa with characteristically increased AChE activity in parasympathetic nerve fibers of lamina propria mucosae (AChE reaction without counterstaining, ×90)

13.2), muscularis mucosae, and circular muscle layer of the muscularis propria.

The LDH reaction is performed according to method of Hess et al. with modification [13, 24, 36]. For the SDH reaction the method of Nachlas et al. [41] is used.

The diagnosis of HD is based on the increase in AChE activity in the parasympathetic nerve fibers of the muscularis mucosae and lamina propria mucosae (Fig. 13.2) of the aganglionic segment, typically the rectosigmoid. The AChE staining of parasympathetic nerve fibers in the lamina propria mucosae is weak in biopsies of immature disease and in ultrashort Hirschsprung segments. It is therefore compulsory that mucosa biopsies of the rectum contain muscularis mucosal (Fig. 13.3). Four mucosal biopsies at increasing distances from the anal ring (0, 1, 3 and 6 cm) are required [3, 26, 36].

It has been known for 35 years that the AChE reaction of a mucosal biopsy is of great practical value in confirming a diagnosis of HD, especially since in ambiguous cases the biopsy can be repeated without any risk to the patient [19, 22, 31]. Furthermore, evidence for the diagnostic reliability of enzyme histochemistry in the morphological identification of HD has recently been put forward by a number of authors [19–21]. Currently, no immunohistochemical staining is equal to the diagnostic value of enzyme histochemistry [12].

13.3 Ultrashort Hirschsprung's Disease (UHD)

The AChE reaction is the single histological procedure which permits a reliable diagnosis of an ultrashort Hirschsprung segment [4, 17, 28, 37]. Over the last 20 years, we have observed an incidence of 13% of UHD among all aganglionoses [37]. UHD resembles classical HD in terms of gender and is twice as frequent in males as in females [37]. In most patients the disease is diagnosed in the second half of the first year of life. The diagnosis of UHD is established in biopsies of only a few patients aged 4 to 34 years. About five children are diagnosed with UHD per year [37].

An absolutely accurate diagnosis of UHD is only possible with an enzyme histochemical AChE reaction. UHD is defined as an aganglionosis with an extension up to 3–4 cm above the pectinate line. The shortest form is limited to the transitional zone of the anal ring. The biopsies must contain muscularis mucosae and submucosa because in most cases nerve fibers with increased AChE reaction are observed only in the muscularis mucosae and the adjacent submucosa, but not in the lamina propria mucosae (Fig. 13.3). Often UHD is limited to the anal ring only with increased ACE activity in nerve fibers of the musculus corrugator cutis ani [26].

It is important to be aware that UHD, diagnosed in the first 3–4 months of life, can grow up to 5–6 cm during the next 18 months because of the caudocranial growth of the distal rectum. A control test 1 year after the first diagnosis is therefore recommended. The same situation arises if a Rehbein or Swenson procedure is performed in the first months of life and the aganglionic centimeter, necessary for the anastomosis, increases in length to 4–8 cm during the following 4 years of life. A short aganglionic rest segment of HD can be differentiated from UHD by the dense net of AChE-positive parasympathetic nerve fibers in the lamina propria mucosae.

13.4 Total Aganglionosis of the Colon

A rectal biopsy in total colonic aganglionosis has more or less the same histochemical appearance as HD. A lower

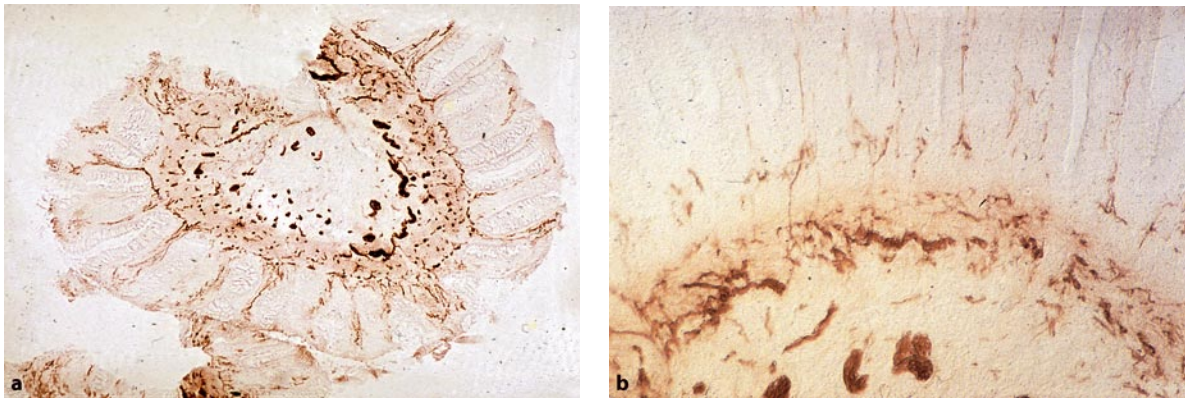


Fig. 13.3a,b Ultrashort HD. **a** Low magnification of a rectum mucosa biopsy 2 cm above the pectinate line with increased AChE activity in the muscularis mucosa and submucosa. There are no AChE-positive nerve fibers in lamina propria mucosae (×35). **b** Higher magnification (AChE reaction without counterstaining, ×150)

density of parasympathetic nerves in the muscularis mucosae and lamina propria mucosae may indicate a total aganglionosis. An obvious decline of nerve fiber density from distal to proximal is fairly characteristic. Total aganglionosis is constantly associated with moderate hypoplasia of the extramural parasympathetic innervation. In hypoplasia of the extramural parasympathetic innervation, morphometry shows a diagnostic, more rapid decline of nerve fibers compared to the classical, short aganglionic Hirschsprung segment (Fig. 13.4).

If the whole colon is resected and available for examination, the extent of sacral parasympathetic innervation of the circular muscles of the distal colon can be determined by histochemical visualization of AChE in a coiled strip of the resected intestine [36]. The density of the network of extramural parasympathetic nerve fibers decreases exponentially from the anal sphincter to the left colonic flexure in accordance with the equation $y=ze-^{0.04}x$ and tends to approach zero above the splenic flexure [32] which has been also proven by biochemical examinations [14]. The exponential distribution of the mean nerve fiber density in the circular muscles shows that it is greatest in the lower rectum. Thus, from the splenic flexure to the anal sphincter there is an increase in the density of nerve fibers in the circular muscles and in their contractile force. It follows that the lower rectum, by virtue of its high contractile potential [15], is responsible for all the clinical features of ganglionic diseases of the colon. This, in turn, explains the similarity of the signs and symptoms caused by colonic aganglionosis of differing segmental lengths. Furthermore, since the density of circular muscle innervation varies exponentially along the distal colon, the visualization of the extramural (sacral) parasympathetic innervation by means of AChE staining—and thus the diagnosis of HD—can only be achieved with tissue taken from the rectosigmoid and the lower distal descending colon. A biopsy from a preternatural anus in the transverse colon in total colonic aganglionosis does not show characteristics of a rectal biopsy. On the contrary, only very few single AChE-positive structures can be observed.

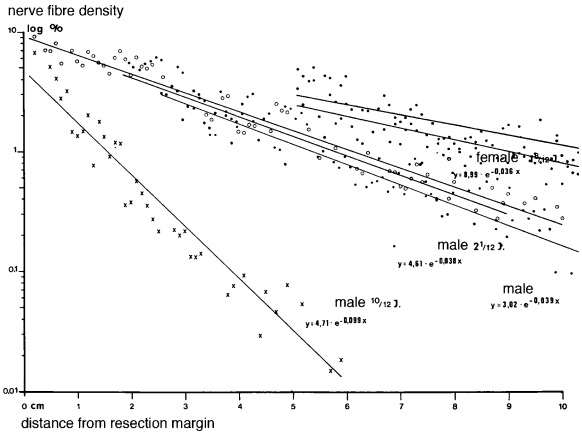


Fig. 13.4 Morphometric measurement of nerve fiber density in circular muscles of aganglionic rectosigmoid. Total colonic aganglionosis shows hypoplasia of (extramural) sacral innervation of the distal colon with a steep decline in nerve fiber density. The other five flat slopes are characteristic of classical short aganglionic segments

13.5 Hypoganglionosis of the Colon

A condition particularly important in this context is hypoganglionosis of the colon. Hypoganglionosis may give rise to megacolon similar to that proximal to the aganglionic segment in HD. Hypoganglionosis of the colon may occur on its own, but may also accompany HD [27, 29, 39, 40].

Hypoganglionosis as an isolated inborn malformation represents only 5% of intestinal neuronal malformations [25]. With systematic investigation of resected specimens, the number of diagnosed hypoganglionoses of the myenteric plexus has increased in recent years.

The diagnosis of hypoganglionosis by means of mucosal biopsy specimens is difficult. Mucosal biopsies with a low level of' AChE activity and scarcely developed submucous plexus can indicate but not prove hypoganglionosis of myenteric plexus. Seromuscular biopsy allows the inspection of the myenteric plexus and is a prerequisite for a reliable diagnosis of hypoganglionosis.

In addition to an AChE reaction, a LDH reaction is mandatory for visualization and assessment of myenteric ganglia. Hypoganglionosis is characterized by a reduction of the number of LDH-positive nerve cells in the myenteric plexus and a scarcely developed net of parasympathetic nerve fibers in the circular and longitudinal muscles with a low AChE activity.

Borderline cases require a morphometric examination of the myenteric plexus. Only cases with hypoplastic hypoganglionosis and hypoplastic nerve cells are simple to recognize by visual inspection (Fig. 13.5). In these cases the number of nerve cells in the myenteric plexus is decreased by a factor up to 10 and the interganglionic distances are significantly increased [27].

13.6 Immaturity of the Submucous and Myenteric Plexus

Immaturity of the submucous plexus can be established objectively only by the SDH reaction. The ganglia show distinct AChE activity and may be highly cellular, but are weakly stained in the SDH reaction (Fig. 13.6). In plexus immaturity, differentiation into glial and nerve cells cannot be recognized. Similarly, in a muscularis propria bi-

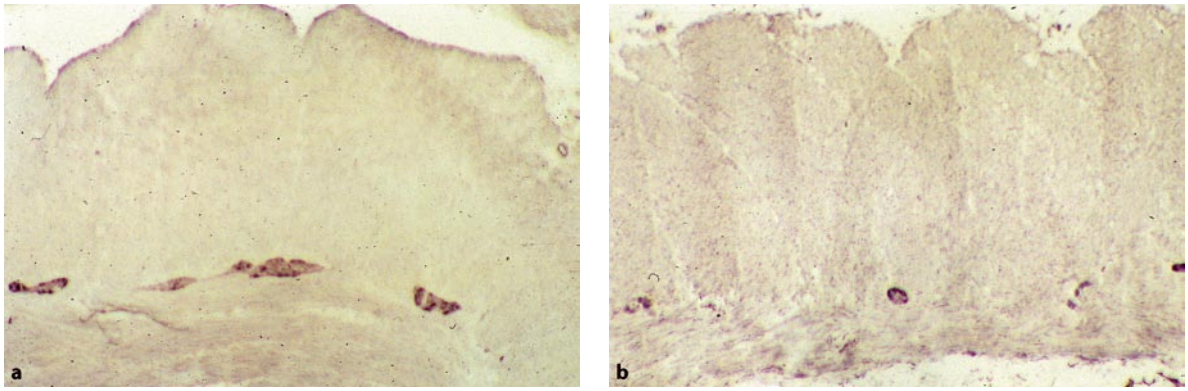


Fig. 13.5a,b Hypoganglionosis of myenteric plexus. **a** Normally innervated myenteric plexus. **b** Hypoganglionosis with sparsely developed myenteric plexus (SDH enzyme histochemistry, ×90)

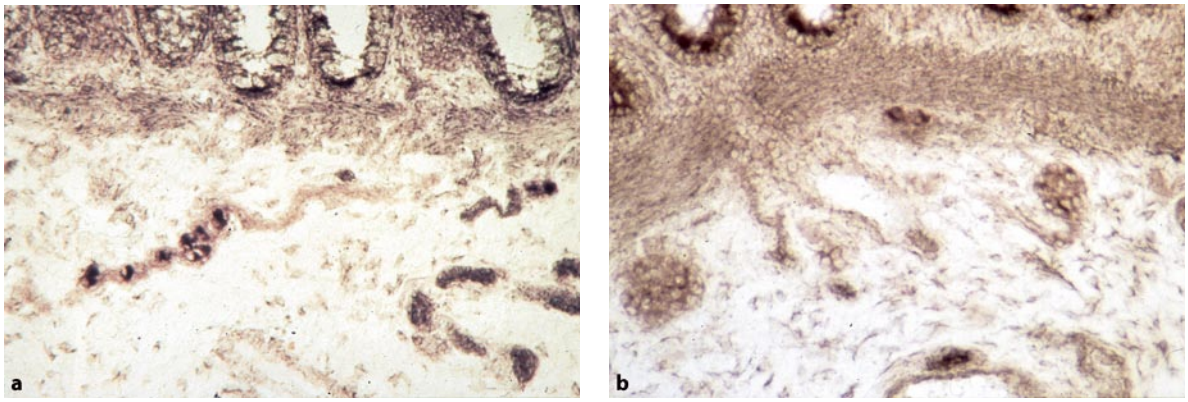


Fig. 13.6a,b Immaturity of submucous plexus. **a** Submucous plexus with mature nerve cells with high dehydrogenase activity. **b** Immature ganglia without any differentiation into dehydrogenase-rich nerve cells and glia cells with low dehydrogenase activity (SDH reaction, ×150)

opsy specimen, the myenteric plexus shows very few and small dehydrogenase-positive nerve cells.

Maturation of nerve cells is best assessed by the SDH reaction. Nerve cells normally show very low SDH activity in the first months of life. Bertoni-Freddari et al. [2] have shown by ultrastructural morphometric measurements that young nerve cells have few small mitochondria that increase in number and size with age. SDH is a specific mitochondrial enzyme. Immaturity of vegetative gut innervation is always accompanied by severe disturbance of bowel motility. Clinically it often simulates HD in early childhood. Maturation of immature nerve cells after full-term delivery is a very slow process. It is only after 2–4 years that originally immature nerve cells become morphologically and functionally normal. In a very convincing morphometric investigation, Munakata et al. [39] showed that neuronal immaturity of the gut is characterized by the presence of small, monopolar intramural nerve cells and is usually found to extend from the jejunum to the rectum. This probably explains the failure of a transverse colostomy in a patient suffering from this abnormality of intestinal motility [39, 40].

13.7 Intestinal Neuronal Dysplasia Type B (IND B)

Intestinal neuronal dysplasia type B (IND B) is currently regarded as a developmental abnormality of the submucous plexus. The most characteristic findings are giant ganglia in the submucosa with more than eight nerve cells (Fig. 13.7). It was shown in morphometric investiga-

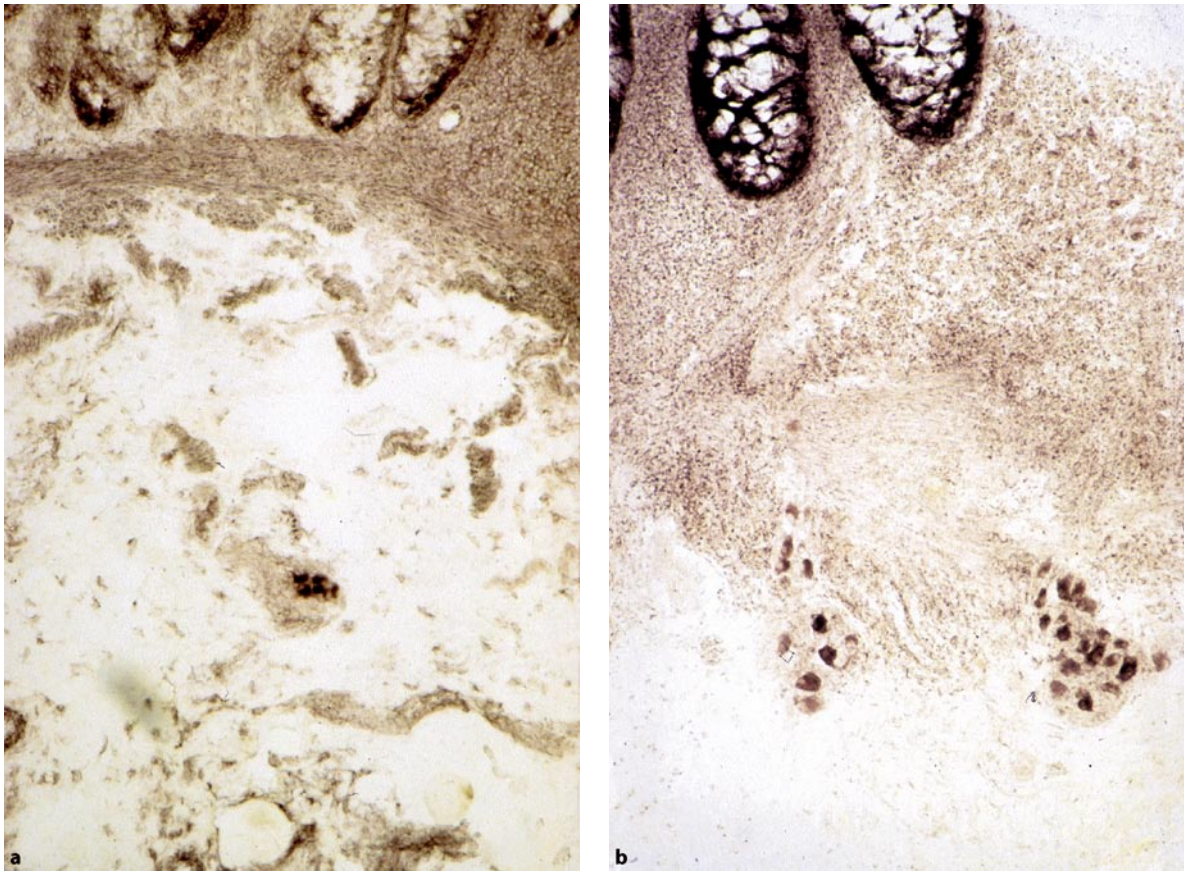


Fig. 13.7a,b Neuronal intestinal dysplasia (IND B) of submucous plexus. **a** Normal submucous plexus. Ganglion with five nerve cells. **b** IND B with giant ganglia in the submucous plexus. Ganglion with more than eight anisomorphous nerve cells (LDH reaction, ×150)

tions that a normally innervated colon mucosa contains 4±2 and IND B 10±2 nerve cells per ganglion [33]. The nerve cells in the giant ganglia are significantly smaller and anisomorphous compared with those in normal biopsies [34, 35].

Occasional giant ganglia by themselves are not specific for IND B but may occur in normal colon in patients without dysmotility. It is their relative increase that appears to indicate a developmental malformation and constitutes the diagnosis of IND B. In IND B, 20–26% of all ganglia are giant ganglia. For the diagnosis of IND B, which is a quantitative diagnosis, at least four to seven giant ganglia should be observed in 25–30 sections [38]. These data were recently confirmed in a morphometric age-related control study [5]. Systematic morphometric measurements in LDH-stained serial sections have shown that 30–55% of the sections contain no ganglia in the submucosa. Only 45–70% of sections show ganglia. Therefore, 25–30 serial sections stained for LDH are a prerequisite for an optimal diagnosis of IND B [37, 40].

IND B exists as an isolated disease or in combination with HD. Morphometric examinations have shown that no significant difference exists between isolated IND B and the combined form with HD [35].

In contrast to previous reports, examination of a sufficiently high number of patients with IND B has shown that hyperplasia of the submucous plexus and an increase in AChE activity in nerve fibers of the lamina propria mucosae are age-dependent findings which disappear during the maturation process of the enteric nervous system [34, 46].

IND B in small infants is mostly combined with immaturity of the vegetative nervous system. In the first year of life, symptomatology of severe constipation quite often appears to be due to more or less pronounced immaturity. Therefore, diagnosis of IND B should be avoided in the first year of life. Children older than 4 years with IND B suffering from chronic constipation or subileus have a small chance of spontaneous improvement. Often, chronic constipation persists into adulthood and around

the age of 30 years these patients finally require surgical intervention due to megacolon.

IND B is an anomaly of the submucous plexus and is diagnosed in biopsies from mucosa with a sufficient amount of submucosa. For diagnosis of IND B, seromuscular biopsies are not required. Only in patients with IND B and therapy-resistant constipation can a seromuscular biopsy of the colon be helpful in recognizing an additional developmental abnormality of the myenteric plexus. If chronic constipation persists beyond the first years of life, seromuscular biopsies for examination of the myenteric plexus are mandatory. Many adults with IND B and chronic constipation since childhood have more or less severe hypoganglionosis of the myenteric plexus as the main reason for chronic constipation.

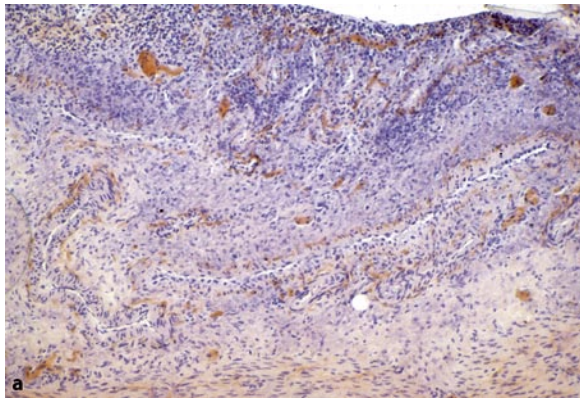
13.8 Intestinal Neuronal Dysplasia Type A (IND A)

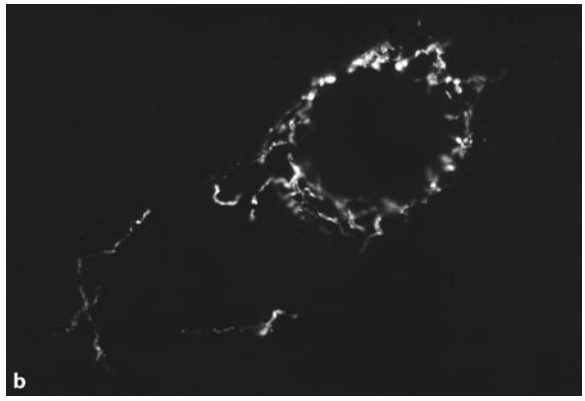
IND A is characterized by a lack or immaturity of sympathetic innervation of the myenteric plexus, arterial vessels and mucosa [23]. It is a rare disease and is only observed in less than 1% of all neuronal malformations of the distal colon [25].

The disease shows a variably diffuse colitis in the first weeks of life (Fig. 13.8a) with increased AChE activity in nerve fibers of the lamina propria mucosae and often signs of immaturity in the submucous plexus. Often the ulcerative colitis destroys the whole muscularis mucosae. The child suffers from spastic diarrhea with bloody stools. The whole symptomatology stops if a colostomy is established in the ascending colon [44, 45]. If the colostomy is closed 8 months later, often no recurrence of colitis is seen, which is typical of immaturity of the sympathetic innervation (Fig. 13.8b, c). If the symptomatology of colitis recurs, the resection of the inflamed part of the distal colon is unavoidable.

13.9 Hypoplasia of Nerve Cells in the Submucous and Myenteric Plexus (Hypoplastic Dysganglionic Oligoneuronal Hypoganglionosis)

Hypoplasia of nerve cells in early childhood seems to be a late outcome of immaturity. However, hypoplasia of vegetative nerve cells can also be found in children and adults who suffer form a symptomatology similar to that of oligoneuronal hypoganglionosis. Hypoplasia of the submucous and myenteric plexus are diagnosed if after 3 years of life the size of the nerve cells is still 50% or less than that of normal controls (Fig. 13.9). In children older than 4 years, hypoplastic dysganglionosis is postulated





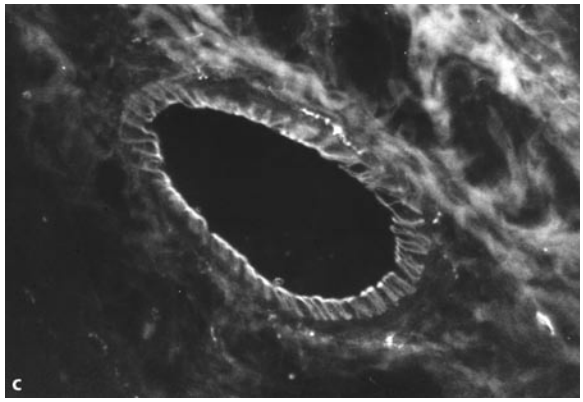


Fig. 13.8a–c Necrotizing enterocolitis (IND A). **a** Inflammatory cells in lamina propria mucosae and necrosis of tubular glands. Moderate increase in AChE activity in parasympathetic nerve fibers (AChE reaction with hemalum counterstaining, ×60). **b** Normal sympathetic innervation in the adventitia of a submucous arterial vessel. **c** No sympathetic nerve fibers in the adventitia of a submucous arterial vessel of mucosa with necrotizing enterocolitis IND A (Falk/Hillarp fluorescence technique for staining of catecholaminergic structures, ×580)

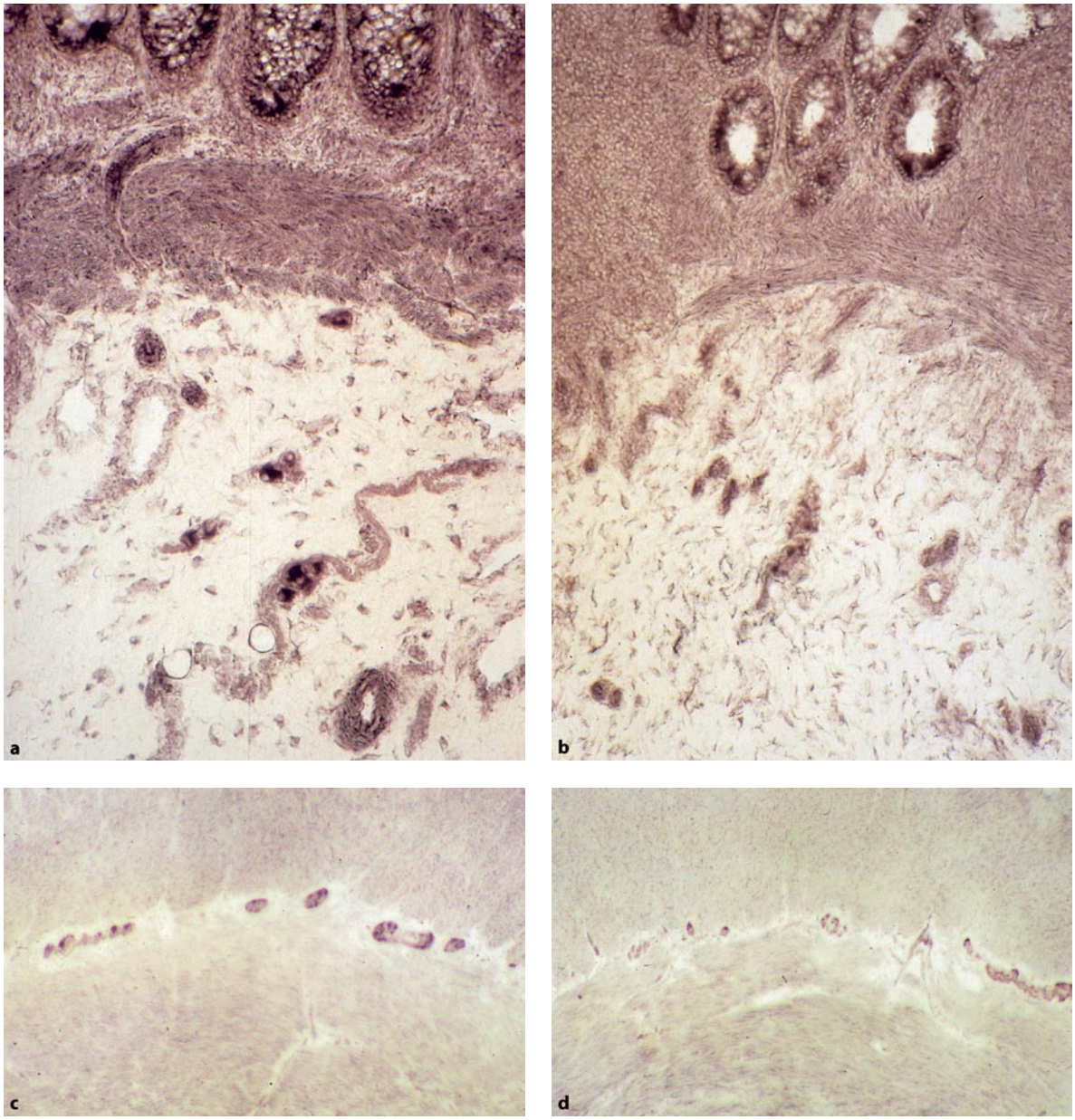


Fig. 13.9a–d Hypoplasia of nerve cells of the submucous and myenteric plexus. **a**, **b** Hypoplasia of submucous plexus: **a** normal rectal mucosa with nerve cells of normal size; **b** hypoplastic nerve cells in submucous ganglia. Nerve cells have a size of 25–30% of normal nerve cells (LDH reaction, ×120). **c**, **d** Hypoplastic oligoneuronal hypoganglionosis of myenteric plexus: **c** normal myenteric plexus; **d** hypoplastic nerve cells in myenteric plexus (SDH reaction, ×150)

to result from a disturbed generation of trophic factors by the accompanying glia cells [26]. Precise diagnosis requires morphometric establishment of the mean nerve cell size. Diffuse hypoplasia of nerve cells of the myenteric plexus, stained with the LDH or SDH reaction, is associated with low AChE activity in the parasympathetic nerve fibers as an insufficient cholinergic force of the circular muscle.

13.10 Desmosis of the Colon

Idiopathic megacolon, colon elongatum, or sigma volvulus often show a malformation of the lattice-like tendinous tissue network of circular and longitudinal muscle layers [15, 25, 26]. Unlike in the normal colon, the myenteric plexus in these patients is not rooted in a tendinous tissue layer between longitudinal and circular muscles, representing the myenteric plexus cleft (Fig. 13.10). Focal displacement of myenteric ganglia is characteristic of a lack of the tendinous tissue layer between the intestinal circular and longitudinal muscle layers (Fig. 13.10). The slow propulsive function in the normal appendix is an example of a complete lack of the tendinous tissue net in circular and longitudinal muscle layers accompanied by displacement of myenteric ganglia into circular and longitudinal muscle layers. Consequently, the ganglia and nerve cells of the myenteric plexus in the veriform appendix are irregularly spread through the circular and longitudinal muscle layers (Fig. 13.11).

These defects in the tendinous tissue net interrupt coordinated movement of colon circular and longitudinal muscle layers [10, 11, 26]. The degree and extent of these tendinous tissue net defects of muscularis propria, which

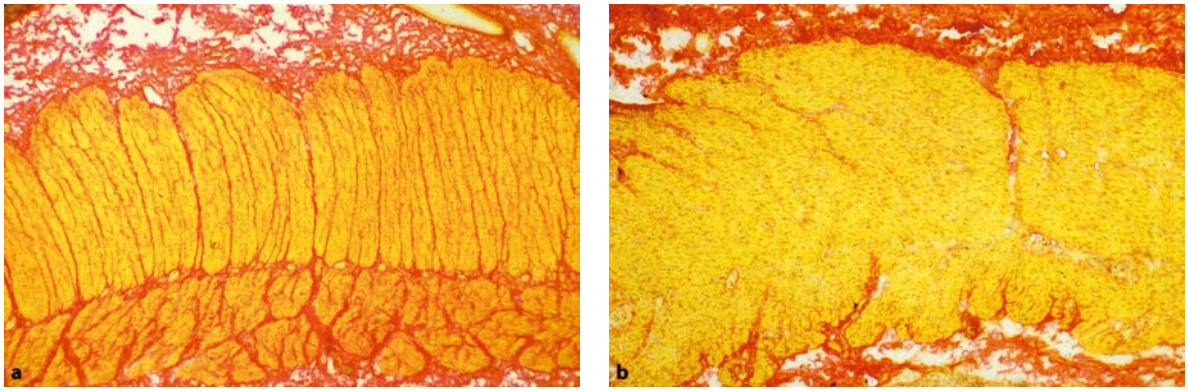


Fig. 13.10a,b Desmosis coli. **a** Normal tendinous tissue layer between circular and longitudinal muscle layers. Regular tendinous tissue nets in circular and longitudinal muscle layers. **b** Desmosis with loss of the tendinous plexus layer and the tendinous tissue structures in longitudinal and circular muscles (picro sirius red staining; cutting angle 45°).

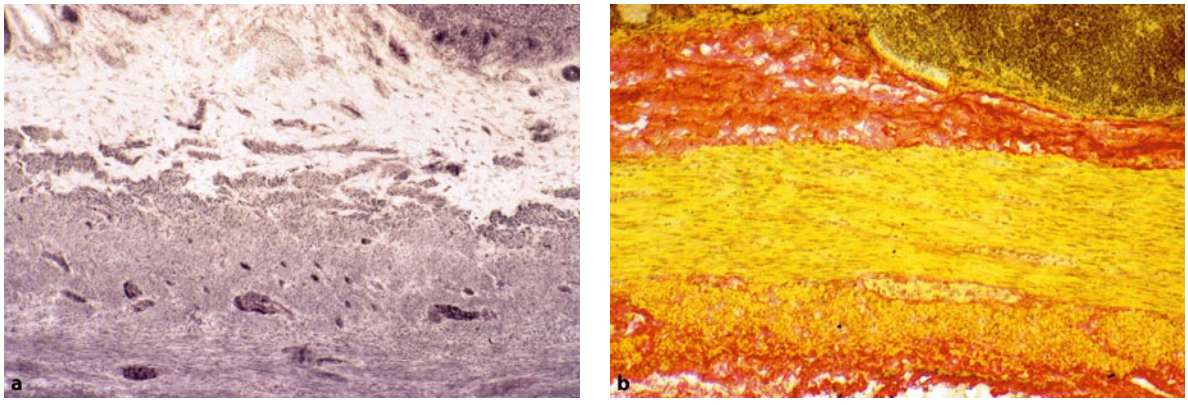


Fig. 13.11a,b Atypical features of normal vermiform appendix. **a** Scattering of myenteric plexus mainly in circular muscles and in part in longitudinal muscles (LDH reaction, ×75). **b** Complete lack of normal tendinous plexus layer and tendinous nets in circular and longitudinal muscles (picro sirius staining, ×75)

is not a neuronal malformation but a desmosis, correlate with the coordinated motoric insufficiency of the colon. It is often observed in a sigmoid volvulus.

Focal atrophic desmoses are found after diverticulitis, tumor irradiation, Crohn's disease, necrotizing enterocolitis, etc., causing symptoms of gut atrophic stenosis.

13.11 Pathogenesis of Hirschsprung's Disease and Related Disorders

HD is currently regarded as prototypical neural crest migration disorder. As reviewed elsewhere in this monograph, the genetic basis of HD is gradually being unraveled. Trophic factors of the mesenchyme seem to be important for the migration of neuroblasts during embryonic life [16]. Gershon et al. [8, 9] and Parikh et al. [42] have shown that an abnormal, early expression of laminin A during embryonic life inhibits neuroblast migration and promotes premature neuroblast differentiation. Therefore, neuroblasts migrating into the submucosa seem to be blocked during embryonic life by laminin A. This process disturbs migration along afferent parasympathetic nerve fibers and induces nerve cell differentiation and mitotic activity resulting in giant ganglia of IND B. This may explain why INB B is associated with HD in so many cases. The time and intensity of laminin expression may be responsible for a premature differentiation of neuroblasts of the myenteric plexus. The variable degree of neuroblast migration impairment may also explain why a hypoganglionic segment of variable length can be observed proximal to the aganglionic segment. Conceivably, the period during embryonic life in which laminin A is expressed may be responsible for the degree of IND B. Therefore, a spectrum of IND B to a nearly normal innervation pattern of the submucous plexus can be observed.

These findings support the hypothesis that the frequently combined IND B, hypoganglionosis of the myenteric plexus and aganglionosis are developmental abnormalities with a common pathogenesis.

In contrast to HD or hypoganglionosis requiring surgical treatment, IND B is only an indicator that the development of the vegetative gut innervation was disturbed. IND B is currently not regarded as an independent disease, but is often accompanied by hypoganglionosis or hypoplasia of myenteric plexus nerve cells.

13.12 Artifacts and Pitfalls in the Enzyme Histochemical Technique

The consensus conference of Borchard et al. in 1991 [3] constituted a first attempt to overcome technical inconsistencies between the different pathological laboratories. Another recent methodological paper referred to the recommendations of Borchard et al. [3] and has tried to further improve histopathological diagnosis of gut dysganglionosis from colonic mucosal biopsies [36].

It must be emphasized that marked deviation from the relatively simple enzyme histochemical techniques (e.g. cryostat sections of 4 µm, incubation for more than 90 minutes, deviation from the recommended pH) will lead to unsatisfactory results [48]. It is important to be aware that a 15-µm thick cryostat section loses 70% of its volume by thawing and drying on a microscopic slide [26], resulting in a final thickness of about 4.7 µm. Furthermore, as has already been pointed out, the results of histochemical visualization of AChE may be misinterpreted. The diagnosis of HD is not based on diffuse staining of the section, but on a histotopochemical identification of deeply stained networks of parasympathetic nerve fibers in the muscularis mucosae and lamina propria mucosae (Fig. 13.12).

Diffuse, brown, cloudy or honeycomb-like staining of the lamina propria mucosae is usually the result of a bleeding artifact induced by the biopsy forceps (Fig. 13.12). Similar changes are seen in suction biopsies with an excessive or unduly prolonged vacuum. A diffuse brown coloration of the muscularis mucosae and that of the lymph nodes of the mucosa is nonspecific. It is important to use tetra-iso-octamethyl pyrophosphoramide (iso-OMPA, Sigma) in the AChE incubation medium to inhibit nonspecific esterase of the muscularis mucosae. An intense nonspecific esterase staining can cover the specific AChE reaction of the parasympathetic nerve fibers. Instead of iso-OMPA, the nontoxic profenamide can also be used. Profenamide is marketed under many different brand names including: Dibutil (Bayer), Lysivane (May and Baker), Parsidol (Specia), Pardisol (Famitalia) [24].

For anatomical reasons (see Section 13.4 Total Aganglionosis of the Colon), the histochemical characteristics of HD are revealed by staining only in material from the rectosigmoid and distal parts of the descending colon. In specimens obtained from the transverse colon, diagnosis must be based on the presence or absence of nerve cells in the submucosal or myenteric plexus (visualized by LDH or SDH staining). For embryological reasons, mucosal biopsy of aganglionic transverse or ascending colon shows absolutely no AChE-positive structures. Since such patients frequently have a colostomy, it is usually easy to biopsy the transverse colon and to obtain samples that include the deeper muscle layers with the myenteric cleft. The diagnosis of IND of the submucous plexus and hypoganglionosis of the myenteric plexus requires an LDH and SDH reaction to electively stain nerve cells (reaction time 8–10 minutes). Similarly, immaturity of submucous ganglia and hypoplasia of submucous and myenteric nerve cells cannot be diagnosed without an LDH and SDH reaction.

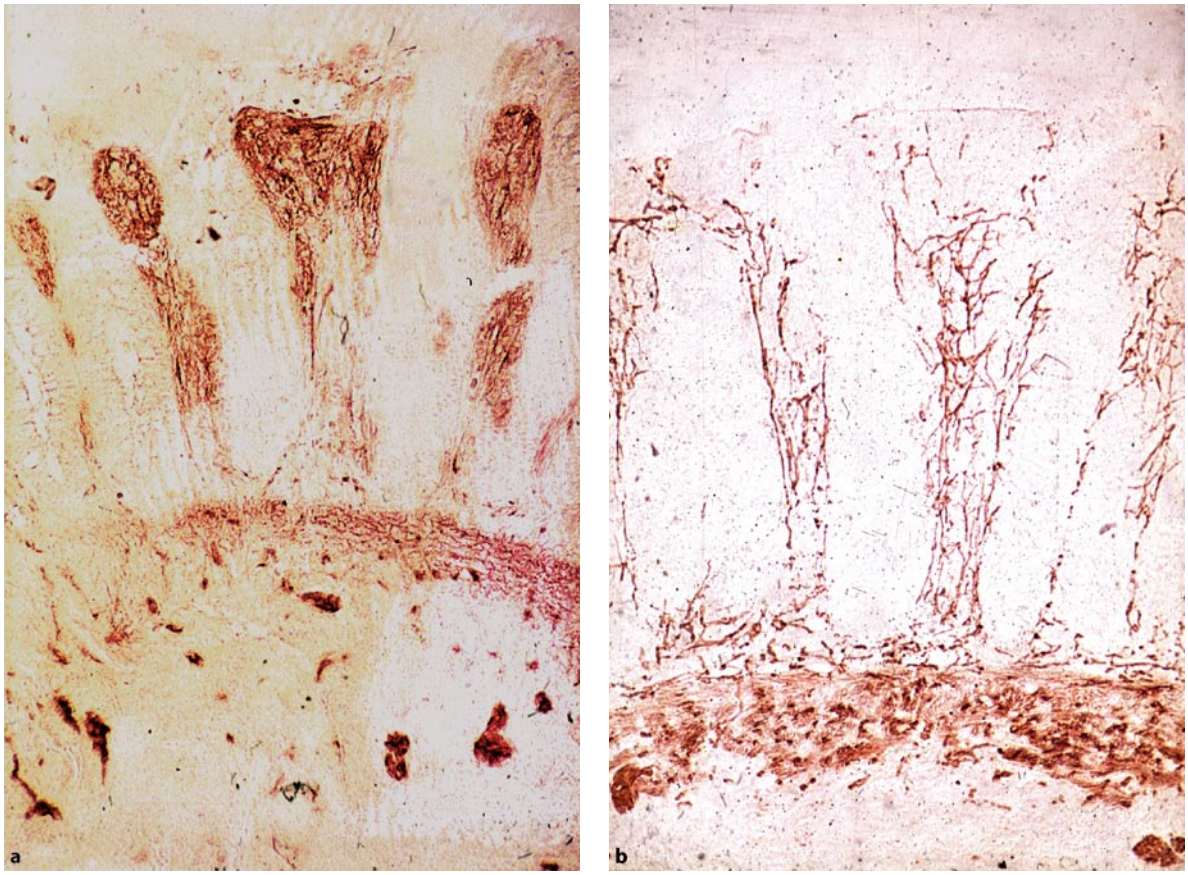


Fig. 13.12a,b Pitfalls in the AChE reaction of rectum mucosa biopsies. **b** Bleeding artifact in the lamina propria mucosae of normally innervated rectum mucosa. The artifact shows a diffuse or spotty amorphous yellow–brown staining of lamina propria mucosae. **b** Elective nerve fiber staining with increased AChE activity in HD (AChE reaction without counterstaining, ×90)

Recognition of mature and immature nerve cells is possible with the SDH reaction. Therefore, in the first months of life, nerve cells of the submucous plexus often show no or a very weak SDH reaction. Mature nerve cells eventually show nearly the same staining intensity in the SDH reaction as in the LDH reaction.

The diagnosis of IND B in the LDH reaction requires at least 25–30 serial sections that must contain at least four giant ganglia each with more than eight nerve cell cross-sections. Since IND B is a malformation of the submucous plexus, a specific nerve cell staining is mandatory. An isolated AChE reaction is not sufficient for a diagnosis of either IND B or other developmental malformations of the submucous or myenteric plexus.

In formalin-fixed paraffin-embedded tissue, immunohistochemistry for PGP 9.5 [18], cathepsin D [1, 47], or S100 [43] (stains glia and leaves nerve cells unstained) can be used for staining submucous and myenteric plexus. However, enzyme histochemical reactions are superior to immunohistochemistry because they yield consistently reliable results from reaction to reaction and are much more elective. In addition, an enzyme histochemical reaction reflects functional differences of a tissue.

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NADPH-Diaphorase Histochemistry

U. Rolle and P. Puri

14

14.1 Introduction

The introduction of rectal suction biopsy, while making the procedure less traumatic for the patient, has made the diagnosis of Hirschsprung's disease (HD) more difficult for the pathologist. Many histopathologists are reluctant to make a positive diagnosis of HD on the basis of suction rectal biopsy results, using conventional H&E stains. This reluctance stems from 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 in accurately identifying smaller and sparse submucosal ganglion cells by comparison with the more compact and familiar ganglion cells of the intermuscular plexus.

The development of histochemical techniques for the detection of acetylcholinesterase (AChE) was a considerable advance in the investigation of HD [1]. However, because occasional false-negative results occur [2, 3], alternative diagnostic neuronal markers have been sought [4–7]. During the last few years, we have used many newer neuronal markers in our laboratory to investigate enteric plexus disorders. We have found nicotine adenine dinucleotide phosphate (NADPH) diaphorase histochemistry a particularly important technique for diagnosis of HD and its allied disorders.

14.2 Nitric Oxide and NADPH-Diaphorase

Nitric oxide (NO) is an important neurotransmitter that mediates relaxation of the smooth muscle within the gastrointestinal tract [8]. Its is synthesized from l-arginine in a reaction catalyzed by NO synthase (NOS). In 1990, Bult et al. [9] provided evidence that NO is released on stimulation of enteric nonadrenergic, noncholinergic (NANC) nerves. Since then, substantial evidence has emerged indicating that NO is the primary mediator of NANC neurotransmission in the intestinal tract in various species [10–12]. Numerous studies have shown the effectiveness of NO in evoking relaxation of the smooth muscle in different parts of the gastrointestinal tract [13, 14]. NO is released from bowel wall and stomach during nerve stimulation [15]. Exogenous NO mimics NANC nerve-evoked relaxation and hyperpolarization in the gastrointestinal muscle in the animal model and human jejunum and colon [16–18]. Inhibition of NO synthesis attenuates the effects of NANC nerve stimulation in animal models and in human sigmoid colon and internal anal sphincter [13, 19, 20]. Furthermore, NO is involved in neurogenic relaxation of the rectum, and NOS immunohistochemistry identified a subpopulation of neurons in the myenteric ganglia and immunoreactive fibers within both layers of the muscularis propria of the human rectum [21]. The mechanism by which NO mediates NANC inhibition of gastrointestinal muscle is understood only partly. NO acting as a neurotransmitter from a final inhibitory neuron binds to cytosolic guanylate cyclase and increases the production of 5'-cyclic guanosine monophosphate (cGMP) with subsequent relaxation of smooth muscle [22].

The above findings suggest that nerves innervating smooth muscle are able to release NO that will penetrate the cells to induce relaxation. Additional sources of NO other than neurons involved in NANC inhibitory transmission have also been proposed, e.g. interstitial cells of Cajal and smooth muscle cells [23].

Deficiency of the nitrergic innervation has been shown in different tissues from patients with infantile hypertrophic pyloric stenosis, HD, and internal anal sphincter achalasia, suggesting that a lack of NO release may be involved in the pathophysiology of these disorders [24–30].

In both brain and peripheral neuronal tissue, NOS has been shown to colocalize with reduced NADPH-diaphorase. Histochemical staining with NADPH-diaphorase, described in brain tissue by Scherer-Singler et al. has facilitated the identification of neuronal NOS [31]. Gabella was the first to describe NADPH-diaphorase staining in gastrointestinal ganglion cells in 1967 [32]. Neuronal NOS catalyses the oxidation of l-arginine to form l-citrulline and NO, a reaction that depends on Ca^{2}+/calmodulin and NADPH. NOS reduces nitroblue tetrazolium to water-insoluble, intensely blue formazan using NADPH as substrate. It has been shown that the activities of NOS and NADPH-diaphorase are identical [33, 34].

14.3 Tissue Preparation for NADPH-Diaphorase Histochemistry

Suction rectal biopsy or full-thickness bowel biopsy tissue is fixed in Zamboni's solution (0.21% picric acid, 2% paraformaldehyde in 0.1 *M* phosphate buffer, pH 7.3, for 15 min at 4°C). After fixation, the specimens are rinsed in 0.1 *M* phosphate buffer with 10% sucrose for 15 min, subsequently snap-frozen and embedded in Tissue-Tek OCT compound (Miles, Elkhart, Ind.). Frozen sections (10 μm thick) are cut serially and processed for NADPH-diaphorase histochemistry.

14.4 Whole-Mount Preparation Technique

Gut innervation has a complex, three-dimensional structure, which is difficult to appreciate on thin sections. The whole-mount preparation technique produces a three-dimensional picture to better show the structure of neuronal networks and the relationship of branching and interconnecting nerve fibers to each other and to the neighboring tissues [35]. This technique, therefore, is especially useful for the investigation of pathological changes in the submucosal and myenteric plexuses such as hyperplasia of the plexuses or giant ganglia. The great advantages for histological evaluation become obvious when whole-mount preparations are compared with regular sections. Histological sections only partially show the morphology of the nerve and glial cells, being dependent on orientation and localization. However, whole-mount preparations show the morphology of the plexuses in full, making possible changes easy to see. Whole-mount preparations of the longitudinal muscle layer and the myenteric plexus are made by separating the muscular layers from the submucosal layer, then removing the circular muscle layer from the longitudinal muscle layer with the adherent myenteric plexus. Subsequently, the mucosa is removed from the submucosal layer.

The submucosal and myenteric plexuses in healthy and diseased bowel can be visualized clearly in whole-mount preparations combined with NADPH-diaphorase histochemistry.

14.5 NADPH-Diaphorase Histochemistry

To stain for NADPH-diaphorase activity, sections or whole-mount preparations are incubated in 10 ml Tris buffer (pH 8.0) containing 0.3% Triton (Sigma), 10 mg β-NADPH (Sigma), and 1 mg nitroblue tetrazolium (Sigma) at 37°C for 60 min. Subsequently the specimens are rinsed and coverslipped with DAKO (Denmark) Glycergel mounting medium.

Recently, several investigators have studied the pattern of NADPH-diaphorase staining in the normal colon and colon from HD patients and have reported lack or deficiency of NOS-containing nerves in the smooth muscle of aganglionic colon [26–29, 36]. There was a strong NADPH-diaphorase staining of submucosal and myenteric plexus in the normal colon and the ganglionic colon of HD patients whereas in aganglionic bowel, weak staining of hypertrophic nerve trunks was found (Figs. 14.1 and 14.2). The lack of NO-producing nerve fibers in the aganglionic bowel contributes to the inability of the smooth muscle to relax, thereby causing the lack of peristalsis in HD. We have used NADPH-diaphorase histochemistry to stain suction rectal biopsies and found it valuable in the diagnosis (Figs. 14.3 and 14.4). There is a considerable lack of NADPH-diaphorase-positive fibers within the muscularis mucosae whereas hypertrophic submucosal fibers stain weakly but are clearly visible.

We have recently employed NADPH-diaphorase histochemistry for the intraoperative evaluation of the extent of the aganglionic segment during pull-through operations for HD [37]. For the intraoperative diagnosis of HD, the sections are incubated in the staining solution for 20 minutes instead of the conventional 60 minutes. NADPH-diaphorase histochemical staining provided 100% diagnostic accuracy regarding the extent of the aganglionosis in HD patients, including newborns. With this technique, it is easier to distinguish the normally innervated bowel segment from the hypoganglionic transitional zone.

Three-dimensional morphology of nitrergic innervation in HD has been investigated using the whole-mount preparation technique [35]. The whole-mount preparation of the ganglionic segment from rectosigmoid HD showed the typical three-dimensional NADPH-diaphorase mesh-like myenteric plexus consisting of nerve bundles with ganglia containing clustered ganglion cells (Fig. 14.5A). In contrast, the aganglionic segment showed absence of the typical architecture of the myenteric plexus

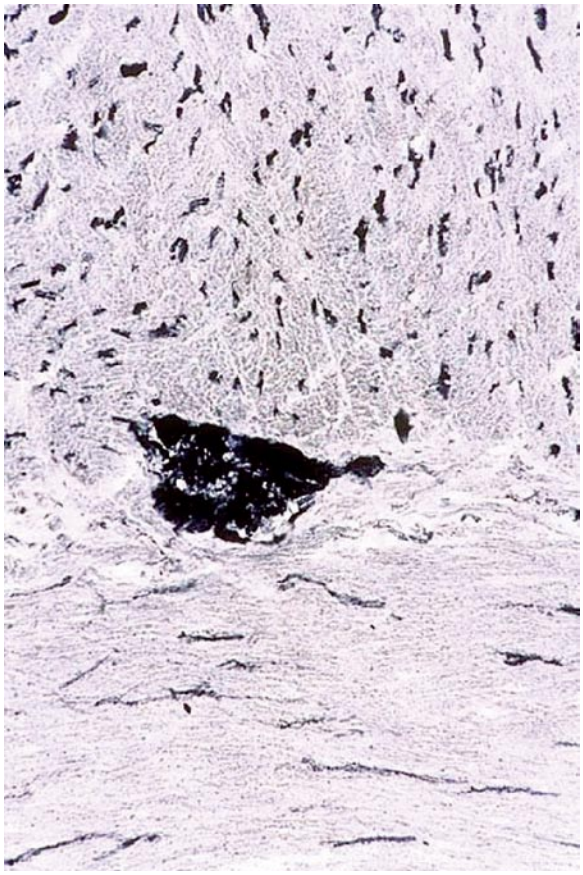


Fig. 14.1 NADPH-diaphorase staining of normal myenteric plexus and intermuscular nerve fibers

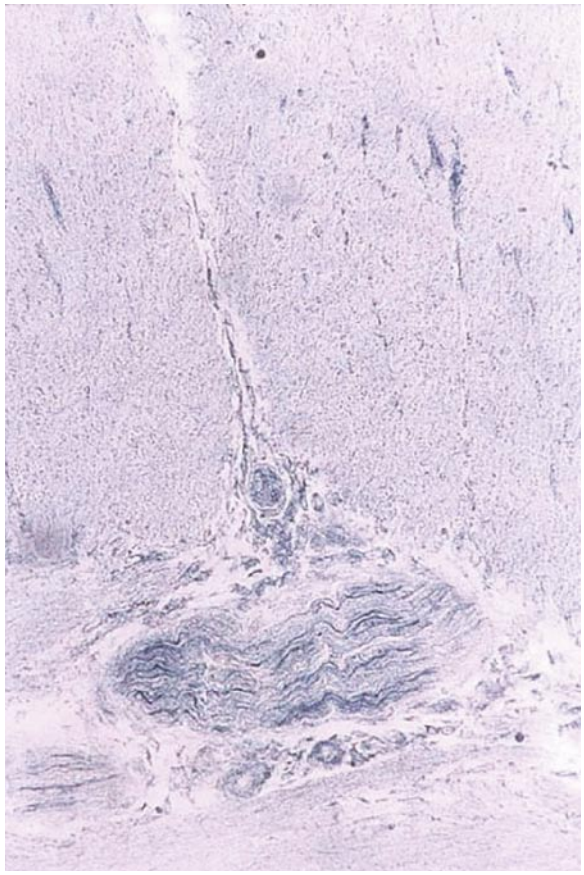


Fig. 14.2 NADPH-diaphorase staining of hypertrophic nerve fibers in aganglionic bowel of HD

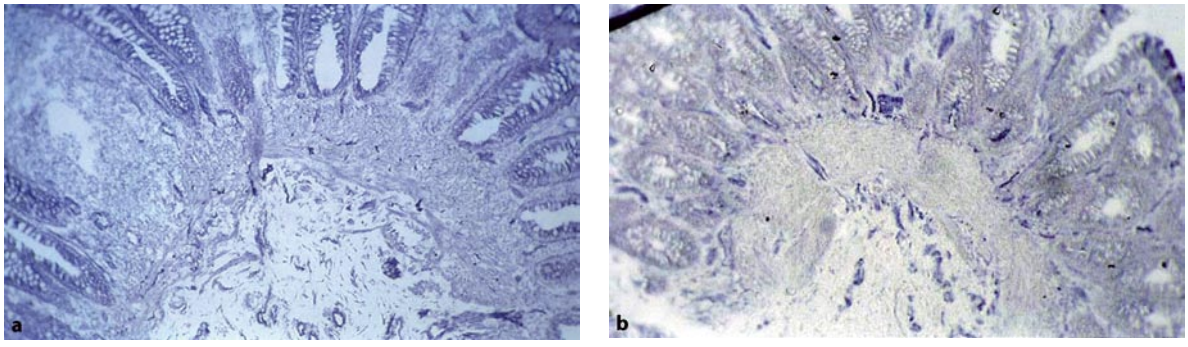


Fig. 14.3 Rectal suction biopsy (RSB). **a** Normal RSB with regular NADPH-diaphorase-positive small submucosal ganglia and normally expressed nerve fibers within the muscularis mucosae. **b** RSB in HD with clearly reduced NADPH-diaphorase-positive fibers within the muscularis mucosae and weakly stained hypertrophic submucosal nerve trunks

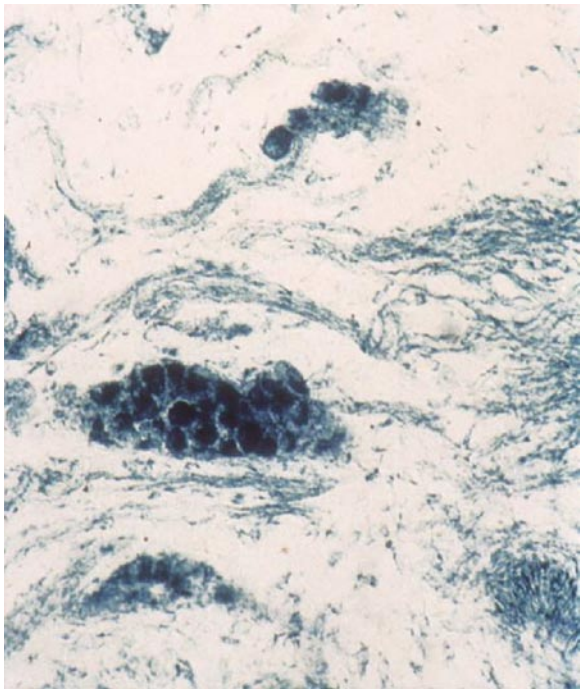
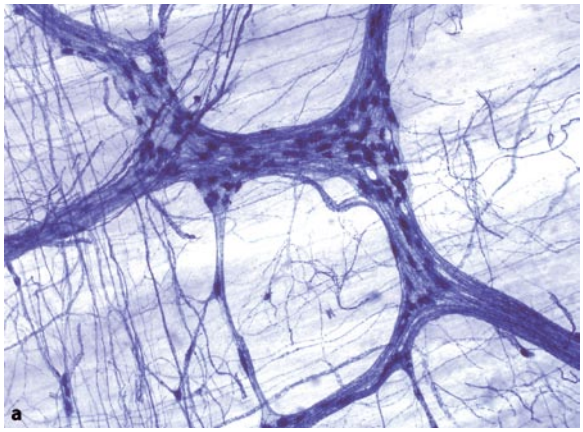
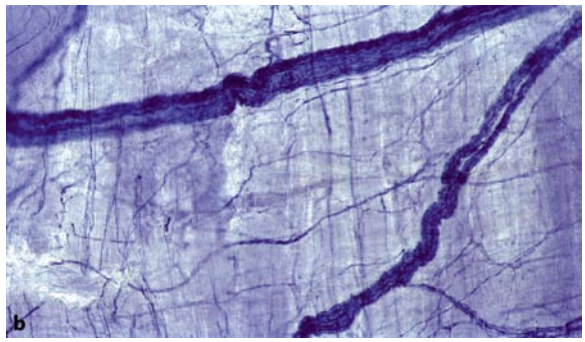


Fig. 14.4 NADPH-diaphorase-positive submucosal giant ganglion





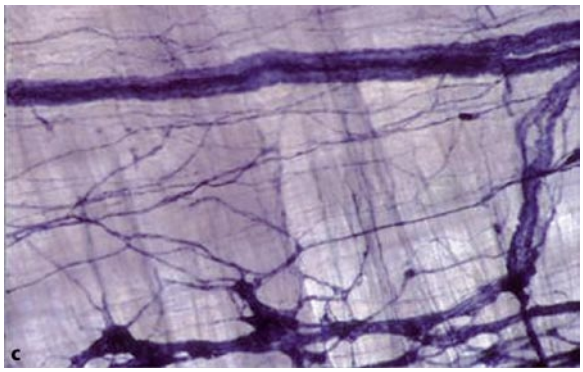


Fig. 14.5 NADPH-diaphorase, whole-mount preparation: **a** normal myenteric plexus; **b** hypertrophic fibers and absent ganglia in the aganglionic zone of HD; **c** hypertrophic fibers and defective small myenteric ganglia in the transitional zone of HD

and the presence of tortuous hypertrophied nerve trunks (Fig. 14.5B). Furthermore, NADPH-diaphorase combined with whole-mount preparation of the specimen is extremely useful to better display the morphological characteristics of the transitional zone in HD, in which there are defective ganglia and still present hypertrophic nerve fibers (Fig. 14.5C).

We have further used NADPH-diaphorase histochemistry in combination with whole-mount preparations to investigate full-thickness bowel biopsies from selected patients with chronic constipation. The specimens showed the characteristic findings of isolated hypoganglionosis [38]. NADPH-diaphorase histochemistry revealed sparse and small myenteric ganglia and a reduced number of nerve fibers in the circular muscle (Fig. 14.6). No hypertrophic nerve trunks were identified in the myenteric or submucous plexuses. Whole-mount preparations of normal bowel stained with NADPH-diaphorase demonstrated a dense mesh-work of nerve bundles in the myenteric plexus containing clusters of ganglion cells.

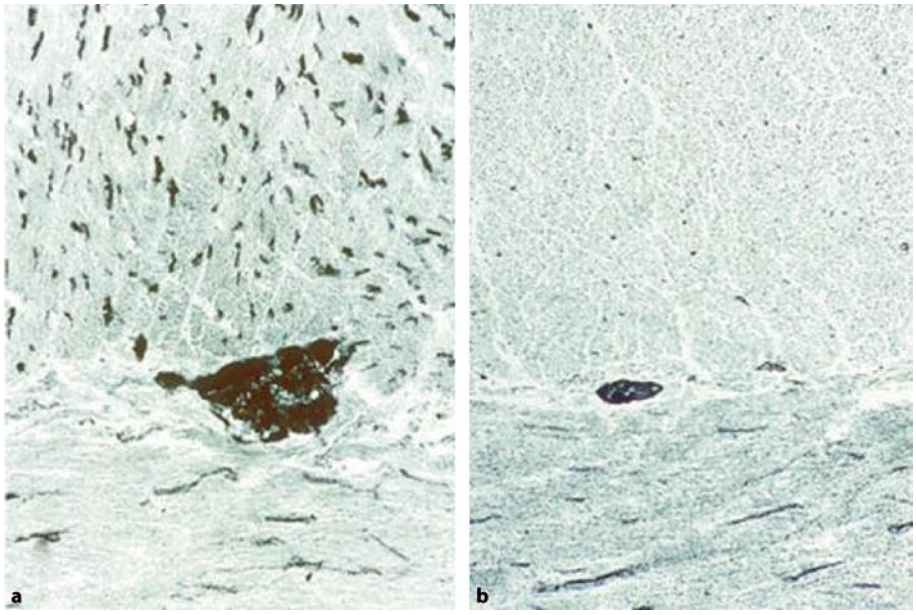


Fig. 14.6 Normal (**a**) and hypoganglionic (**b**) myenteric plexus (section, NADPH-diaphorase histochemistry)

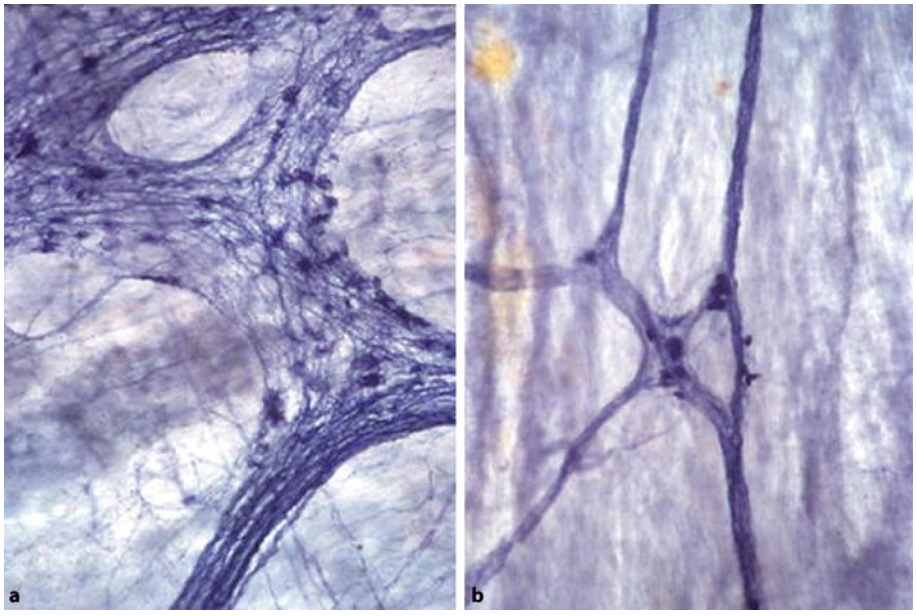


Fig. 14.7 Normal (**a**) and hypoganglionic (**b**) myenteric plexus (whole-mount preparation, NADPH-diaphorase histochemistry)

In hypoganglionosis the number of ganglion cells and thickness of nerve bundles in the myenteric plexus were markedly reduced compared to controls (Fig. 14.7).

Combination of whole-mount preparation and NADPH-diaphorase staining has been shown to be very valuable in patients with chronic constipation and histological signs of intestinal neuronal dysplasia (IND). We have used this technique to assess bowel specimens during and after surgery for persistent symptomatic constipation. Whole-mount preparations combined with NADPH-staining elegantly show the three-dimensional morphology of the normal submucous plexus (Fig. 14.8) and myenteric plexuses (Fig. 14.10) compared to the submucous and myenteric plexus in IND which demonstrate markedly a increased number of ganglion cells compared to controls (Figs. 14.9,11) [39]. This technique accurately identifies the hyperganglionosis of the myenteric and submucous plexuses which is characteristic of IND.

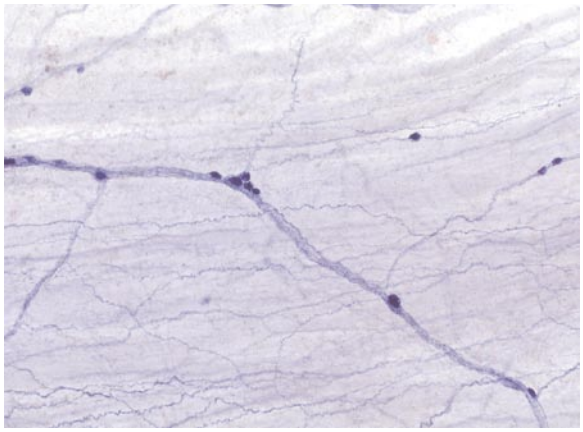


Fig. 14.8 Normal submucous plexus (whole-mount preparation, NADPH-diaphorase histochemistry)

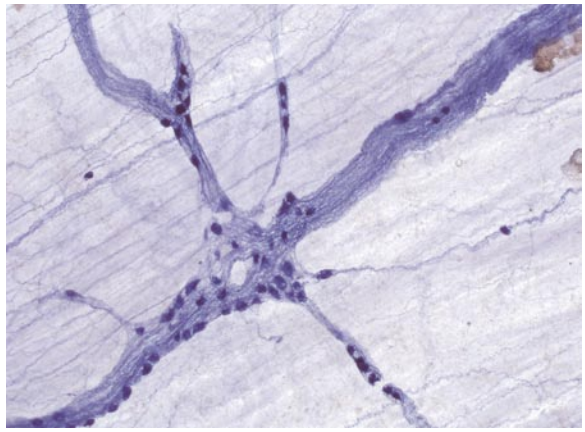


Fig. 14.9 Hyperganglionic submucous plexus (whole-mount preparation, NADPH-diaphorase histochemistry)

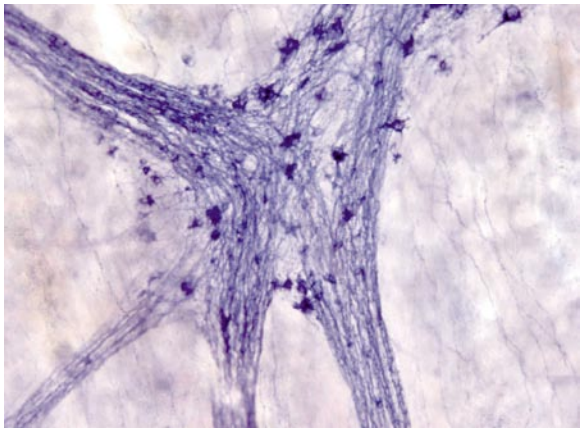


Fig. 14.10 Normal myenteric plexus (whole-mount preparation, NADPH-diaphorase histochemistry)

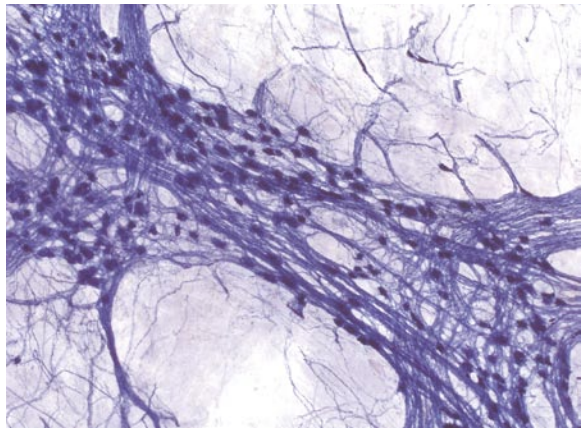


Fig. 14.11 Hyperganglionic myenteric plexus (whole-mount preparation, NADPH-diaphorase histochemistry)

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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

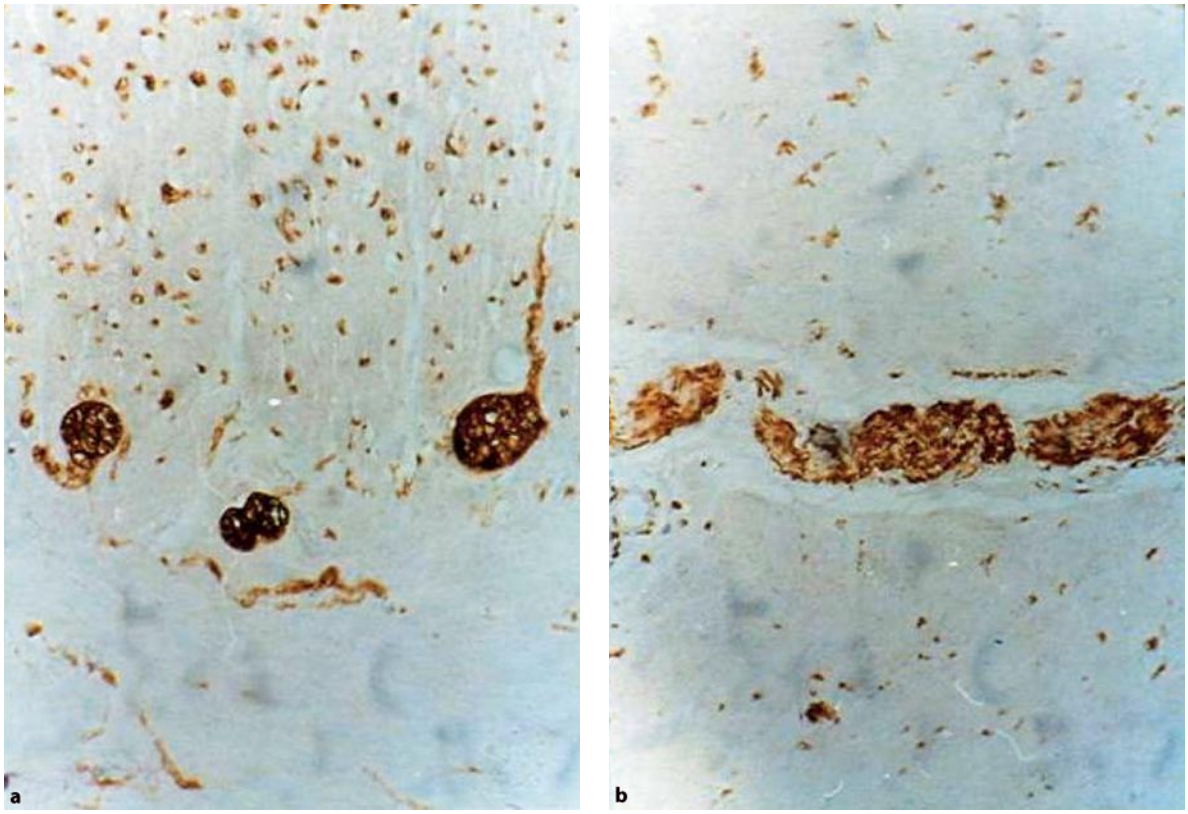

Fig. 15.1 PGP9.5 immunostaining: **a** myenteric ganglia in normal bowel; **b** hypertrophic fibers in HD bowel

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-

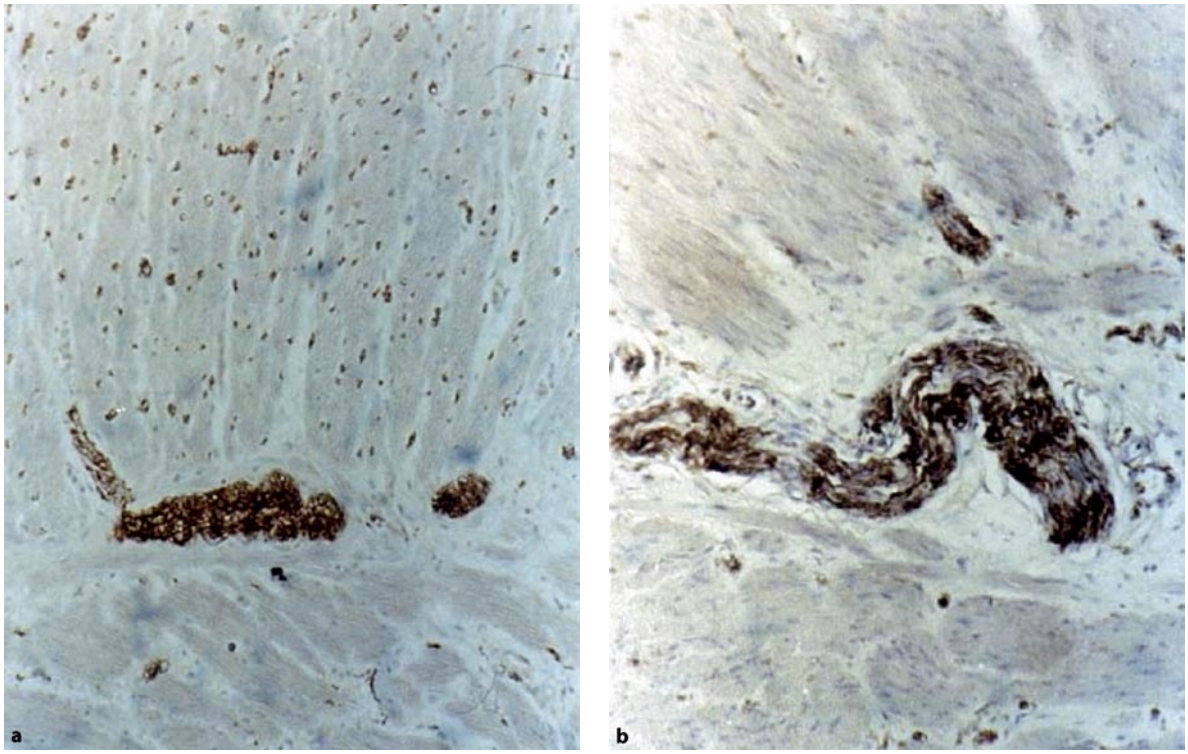

Fig. 15.2 NCAM immunostaining: **a** myenteric ganglia in normal bowel; **b** hypertrophic fibers in HD bowel

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

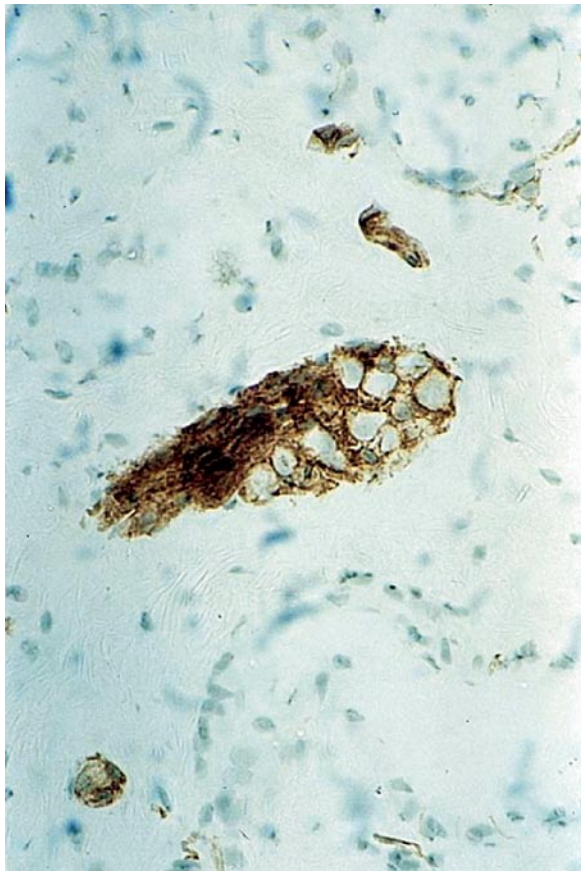

Fig. 15.3 NCAM immunostaining. Suction rectal biopsy with giant ganglion in IND

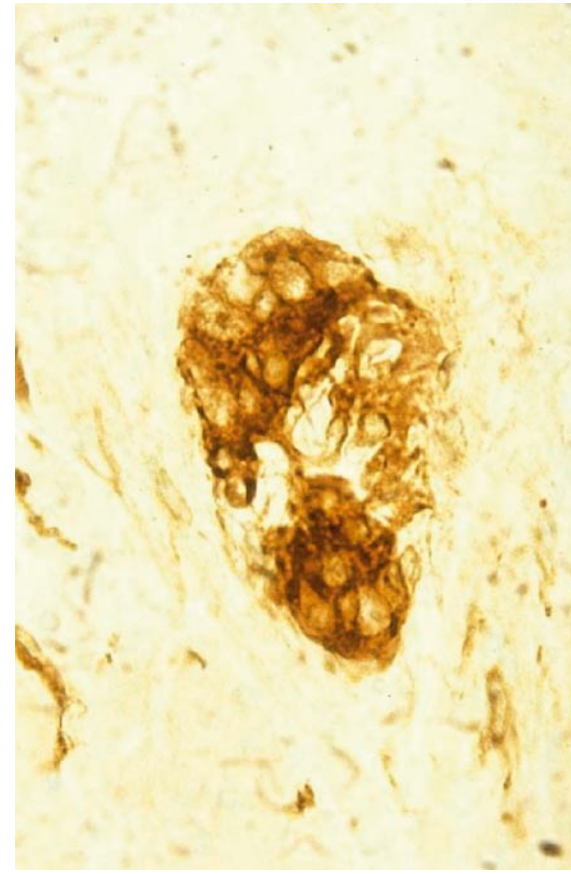

Fig. 15.4 NGFR immunostaining. Suction rectal biopsy with giant ganglion in IND

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

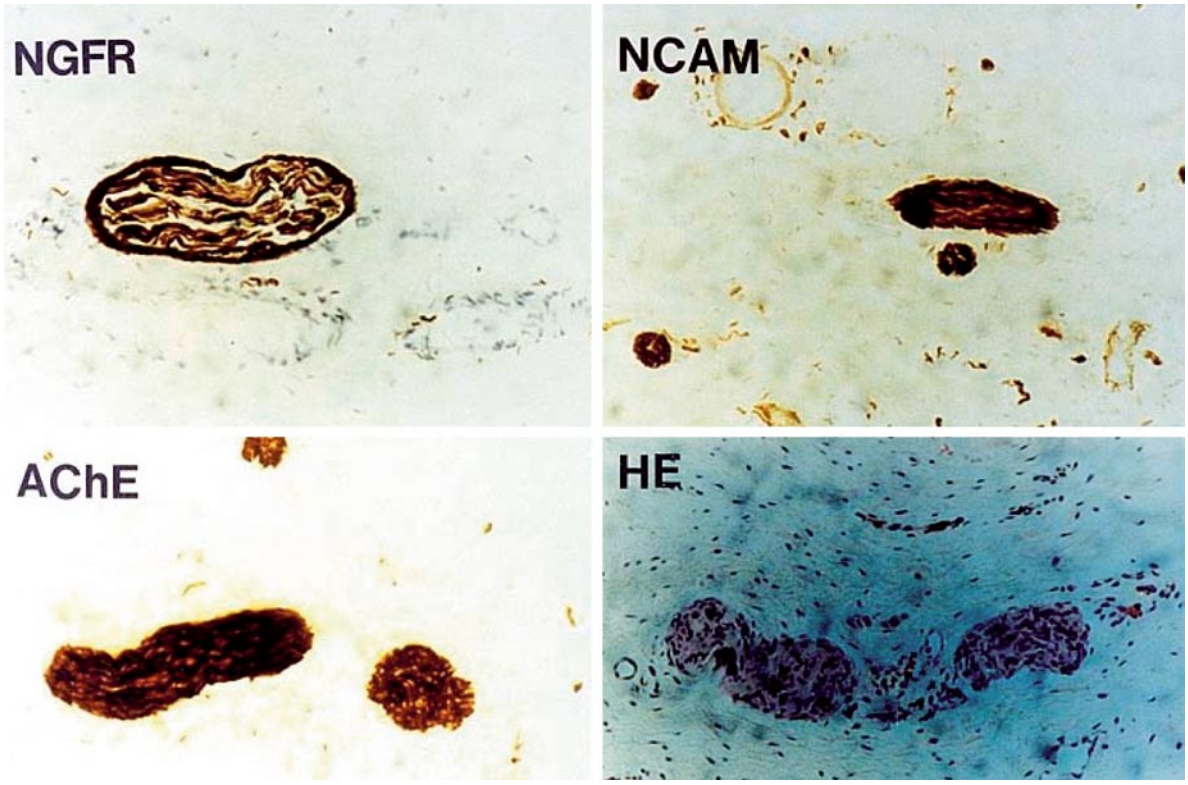

Fig. 15.5 Suction rectal biopsy. Staining with NGFR, NCAM, AChE and HE reveals submucous hypertrophic nerve trunk with perineurium only stained with NGFR

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].

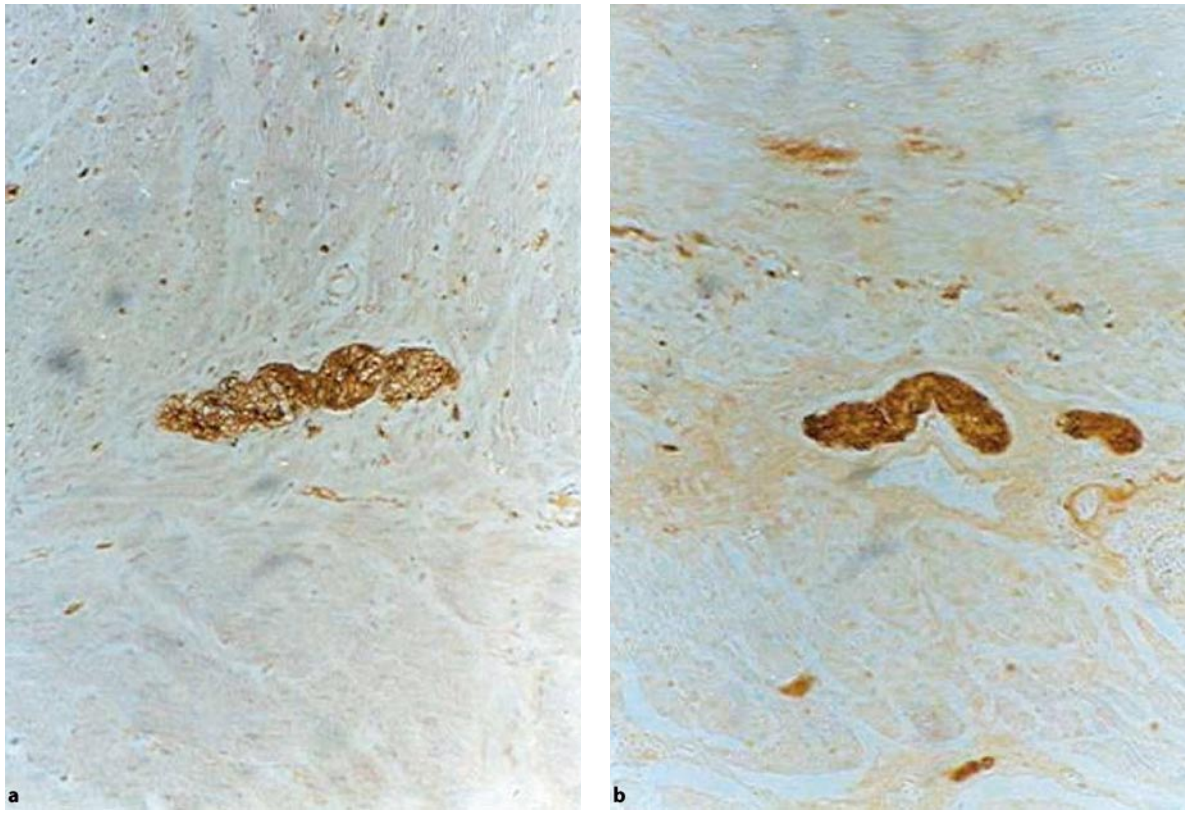

Fig. 15.6 S-100 immunostaining: **a** glial cells surrounding normal myenteric plexus; **b** glial cells around hypertrophic nerve fibers in HD

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.

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Electron Microscopic Studies of Hirschsprung's Disease

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16

16.1 Introduction

The histopathology of Hirschsprung's disease is defined by a complete absence of intramural nerve cells and a concomitant hypertrophy of nerve fascicles in caudal regions of the gastrointestinal tract. These pathomorphological criteria are readily recognized by standard enzyme and immunohistochemical techniques in establishing the diagnosis of Hirschsprung's disease. In contrast, electron microscopic studies are more time-consuming and require several procedures prior to the assessment of the specimens (e.g. fixation, postfixations, semi- and ultrathin sectioning, and specific stainings with heavy metal compounds). Therefore, in clinical routine, electron microscopy is not the tool of first choice to confirm the histopathology of Hirschsprung's disease.

Nevertheless, electron microscopic examination allows a subtle assessment of the ultrastructural peculiarities (e.g. nerve fiber and glial cell morphology, subcellular and extracellular components) not discernible in detail at light microscopic level. Whereas the first description of the histopathology of Hirschsprung's disease [1] gave rise to a variety of light microscopic studies, reports on the ultrastructure are comparatively rare. In 1970, Howard and Garrett [2] documented the electron microscopic findings in human Hirschsprung's disease describing several of the ultrastructural features of the aganglionic bowel wall. Baumgarten et al. [3] ultrastructurally analyzed the nervous elements in human Hirschsprung's disease and offered comparative remarks on the normal colon and internal anal sphincter. Later on, electron microscopic studies were combined with immunohistochemical techniques to investigate distinct neurochemically defined subpopulations of enteric nerves [4].

In this chapter, the typical ultrastructural features of human Hirschsprung's disease are outlined, discussed in relation to the morphology of the unaffected intestine and compared to the findings obtained from animal models of this intestinal innervation disorder.

16.2 Ultrastructural Features of Intestinal Aganglionosis

16.2.1 Hypertrophic Nerve Fascicles

In contrast to the normally configured enteric nerve plexus composed of ganglia and interconnecting nerve fascicles, in Hirschsprung's disease the bowel wall is characterized by a complete lack of intramural nerve cells and, thus, the absence of ganglia. Conversely, the remaining nerve fascicles passing within the intermuscular zone, the submucosal and mucosal layer are considerably thickened. This striking nerve trunk hypertrophy has also been reported in light microscopic studies [5] using either conventional hematoxylin-eosin or immunostaining of the glial marker protein S-100. As 90% of rectal suction biopsies contain nerve fascicles greater than 40 µm in diameter, this feature is considered to be highly predictive of aganglionosis and represents an important additional parameter in the diagnosis of Hirschsprung's

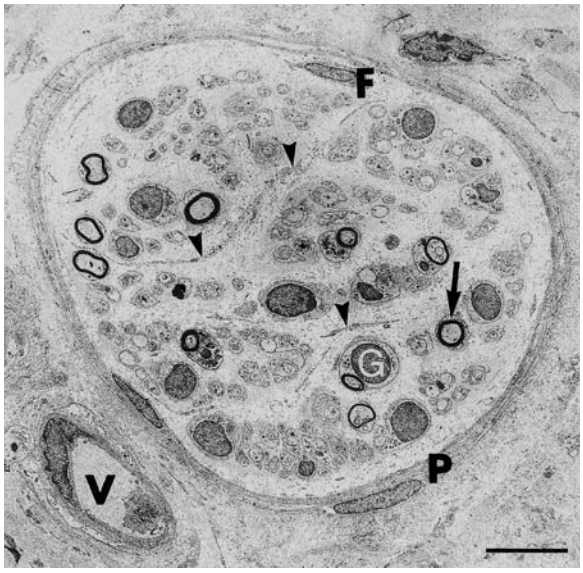

Fig. 16.1 Hypertrophic intermuscular nerve fascicle within the aganglionic segment. The nerve fascicle is composed of a prominent perineural sheath (*P*), numerous glial cells (*G*), naked and myelinated (*arrow*) nerve fibers. Abundant endoneural connective tissue composed of collagen and fibroblasts (*F*) with elongated processes (*arrowheads*) subdivide the nerve fascicle into different compartments (*V* adjacent blood vessel; *bar* 10 μm)

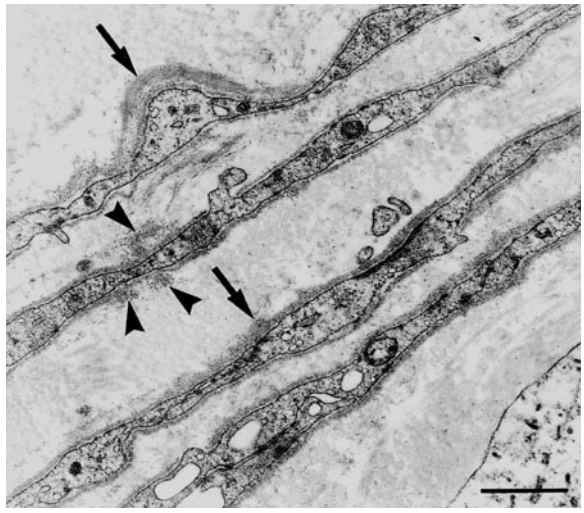

Fig. 16.2 Perineurium of a hypertrophic nerve fascicle within the aganglionic segment. Perineural cells exhibit a discontinuous thickening of basal laminae (*arrows*) and flocculent accumulations of basal lamina material (*arrowheads*) (*bar* 1 μm)

disease. The hypertrophic nerve fascicles are composed of a surrounding perineurium, abundant nerve fibers, distinctly shaped glial cells and a well-developed endoneurium (Fig. 16.1).

16.2.2 Perineurium

The prominent perineural sheath consists of multiple layers of flattened perineural cells connected by close intercellular contacts. Their cell borders are covered by a basal lamina delimiting the collagen-filled extracellular space between adjacent perineural cell layers (Fig. 16.2). Smaller nerve fascicles gradually lose their thick perineural sheath and possess a single-layered (Fig. 16.9), in some instances, discontinuous perineural envelope. When ramifying within the smooth muscle layers, the nerve fiber bundles are bare of a surrounding perineurium. The perineurium of hypertrophic nerve fascicles is richly supplied with blood vessels located adjacent to or intercalated within the perineural cell layers resembling typical features of vasa nervorum encountered in conventional peripheral nerves (Fig. 16.1). Large numbers of collagen fiber bundles and fibroblasts surround the outer border of the perineurium. The thin and remarkably elongated fibroblast processes frequently form loop-like, almost completely closed extensions ("collagen pockets") engulfing bundles of collagen fibers (Fig. 16.3).

16.2.3 Endoneurium

The nerve fibers within the hypertrophic nerve fascicles are not directly apposed but separated by endoneural connective tissue. The widened endoneural space is filled with densely packed collagen fibers and elongated fibroblasts subdividing the entire nerve fascicle into different endoneural compartments (Fig. 16.1). Whereas the majority of endoneural connective tissue fibers exhibit a typical periodical cross-banding (collagen fibers), a minor portion forms a hairy web of interdigitating reticular fibers (Fig. 16.4).

16.2.4 Glial Cells

The glial cell population within the hypertrophic nerve fascicles shows a remarkably homogeneous ultrastructure characterized by an oval nucleus lacking chromatinic condensations, a translucent cytoplasm poorly equipped with organelles and gliofilaments, and an individual basal lamina envelope (Fig. 16.5). In hypertrophic nerve fasci-

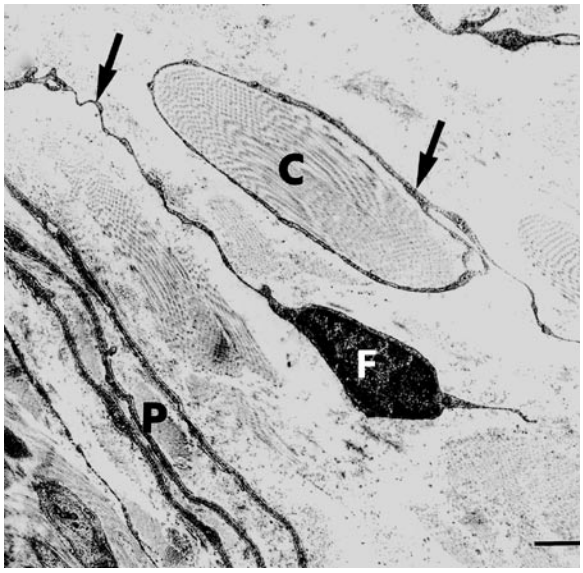

Fig. 16.3 Fibroblast (*F*) in close proximity to the perineurium (*P*) of a hypertrophic nerve fascicle within the aganglionic segment. Elongated fibroblast processes (*arrows*) engulf bundles of collagen fibers (*C*) forming "collagen pockets" (*bar* 2 μm)

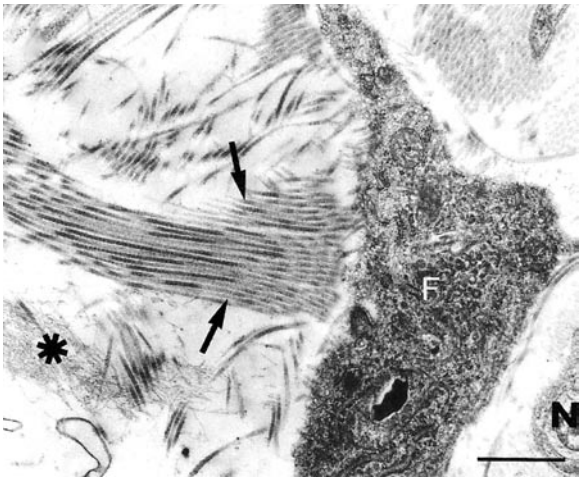

Fig. 16.4 Endoneural fibroblast (*F*) located within a hypertrophic nerve fascicle of the aganglionic segment. Collagen fibers extend from the cellular border into the endoneural space and aggregate to bundles (*arrows*). Reticular fibers form a hairy web of thin interdigitating filaments (*asterisk*) (*N* adjacent nerve fiber; *bar* 1 μm)

cles encountered in the aganglionic segment the glial cell processes generally ensheath a reduced number of nerve fibers forming mono- or oligoaxonal units (Fig. 16.6). Multiaxonal units are confined to smaller nerve fiber strands and their intramuscular ramifications. The observed ultrastructural characteristics correspond to the morphology typical of Schwann cells rather than of normal enteric glial cells: glial cells of the unaffected colonic wall possess a heterochromatinic nucleus with multiple indentations and a rich supply of cytoplasmic organelles and gliofilaments. Numerous nerve fibers are enveloped by extensively dividing glial cell processes resembling multiaxonal units (Fig. 16.7).

16.2.5 Nerve Fibers

The diameter of nerve fibers varies widely from 0.2 μm up to 8 μm. Their axoplasm is electrolucent and contains a reduced number of neurofilaments, microtubules and mitochondria (Figs. 16.5 and 16.6) in comparison to nerve fibers of normal interganglionic nerve fascicles (Fig. 16.7). While the axonal extensions of hypertrophic nerve fascicles within the intermuscular zone and the submucosal layer are almost completely bare of synaptic vesicles, the axoplasm of thinner nerve fibers enter-

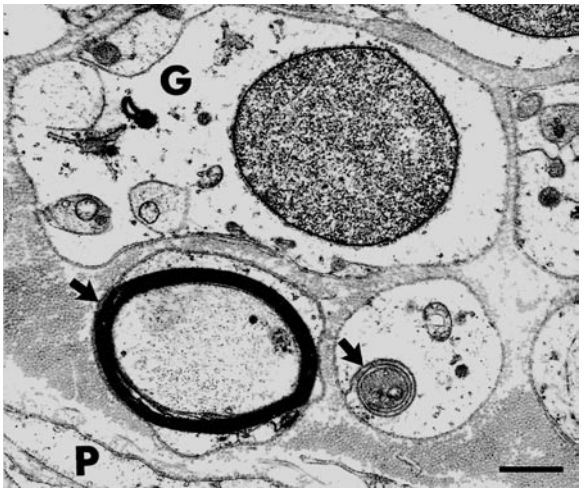

Fig. 16.5 Hypertrophic intermuscular nerve fascicle within the aganglionic segment. The glial cell (*G*) is characterized by a round euchromatinic nucleus and an electrolucent cytoplasm with a reduced number of organelles and gliofilaments. Two monoaxonal units display different stages of myelination (*arrows*) (*P* perineurium; *bar* 1 μm)

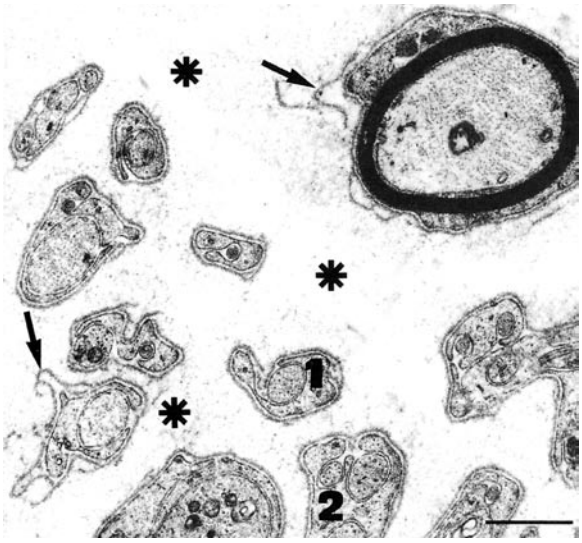

Fig. 16.6 Hypertrophic submucosal nerve fascicle within the aganglionic segment. Nerve fibers are separated by the widened collagen filled endoneural space (*asterisks*) and arranged in monoaxonal (*1*) and oligoaxonal (*2*) units. The basal lamina of glial cells enveloping naked or myelinated nerve fibers is frequently multilayered (*arrows*) (*bar* 1 μm)

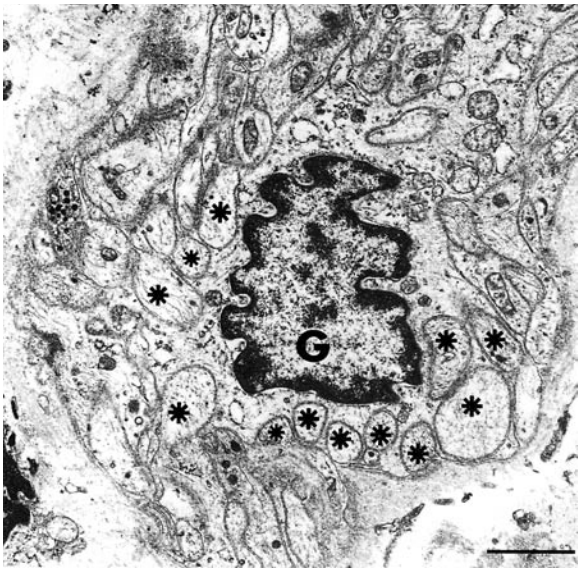

Fig. 16.7 Nerve fascicle of the myenteric plexus of a control specimen. Numerous nerve fibers (*asterisks*) are enclosed by one glial cell resembling a multiaxonal unit. The glial cell (*G*) is characterized by an indented heterochromatinic nucleus and a cytoplasm richly equipped with organelles and gliofilaments. The nerve fascicle lacks a prominent perineural sheath (*bar* 1 μm)

ing the smooth muscle layers exhibit varicose swellings predominantly filled with small empty, numerous small electrolucent and medium to large-sized electrodense vesicles. It has been shown that this distinct subpopulation of synaptic vesicles contrasts with the highly heterogeneous synaptic vesicle population of the unaffected bowel wall [3]. Immunohistochemical studies in human Hirschsprung's disease have confirmed a decreased number of synaptophysin-positive nerve fiber endings [6, 7]. Ultrastructural studies [8] have shown that the remaining neurotransmitters mainly correspond to acetylcholine (reaction deposits of acetylcholinesterase) observed between nerve terminals and smooth muscle cells, suggesting a direct innervation by extrinsic nerve fibers.

16.2.6 Myelination

An additional feature of hypertrophic nerve fascicles is the presence of myelinated nerve fibers (Fig. 16.1). Myelination of intramural nerve fibers is not confined to the intermuscular zone but also extends up to the inner submucosal layer with the ratio of myelinated nerve fibers to nonmyelinated nerve fibers ranging from 1:20 to 1:40. Whereas some nerve fibers are surrounded by a few lamellae probably indicating the initiation of myelination (Fig. 16.5), the majority of myelinated nerve fibers possess a myelin sheath composed of multiple apposed lamellae (Figs. 16.6 and 16.8).

16.2.7 Basal Laminae

Within the aganglionic bowel wall distinct basal lamina abnormalities can be observed. The basal lamina of perineural cells surrounding large and medium-sized nerve fascicles shows a discontinuous but marked thickening (Fig. 16.2). The width, measured from the perineural plasmalemma to the collagen-filled intercellular space, ranges from 50 nm to 200 nm. Additionally, flocculent accumulations of amorphous material exhibiting an electron density similar to the thickened perineural basal lamina are frequently disseminated along the perineural cell layers and protrude from the basal lamina into the extracellular space. Moreover, multilamination of basal laminae is found covering the glial cell plasmalemma of small monoaxonal units (Figs. 16.6 and 16.9) and myelinated nerve fibers of small- to medium-sized diameters including the nodes of Ranvier (Figs. 16.6 and 16.8). The basal lamina layers are irregularly apposed and show wriggling ramifications either connecting two adjacent basal laminae or blindly ending between endoneural

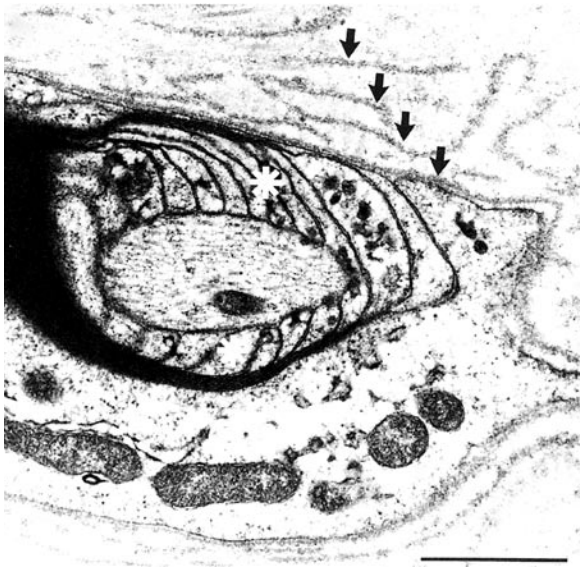

Fig. 16.8 Hypertrophic submucosal nerve fascicle within the aganglionic segment. A myelinated nerve fiber displays a node of Ranvier characterized by terminal loops (*asterisks*) of the glial corona and is surrounded by a multilayered basal lamina (*arrows*) (*bar* 1 μm)

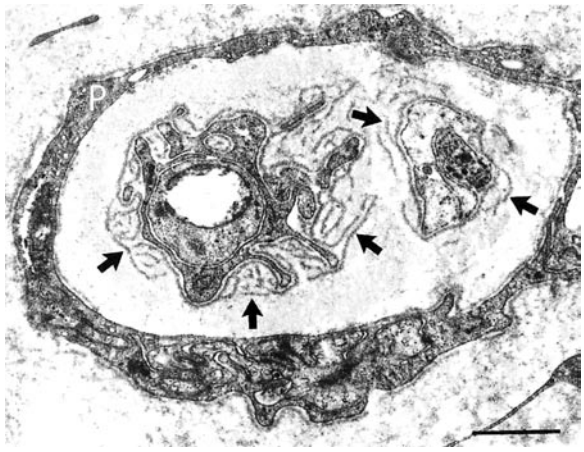

Fig. 16.9 Small submucosal nerve fascicle within the aganglionic segment. Glial cell processes of two monoaxonal units are concentrically surrounded by highly multilayered basal laminae (*arrows*) ramifying throughout the collagen-filled endoneurium (*P* single-layered perineurium; *bar* 1 μm)

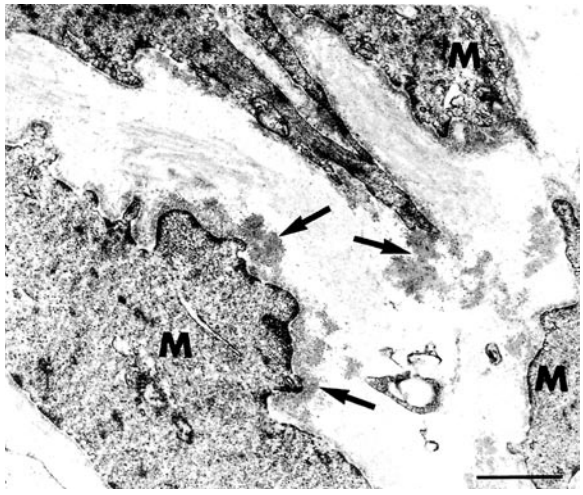

Fig. 16.10 Irregularly contoured smooth muscle cells (*M*) of the lamina muscularis mucosae of the aganglionic segment. Flocculent accumulations of basal lamina material (*arrows*) protrude into the interstitial space (*bar* 1 μm)

collagen fibers. Similar morphological abnormalities as observed in the perineural basal lamina are also discernible in the basal lamina surrounding smooth muscle cells, in particular those of the lamina muscularis mucosae. Prominent flocculent protrusions of amorphous basal lamina material are discontinuously distributed along the borders of the irregularly contoured smooth muscle cells (Fig. 16.10).

16.2.8 Subserosal Nerve Fascicles

Subserosal nerve fascicles approaching the aganglionic bowel wall via the mesentery exhibit ultrastructural characteristics very similar to those observed in intramural nerve fascicles located in the intermuscular and submucosal layer. Although the perineural sheath is more prominent, the amount of endoneural tissue is larger and

myelination occurs more frequently, both glial cells and nerve fibers virtually show the same ultrastructural arrangement.

16.2.9 Transitional Zone

Between the aganglionic and normoganglionic segment extends a transitional zone of varying length. This hypoganglionic region is characterized by small oligoneuronal ganglia and an irregular network of interganglionic nerve fiber connections. Although the hypertrophic nerve fascicles of the aganglionic segment enter the transitional zone, their number and diameter gradually decreases in the oral direction. The perineural sheath is thinner, the amount of endoneural tissue is diminished, and myelination is only rarely discernible. However, their ultrastructure still differs from the normal nerve plexus morphology by the predominance of mono- and oligoaxonal units and the Schwann cell-like appearance of glial cells.

16.3 Pathogenetic Implications

16.3.1 Extrinsic Origin of Hypertrophic Nerve Fascicles

The hypertrophic nerve fascicles encountered within the aganglionic bowel wall exhibit histological and ultrastructural features resembling those of extrinsic rather than intrinsic nerves. The presence of a thickened perineurium, wide endoneural spaces, vasa nervorum, Schwann cell-like glia, mono-/oligoaxonal units, myelination, and additionally the similarity of subserosal and intramural nerve fascicles suggest an extraenteric origin.

Indeed, whole-mount studies on the aganglionic colon from patients with Hirschsprung's disease [9] and of aganglionic spotted lethal rats [10] have confirmed the extrinsic origin of both intermuscular and submucosal nerve fascicles. Moreover, retrograde tracing experiments in aganglionic lethal spotted mice have revealed that the majority of nerve fibers originate from the inferior mesenteric ganglion and dorsal root ganglia, whereas only a minor proportion of intrinsic fibers seem to penetrate into the aganglionic segment [11].

Since Stach's [12] description of the "ascending nerves of the pelvic plexus" in various mammals, it is now well established that the distal colon is penetrated by large nerve bundles passing through the intermuscular zone and giving off branches to the myenteric plexus. It has been suggested that the over-abundance of thickened nerve fascicles found in the aganglionic segment results from a hypertrophy of these ascending pelvic nerves [10].

Experiments on extrinsically denervated cat colon have shown that the entire population of myelinated nerve fibers enter the colon from the pelvic plexus and, in part, from pudendal nerves [13]. As the stimulation of pelvic nerves induces contraction in the distal colon [14], it is suspected that the over-abundance of myelinated intramural nerve fibers originating from the pelvic plexus contributes to the functional colonic obstruction besides other intrinsic pathophysiological mechanisms. Constrictive influences mediated by hypertrophic nerves on nerve cell-deprived colonic segments have also been claimed by Baumgarten et al. [3] and Howard and Garrett [2], as these nerve fibers do not resemble blind endings, but form axonal varicosities richly supplied by synaptic vesicles in close proximity to smooth muscle cells.

16.3.2 Basal Lamina Abnormalities

As outlined above, ultrastructural peculiarities encountered in the aganglionic bowel wall include distinct morphological basal lamina abnormalities of perineural, glial and smooth muscle cells. These observations are in accordance with the light microscopic demonstration of an abnormal distribution of basal lamina-specific components such as collagen type IV, laminin and fibronectin in the aganglionic bowel wall [15, 16]. In particular, the extensive production of basal lamina material within the hypertrophic nerve fascicles provides an ultrastructural correlate of the findings reported by Parikh et al. [16] who have demonstrated intense immunoreactivity of the basal lamina constituent fibronectin in thickened nerve fascicles within the aganglionic segment.

However, morphological alterations of the basal lamina are not specifically related to Hirschsprung's disease, as they have also been documented in other neurological diseases such as diabetic autonomic [17] and hereditary peripheral neuropathies [18, 19]. In infantile hypertrophic and hereditary motor and sensory neuropathy type III the reduplication of the glial basal lamina has been attributed to reactivated Schwann cells from which the basal lamina material originates [20]. Thus, in Hirschsprung's disease the over-production of basal lamina material in hypertrophic nerve fascicles may reflect an increased activity of proliferating glial cells. In diabetic enteroneuropathy both a thickening and a reduplication of the glial cell basal lamina has been observed and is considered to represent a diffusion barrier for neurotransmitters impairing their release to neuroeffector sites [21]. However, this assumption may not apply to patients with Hirschsprung's disease, as in the aganglionic segment most of the axonal swellings located adjacent to smooth muscle cells are bare of a basal lamina thickening. Furthermore, it cannot be excluded that basal lamina abnormalities observed in Hirschsprung's disease may represent secondary defects resulting from the chronic mechanical distension resting upon the dilated bowel wall. In fact, intraluminal tension forces

are capable of provoking both basal lamina thickening and reduplication as demonstrated in the endothelium of blood vessels exposed to hypertension [22].

Abnormalities of the basal lamina in human Hirschsprung's disease remain of special interest, as they have also been found in mouse strains developing congenital megacolon [23, 24]. In particular, the thickening and reduplication of basal laminae surrounding smooth muscle cells of the lamina muscularis mucosae have been considered to reflect microenvironmental abnormalities during embryogenesis persisting into adult life. It has been proposed that the increased production of extracellular matrix components within the presumptive aganglionic segment may provide a good substrate for the ingrowth of extrinsic nerves, but seems to impair the colonization by nerve cell precursors [25–27]. These findings suggest that the pathogenetic mechanisms leading to aganglionosis in murine models of Hirschsprung's disease are not entirely related to the neural crest, but include microenvironmental abnormalities intrinsic to the colonic wall.

In summary, morphological alterations of basal laminae in human Hirschsprung's disease involve both neuronal and non-neuronal elements and provide further evidence that extracellular matrix components are abnormally distributed within the affected bowel wall. Moreover, the increased amount of collagen observed in the endoneurium, the perineurium and in areas adjacent to hypertrophic nerve fascicles indicates that the histopathology of the aganglionic intestine is not exclusively confined to nervous tissue alterations, but also includes an impressive over-production of connective tissue components.

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Intestinal Neuronal Malformations (IND): Clinical Experience and Treatment

A. M. Holschneider, P. Puri, L. H. Homrighausen, and W. Meier-Ruge

17

17.1 Introduction

Neuronal intestinal dysplasia (IND or NID) was first described by Meier-Ruge in 1971 as a condition of unknown pathogenesis which is usually associated with obstruction of the lower intestine [1]. It may be induced, like Hirschsprung's disease (HD), by an underlying autoimmune mechanism [2], a deficient production of trophic factors such as laminin A [3] or a genetic defect. The main problem is that it remains controversial as to whether or not there is a causal relationship between specific histological findings and clinical symptoms, in particular since so-called pathological innervation patterns have been described in the proximal colon of patients with fetal obstruction [4, 5] and in normal controls [6].

Many investigations have sought to determine whether IND is a separate entity [6–8]. In these studies, on analyzing the number of ganglion cells, the histological picture of IND has been found even in normal individuals without any symptomatic constipation [9, 10], or a high interobserver variation has been found using the usual diagnostic criteria on rectal biopsies [11] (see Section 17.11). It has been suggested that the pathological changes seen in IND may be part of normal development or may be a secondary phenomenon induced by congenital obstruction and inflammatory disease [4, 12]. Due to this inconsistency in diagnostic pattern, the view on IND has changed over the years.

Meier-Ruge, who was the first to describe IND, modified his criteria for the definition of IND over the years [3, 13–16]. Investigating 30 sections per biopsy in patients with IND, he found normal ganglion cells, hyperplasia or absence of ganglion cells in the same specimen. Munakata et al. [17] used monoclonal antibodies to synaptic vesicles and other specific techniques and identified neuron degeneration. Csury and Pena [18] reviewed 25 studies including 322 patients with IND. Seven different histochemical methods had been used with varying frequency leading to a confusing variety of histochemical findings. Even the consensus conference of Borchard et al. in 1990 [19] could not overcome this inconsistency although specific morphological criteria for IND were established and acetylcholinesterase (AChE) staining, lactic-dehydrogenase (LDH) reaction, and succinic dehydrogenase (SDH) reaction became the preferred enzyme-histochemical methods [6, 14–16, 20, 21].

17.2 Genetic Observations

17.2.1 Ncx/Hox11L1 Deficiency

The possibility that there is a genetic component in IND as well as in HD is recognized. Different studies showed

a correlation between Ncx/Hox11L.1 deficiency in mice and rats. Homozygous Ncx$^{-/-}$ mice develop megacolon with massive distension of the proximal colon and the histopathological findings of IND [22, 23].

The Ncx/Hox11L1 gene belongs to the Hox11 gene family of mice [24] and is expressed in neural crest-derived tissues such as dorsal root ganglia, cranial nerve ganglia, sympathetic ganglia and enteric nerve ganglia in embryos between days 9.5 and 13.5. In adult mice, the gene is expressed in enteric nerve ganglia and adrenal glands [23, 25]. Hatano et al. and as well as Shirasawa et al. found megacolon and myenteric neuronal hyperplasia in homozygous Ncx/Hox11L1-deficient mice [22, 23]. After inactivating the Ncx/Hox11L1 gene, Hatano et al. [22] described hyperinnervated enteric neurons in the narrow segment of megacolon and cell degeneration and neuronal cell death which occurred in later stages of age. Shirasawa et al. [23] described a similar picture with an increased number of myenteric ganglia, total neurons per ganglion and NADPH diaphorase presumptive inhibitory neurons per ganglion in the proximal and distal colon and decreased findings in the distal ileum of all homozygous Nox/Hox11L1-deficient mice. They interpreted these findings as the classical picture of IND, even if it also involves the plexus myentericus and stated that a human homologue of Ncx/Hox11L1 might be a candidate in understanding the development of IND.

Yamataka et al. studied ten specifically created homozygous Nox/Hox11L.1-deficient mice showing megaileococecocolon with a caliber change in the proximal colon. Five age-matched wildtype mice were used as control group. They investigated AChE and NADPH histochemistry in half of the specimens and H&E staining, immunohistochemistry for protein gene product 9.5 (PGP 9.5) antibody (neuronal marker), C-kit antibody (marker of intestinal pacemaker cells) and stem cell factor antibody (C-kit ligand marker) in the other half. In the Ncx$^{-/-}$ mice they found the typical patterns of human IND with hyperganglionosis in the neuronal plexus, and ectopic ganglia in the mucosal and muscular layers and ghost-like ganglia [26].

Yanai et al. [27] found a bowel dysmotility related to acetylcholine (ACh) in homozygous mutant Ncx/Hox11L.1-deficient mice. They examined the contractile responses to ACh, histamine, serotonin and barium chloride in Ncx$^{-/-}$ compared to age-matched control mice. They found decreased distal colon circular muscle contractions for lower ACh doses and decreased distal colon longitudinal muscle contractions for all ACh doses in the Ncx$^{-/-}$ mice compared to the controls. In the proximal colon of the Ncx$^{-/-}$ mice increased circular muscle contraction was found at higher doses and decreased longitudinal muscle contraction at lower doses compared to the controls. There were no effects of ACh found in the jejunum and no significant effects in the ileum. Responses to histamine and serotonin were not found, and responses at barium chloride were the same in Ncx$^{-/-}$ and control mice. The question is whether these findings can be transferred to humans. Costa et al. [28] performed a mutation screening of the whole Hox11L1 coding region in 48 patients affected by IND or HD and could not show sequence variants, causative missense mutation or neutral substitution. The linkage analysis excluded other molecular defects as well. Even an analysis of non-coding promoter regions of Hox11L1 in affected patients, performed by Fava et al. [29], was unable to show alterations such as nucleotide variants, small deletions or cytogenic alterations. These authors stated that Hox11L1 might influence the development of human intestinal motility disorders but is not directly involved.

17.2.2 Endothelin-B Receptor Deficiency

Another possible genetic pathway of IND was found in a described deficiency of the endothelin-B receptor (EDNRB). The endothelin system (EDN) is a ubiquitous, well-balanced network of components which interact with each other to influence vascular smooth muscle cells to cause potent long-lasting vasoconstriction [30]. One of the endothelin receptors (END 3) plays a key role in the development of the enteric nervous system in mice, horses and also in humans. In rats with homozygous deficiency of EDNRB, HD with long-segment aganglionosis occurs. Holland-Cunz et al. compared heterozygous (+/sl) EDRNB-deficient rats with homozygous (sl/sl) and wildtype rats (+/+). As expected distal aganglionosis was found in the sl/sl rats but not in the +/sl or wildtype rats. In the heterozygous EDNRB-deficient rats the picture of IND with submucosal giant ganglia and hypertrophied nerve fiber strands was found [31–34].

Puri has emphasized the necessity for future investigation of these genetic pathways because of the strong evidence that has emerged from the models discussed above to find new insights into the etiology of IND [35, 36].

17.3 Occurrence

Schärli and Meier-Ruge [37] divided IND into a localized form and a disseminated form. Kunde et al. [38] described ten patients with involvement of the small bowel. Two of them had additional gastroschisis. Gittes et al. [39] reported one patient with diffuse hyperganglionosis of the myenteric plexus. Stoss considered a variable degree of dysganglionosis of the submucous plexus to be responsible for primary chronic constipation in 18 adults [40]. Kobayashi et al. found abnormal innervation of the internal anal sphincter (IAS) not only in patients with HD and hypoganglionosis but also in five children with isolated IND [41]. The alterations in IND can therefore

involve the whole enteric nerve system including both plexus and the IAS and can be observed in all age groups.

17.4 Classification

Schärli and Meier Ruge distinguished what they called "myenteric hyperplasia" in disturbances of sympathetic and parasympathetic origin in 1981. In 1983, Fadda et al. classified IND into two subtypes [42]. Type A which occurs in less than 5% of patients is characterized by congenital aplasia or hypoplasia of the adrenergic innervation especially of the blood vessels, and presents acutely in the neonatal period with episodes of intestinal obstruction, diarrhea and bloody stools. Type B is characterized by a malformation of the parasympathetic submucous plexus and accounts for over 95% of cases of isolated IND. Clinically, it shows a varied picture of chronic constipation, in severe cases mimicking HD (Fig. 17.1)

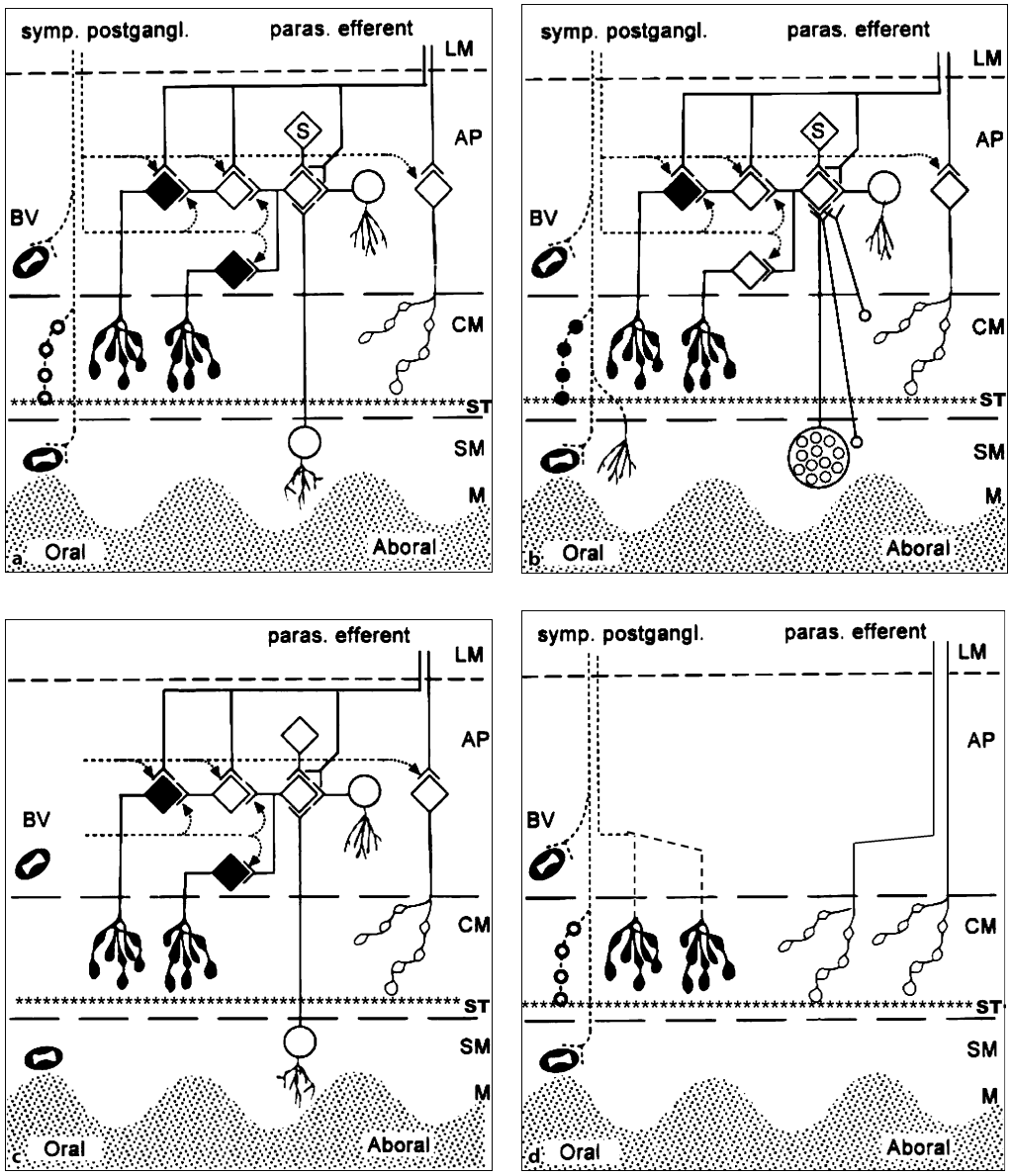

Fig. 17.1a–d Schematic drawing of normal and pathological innervation of the colon: **a** normal innervation, **b** IND type B, **c** IND type A, **d** aganglionosis (*AP* Auerbach's plexus, *CM* circular muscle, *LM* longitudinal muscle, *M* mucosa, *SM* submucosa, *ST* Stach's plexus)

Table 17.1 Classification of congenital neuronal malformations of the bowel (according to Holschneider, 1994)

Aganglionosis (Hirschprung's disease)	**Ultrashort segment**
	Short-segment (classical HD)
	Long-segment (above colon descendens)
	Zuetzer-Wilson syndrome
	Aganglionosis of the colon and parts of the small intestine
	Aganglionosis of the whole bowel
Hypoganglionosis	**Isolated hypoganglionosis**
	Hypoganglionosis and heterotopia
	Hypoganglionosis and immaturity of ganglion cells (=hypogenesis)
Intestinal neuronal dysplasia	**IND A**
	IND B
	Abortive IND
Isolated heterotopia of nerve cells	**Heterotopia of the submucous plexus**
	Heterotopia of the myenteric plexus
	Heterotopia of both plexus
Immaturity of nerve cells	**Immaturity of the submucous plexus (<1.5 years)**
	Hypogenesis of nerve cells of the submucous plexus (>2 years)

Holschneider in 1994 [21] and Schärli in 1995 [16] proposed a classification of all intestinal innervation disorders including HD (Table 17.1). Moore et al. [43] introduced a histological grading system for the evaluation of IND coexisting with HD.

17.5 Symptoms

Symptoms associated with IND were reviewed by Csury and Pena [18] who analyzed reports of 279 patients. Constipation was reported in 53%, obstruction in 20%, "colitis" in 12%, and bloody stool, diarrhea, or vomiting in less than 10% each. However, no pathognomonic symptoms for any specific neuronal intestinal disorder were identified in 203 patients by Ure and Holschneider [44]. Koletzko et al. [45] investigated 6 children with IND type B, 18 with "abortive" IND with heterotopic ganglion cells without hyperganglionosis, and 22 normal controls. The mean colonic transit time and the symptomatic course did not differ significantly between the groups. Therefore, in patients with IND type B a great variability of symptoms may be expected, but chronic constipation is the leading complaint.

Isolated IND is rarely associated with severe symptoms. Sacher et al. [46] reported six children with isolated IND and meconium peritonitis, volvulus or intussusception and concluded that the symptomatology is correlated with the degree of functional defect of gut motility. Ure and Holschneider in 1994 found ileus in 2 out of 141 patients with isolated IND type B, in 1 combined with hypoganglionosis, in another one combined with heterotopia of the submucous and of the myenteric plexus each. Therefore, in the individual patient with IND, hypoganglionosis or heterotopia the severity of symptoms may not be derived from specific histochemical findings alone.

In our recent investigations constipation and the consequent secondary symptoms were the main symptoms of isolated IND B. Out of 81 children with isolated IND B, one suffered from ileus, three from subileus and three from enterocolitis (see Sect. 17.14.2)

Montedonico et al. compared 44 patients with severe IND and 16 with mild IND with 37 patients with functional constipation. The aim of their study was to determine the presence of specific clinical symptoms in IND and whether there is a correlation with the severity of histopathological findings. They found a higher presence of intestinal obstruction in the patients with severe IND, while fecaloma and soiling were more frequent in patients with mild IND and functional constipation. In comparison, the patients with severe IND showed a lower incidence of rectosigmoid distension on barium enema. Internal sphincter relaxation was frequently absent in these patients compared to those with functional

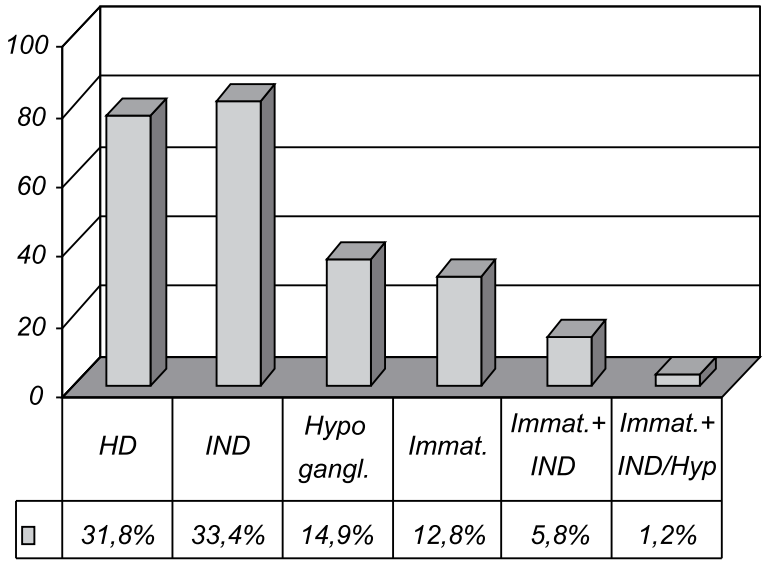

Fig. 17.2 Distribution of intestinal neuronal disorders in 242 children in the Hospital for Sick Children Cologne (1993–2004)

constipation. They concluded that clinical, radiological and manometric presentation of IND correlates with the severity of histochemical findings [47].

17.6 Incidence

The pathomorphology of IND can frequently be observed. Meier-Ruge et al. [48] evaluated 3,699 colonic mucosal biopsy specimens obtained from 773 patients over a 5-year period. A neuronal defect was present in 46.3% of the patients, 52.2% of them showing aganglionosis, 40.6% IND type B, 5% hypoganglionosis and 2.2% IND type A. In 29.6% of the patients the defect was not classifiable. This group included those with slight dysplasia, immaturity or hypogenesis of the submucous plexus and heterotopia of nerve cells to different degrees. Münteferring [49] reported aganglionosis in 64% of 631 specimens, IND in 24%, ultrashort HD in 3%, hypoganglionosis in 0.3%, HD associated with IND in 8%, and IND with colitis in 0.3%. In our own series of 242 patients, treated between 1993 and 2004, we found HD in 31.8% (n = 77) and IND in 33.4% (n = 81). Hypoganglionosis was seen in 14.9% and immaturity in 12.8%. A combination of immaturity and IND was seen in 5.8% of our patients and 1.2% showed immaturity with IND and hypoganglionosis in the histopathological evaluation of mucosal biopsies. In 28 of the 77 children with HD, the HD was combined with IND (36.6%) (Fig. 17.2).

In general, the incidence of isolated IND varies from 3% to 40% of all suction rectal biopsies in different centers [13, 16, 50, 51] but varies considerably between different countries. IND combined with HD occurs in 25–35% of patients with HD [13, 16, 44, 52], but HD occurs in only 17.9% of children with IND [13, 48]. Schärli [15], using the staining techniques of Meier-Ruge, found IND type B in 62% of his patients compared to 14% in our 1994 series of (n = 141 [52]) and 33% in our recent review of 2004.

The uncertainty regarding occurrence, classification, incidence, and clinical significance of the histological observations resulted from considerable confusion concerning the essential diagnostic histological and histochemical criteria, the most appropriate biopsy procedure, staining techniques and the age-dependence of the findings. Meier-Ruge [3] stated that there is often a combination of histochemical findings and "children older than 4 years with IND type B often had, in addition to giant ganglia, hypoganglionosis, hypogenesis or heterotopia of the myenteric plexus". This may confirm his hypothesis that the primary cause of intestinal neuronal malformations is a disturbance in the development of the embryological mesenchyme of the bowel.

Bandyopadhyay et al. reported seven patients with a suspected innervation disorder. Four patients ful-

filled the IND criteria laid down by Kobayashi and his co-workers (hyperganglionosis, giant ganglia and ectopic ganglion cells in the lamina propria), while the other three were highly suggestive of the diagnosis of IND following the criteria of other workers [53].

17.7 Biopsy Technique

Meier-Ruge [14, 48] emphasized the importance of taking a series of suction biopsies at distances in the geometric sequence 1, 2, 4, 8, and 16 cm above the dentate line and reported a detailed methodological survey for optimal histopathological diagnosis with special regard to a-, hypo- and dysganglionoses. In principle, he followed the recommendations of a consensus conference of three pediatric pathologists and a few pediatric surgeons (among them the author) from 1991 [19]. However, these widely used criteria were not able to enlighten the situation. Kobayashi and co-workers found giant ganglia in all their patients investigated with full-thickness rectal biopsies, whereas less than 60% of patients investigated with suction biopsies had giant ganglia [6].

Therefore, Krammer et al. in 1994 [54] and Smith [55] recommended whole-mount preparations as the most suitable section technique for histopathological evaluation and interpretation. They emphasized that the enteric nervous system consists of a three-dimensional plexus lying within the different layers of the intestinal wall. Cross-sections reveal only a part of the ganglia, neurons and glial cells, whereas whole-mount preparations show the three-dimensional morphology as a whole (Fig. 17.3).

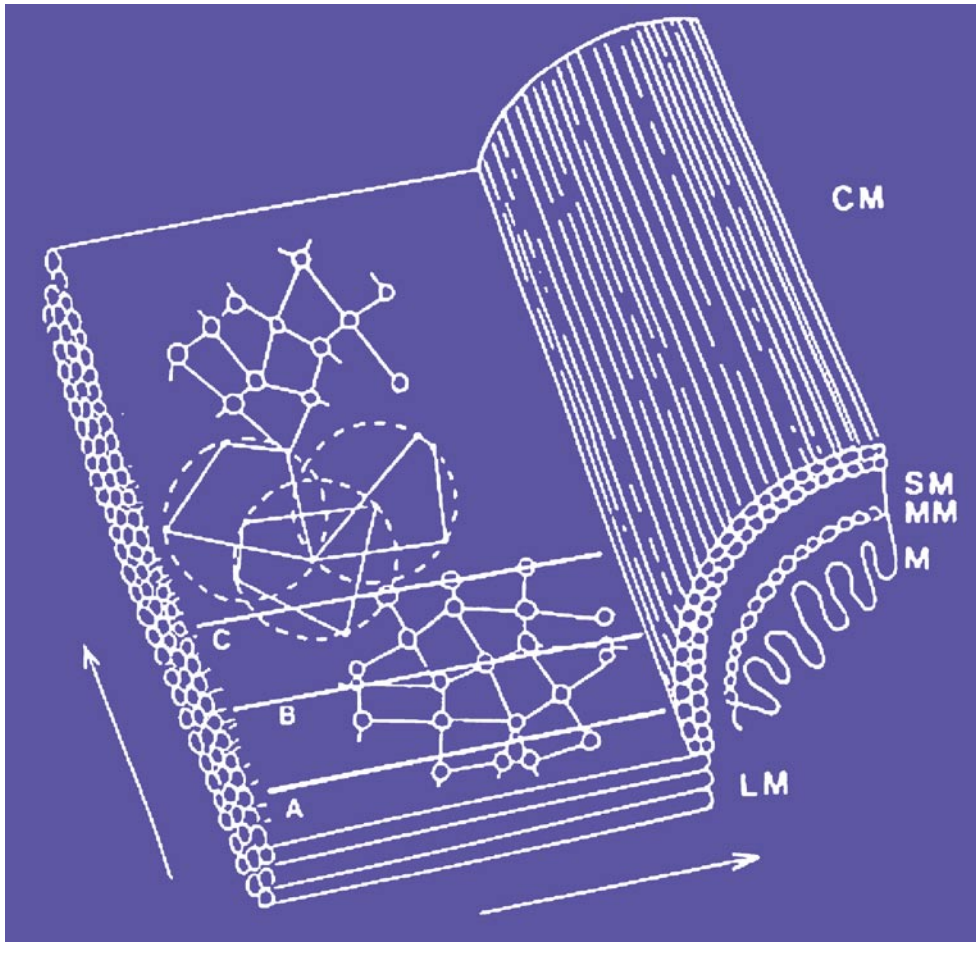

Fig. 17.3 Diagrammatic representation of the plexus myentericus [65]. Random sections in planes indicated by lines *A*, *B* and *C* will miss some of the ganglia (*B*), all of the ganglia (*A*), or none of the ganglia (*C*) if short lengths of bowel are analyzed (*LM* longitudinal muscle, *CM* circular muscle, *SM* submucosa, *MM* muscularis mucosa, *M* mucosa)

17.8 Diagnostic Criteria

Besides the lack of agreement concerning the optimal handling of biopsies, there is considerable confusion regarding the essential diagnostic criteria for IND. In 1971, hyperplasia of the submucous and myenteric plexuses and increased ACE activity in parasympathetic nerve fibers in the lamina propria were regarded by Meier-Ruge as the most characteristic histological findings of IND [1]. In 1981, Schärli and Meier-Ruge [37] considered an increase in AChE activity in the lamina propria and circular muscle layer as the most significant criterion.

Hyperplasia of the submucous and myenteric plexus, however, and heterotopia of neuronal cells into the lamina propria were described as characteristic but less-reliable features. Fadda et al. [42] in 1983 described an only moderately increased AChE activity in the lamina propria mucosae and muscularis mucosae and a stronger AChE reaction in the circular muscle layer as important criteria. The most important criterion according to these authors was an increase in the parasympathetic nerve fibers in the ascending and transverse colon, hyperplasia of the submucous plexus and giant ganglia with an increased number of ganglion cells. Schofield and Yunis [56] collected the diagnostic features of 13 different authors from the literature and showed marked differences, especially concerning AChE activity, in the lamina propria, muscularis mucosae and circular muscle. Giant ganglia were not mentioned in that review. To overcome this confusion the above-mentioned consensus conference from 1991 [49] established guidelines for identifying IND in mucosal rectal biopsies. The obligatory criteria were hyperplasia of the submucous plexus, giant ganglia with multiple small ganglion cells, bud-like nerve cell groups along nerve fibers and increased AChE activity in the adventitia of submucous arteries. In full-thickness biopsies the AChE activity in the muscularis propria was considered to be increased. Two additional facultative criteria were proposed: increased AChE activity in the mucosa decreasing with age and lacking in adults, and heterotopia of ganglion cells in the mucosa, and tunica muscularis propria (Table 17.2).

Furthermore, Meier-Ruge et al. [3] described giant ganglia of the submucous plexus with more than seven LDH positive nerve cells ($n = 9\pm3$) as the most relevant and age-independent criterion for IND. In contrast, hyperplasia of the submucous plexus, increase in AChE activity in nerve fibers of the lamina propria mucosae and the lack of SDH in nerve cells disappear with age [3] (Figs. 17.4 and 17.5).

In a more recent report [57], and with special regard to the three-dimensional network of the plexus, Meier-Ruge recommended 40 serial sections stained for LDH reaction as an important prerequisite for an optimal diagnosis because 30–55% of sections contain no ganglia in the submucosa, only 45–70% show ganglia and only 20–26% of all ganglia are giant ganglia. For the diagnosis of IND, at least four giant ganglia in 30 sections must be observed. In an earlier report, however, he stated that only 3–5% of ganglia in IND are giant ganglia [3]. These reports describe hyperganglionosis and increased AChE activity in the lamina propria as age-dependent findings, whereas bud-like nerve cell groups along nerve fibers and heterotopic nerve cells in the muscularis mucosae or lamina propria were regarded as age-dependent findings. Finally in 2004 Meier Ruge et al. described the quantitative IND diagnosis: IND is indicated in 30 sections by 15–20% submucosal giant ganglia with more than eight nerve cells with an average of 10±2 nerve cells per ganglion. In children under 1 year of age a sure diagnosis of IND is not possible because of apoptosis and maturation [58] (see Sect. 17.11)

Kobayashi et al. [6] came to different conclusions. Only 8 of their 19 patients with IND showed increased AChE-positive nerve fibers around submucosal blood vessels, and only 12 of the 19 patients showed moderate increases in AChE activity in the lamina propria and muscularis mucosae. The demonstration of neuronal het-

Table 17.2 Morphological criteria for IND (according to the Consensus Conference of the Division of Gastroenteropathology of the German Association of Pathology, Frankfurt 1990 [19])

Obligatory criteria	Optional criteria
Hyperplasia of the submucous plexus	Increased AChE activity in the lamina propria mucosae and muscularis mucosae (until 2nd year of life)
Giant ganglia (hyperganglionosis)	Heterotopia of ganglion cells
Bottom-like connection of groups of nerve cells to parasympathetic nerve fibers	
Increased AChE activity in the muscularis propria	
Increased AChE activity in the adventitia of submucous arteries	

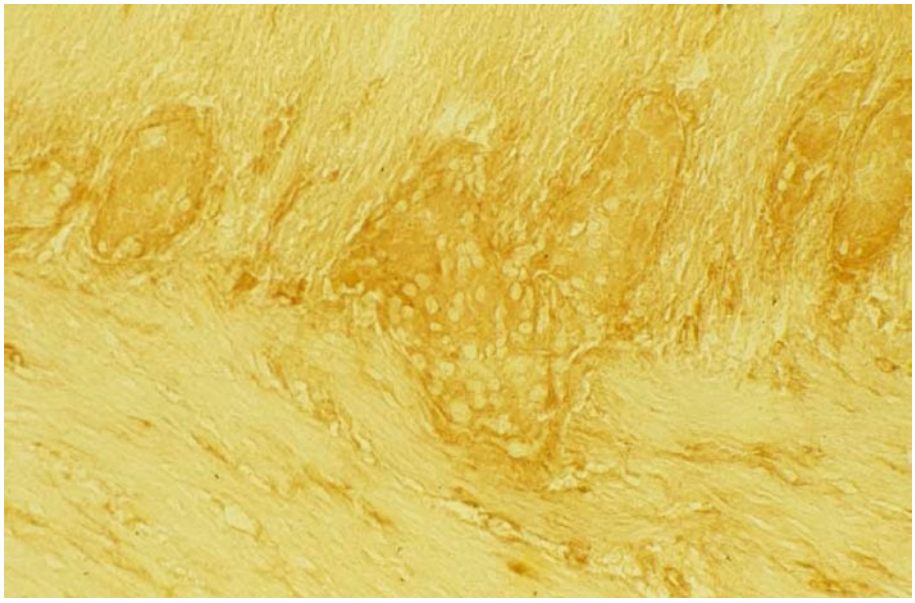

Fig. 17.4 Enlarged ganglia of the submucous plexus (AChE staining)

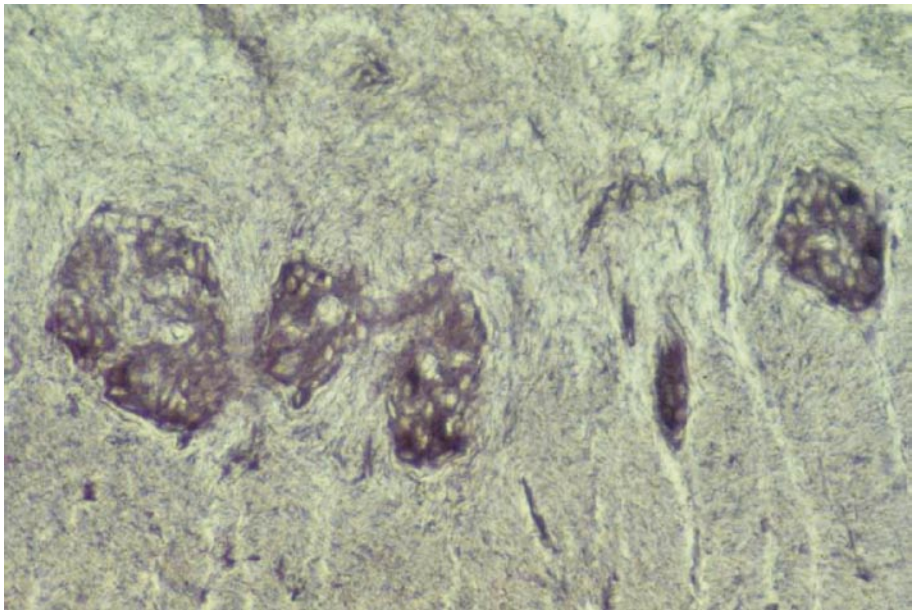

Fig. 17.5 Enlarged ganglia of the submucous plexus (LDH staining)

erotopia and giant ganglia in IND depends upon whether the biopsy was full-thickness or a suction rectal biopsy. In full-thickness biopsies ganglion cell heterotopia is more frequently detected. Besides, even in normal colon biopsies a moderate increase in AChE activity in the lamina propria and muscularis mucosae is found in four, giant ganglia in two, and hyperganglionosis and ectopic ganglion cells in 2 out of 21 normal individuals.

17.9 Newer Staining Techniques

Because no clear consensus could be obtained with the classical staining techniques such as H&E, LDH, SDH and AChE, newer markers for neuronal structures have been investigated. Kobayashi et al. [59] used monoclonal antibodies to growth associated protein 43 (GAP-43), synaptophysin and neuronal cell adhesion molecule

(NCAM) as neuromuscular junction markers. The submucous and myenteric plexus of all patients with IND displayed strong immunoreactivity for these markers demonstrating that patients with IND have defective innervation of the neuromuscular junction of the affected bowel. However, not all the three markers were absent in an individual patient. Krammer et al. [60] performed whole-mount preparations in patients with IND and used a polyclonal antibody to PGP 9.5, which is a novel cytoplasmatic marker specific for the nervous system. They found typical giant ganglia in the tunica submucosa with hyperganglionosis. Nerve cell buds were observed along large axonal bundles, enlarged bundles of nerve fibers running through the tunica submucosa and nerve cells within the nerve trunks. Heterotopic nerve cells were also observed in the lamina propria mucosae which is normally free of nerve cells.

Klück et al. [61] recommended monoclonal antineurofilament antibodies and revealed six different distinctive and divergent pictures, but with abundant and stained hyperplastic axon bundles. Dudorkinova et al. [62] recommended the NADH tetrazolium reductase reaction for the demonstration of ganglion cells in IND as a quick staining test. Kobayashi et al. found that the number of NCAM and nerve fibers positive for nerve growth factor receptor (NGFR) in the lamina propria and muscularis mucosae were markedly decreased in IND. There was also decreased expression of NAPDH diaphorase, NCAM, and NGFR in the circular and longitudinal muscle layers in full-thickness rectal biopsies from IND patients. These findings were considered particularly helpful in the diagnosis of IND in neonatal patients in whom hyperganglionosis may by a normal finding. Further stains were NAPDH-diaphorase [41], substance P [63] and others. In general, S-100 protein and glial fibrillary acidic protein (GFAP) can be recommended as markers for glial proteins, whereas PGP9.5, neuron-specific enolase (NSE), neurofilament protein 200 (NFP) and microtubule-associated proteins (MAPs) are useful as neuronal markers [54].

17.10 Age

One of the most important factors, not only in IND, is that some of the histomorphological findings are age-dependent. Smith [55] reported neuronal findings in the intestinal nerve system of 21 patients with non-gut-related diseases ranging in age from 4 weeks to 10 years and in 8 adults ranging in age from 16 to 83 years. The neuron density was lowest in the jejunum and highest in the colon. No significant variation could be shown in neuron density with age. However, different assessments of neuron densities in the myenteric plexus using various methods have resulted in a wide range of normal values.

In 1994 Meier-Ruge et al. [64] compared normal controls and patients with IND below and above age 1.5 years. They found that the size of the ganglia and the number of nerve cells were not dependent on age in IND patients as well as in normal controls. However, the ganglion cell size increased and the neural density of the submucous plexus decreased significantly with age in both groups. Wester et al. [65] reported similar results. Meier-Ruge et al. and others therefore concluded that in neonates and premature infants the diagnosis of IND cannot be established. A maturation to normal findings as well as a development to significant IND is possible within 2–4 years [57, 66]. In contrast, Munakata et al. [67] demonstrated that the characteristic findings of IND present in repeated rectal biopsies taken after various intervals show little change with time.

The uncertainty regarding age also concerns AChE staining. According to Hinkel et al. [68] and Goto and Ikeda [69], the intensity of AChE activity depends on the age of the patient and on the length of the involved segment: the more advanced the patient's age at the time of operation and the more intense the AChE activity in rectal biopsies, the more proximal is the extent of increased AChE activity. Normal ganglion cells and increased AChE activity are characteristic of ultrashort-segment HD, whereas normal AChE and missing ganglion cells are characteristic of long aganglionic segments.

17.11 Correlation Between Histological Findings and Clinical Symptoms

In 1992, Schärli distinguished four groups of patients with IND type B according to their histological features [15] and correlated the spectrum of the histopathological changes in each group with their clinical symptoms. However, Smith [50] reviewed suction rectal biopsies from 85 children and found, according to the criteria used by Schärli [15], and emphasized by the consensus conference [19], that 60 patients had some features of IND, whereas the obligatory criteria were found in only 11% of the patients.

In a more recent study by Coerdt et al. [9] examined post-mortem colon segments or colon tissue specimens from 36 patients obtained from different pathology or pediatric pathology units as a control group with no clinical history of constipation or motility disorders of the intestine. They tried to create a new diagnostic procedure using certain anatomical structures as internal references and to document the distribution of ganglion size over a patient's life. They used enzymatic histological staining for AChE, LDH, SDH and NADPH-diaphorase, and a morphometric analysis to evaluate the number of ganglion cells per ganglion and the distance between ganglia. Looking at the variability of size and distribution

of ganglia in the submucous plexus during development, they found giant ganglia with more than seven ganglion cells in four age-related groups (premature birth to 35th week, mature birth to 1 year, 1–14 years, 15–70 years). They found a decrease in giant ganglia and an increase in the distance between ganglia in the different age groups. Their findings indicate that all specimens of the 36 clinical and anamnestic symptom-free patients would have been pathological according to the criteria of the Borchard consensus conference [19] (see Sect. 17.8)

With a similar objective, Tafazzoli et al. [70] investigated specimens from 15 patients between 32 and 89 years of age without diseases of the gastrointestinal tract to provide reference data on the quantitative distribution of nerve cells and ganglia within the submucous plexus of the human anorectum. While Coerdt et al. [9] demonstrated the above-described alterations in relation to different age groups, Tafazzoli et al. [70] found segment-specific alterations of the intramural nerve plexus. There was a continuous decrease in ganglionic density towards the anal canal from 93.7 ganglia/100 mm intestinal length in the upper rectum to 12.4 ganglia/100 mm in the anal canal and 179.4 nerve cells/100 mm to 15.7 nerve cells/100 mm in the anal canal. Giant ganglia with more than seven nerve cells were not found in the distal anorectum, but significantly were found in the parts of the upper rectum in all specimens of the investigated healthy patients.

From the clinical point of view, there are many doubts about the existence of IND as a distinct clinical entity. Berry wrote in 1993 "the term IND is at best a descriptive histopathological appearance rather than a unique clinicopathologic entity" [71]. Cord-Udy et al. [72] compared the histological criteria of the consensus Conference with clinical dysmotility symptoms in individual patients. They came to the conclusion that the consensus criteria were unhelpful in predicting clinical outcome and should therefore not influence clinical management. Besides, hyperplasia of the submucosal plexus was significantly more common in neonates.

Koletzko et al. [45] found a high interobserver variation among three pediatric pathologists evaluating independently the coded 23 features of IND in 377 biopsies of 108 children. There was full agreement between the three pathologists with respect to the final diagnosis in all children with HD, but only in 14% of the remaining patients. There was no correlation between the histological findings and clinical symptoms. This corresponds well with our clinical experience [44]. In a retro- and prospective study of 203 patients with neuronal intestinal malformations, we found no correlation between clinical symptoms, radiographic findings and electromanometric findings and the IND criteria of the consensus conference, but a close correlation with the histological findings in HD and hypoganglionosis. Even the transit time is not an absolutely relevant clinical parameter for IND. In a recent study [73], we found a prolonged transit time in all children with HD, in 90% of the patients with hypoganglionosis and in those with reduced parasympathetic tone, but in only 50% of those with IND. Therefore, no resection was performed in these patients. Only in 2 out of 17 patients with a highly retarded transit time, high anorectal resting pressure profile and missing internal anal sphincter relaxation was a sphincteromyectomy performed.

17.12 Maturation and Apoptosis

Another observation found in patients with IND over the years is a change in the first histological picture due to maturation or apoptosis of ganglion cells in the submucosa. In most patients with immaturity or IND, conservative treatment is successful, but in a few it is not. We suggest that the possible pathways of development shown in Fig. 17.6 are involved. The problem with this suggestion is that not all of the IND patients underwent repeated suction biopsies, because of the more or less symptom-free course under conservative treatment. It is ethically problematic to take another set of biopsies in subjectively healthy children (subjectively meaning in the eyes of the parents and their children).

While patients with IND treated conservatively normally do not need a biopsy follow-up, patients with HD do. They usually undergo suction biopsies or full-thickness biopsies several times: at enterostomy, again (suction biopsy) to confirm suspected HD, again during pull-through surgery, and sometimes again due to persistent constipation.

By reviewing the case histories of 77 of our HD patients we observed that a previously normal upper border of anastomosis changed to IND in one patient, and to hypoganglionosis in another. Four patients showed IND at the lower border of the resected segment but without clinical symptoms. Dysganglionosis is defined as a pool of different steps of neuronal development which cannot clearly be classified histologically. In 26 of our HD patients the upper border of the first suction biopsies showed an undefined picture of dysganglionosis at first and changed to immaturity in 23 and to IND in 3 patients. In further suction biopsies the described histological picture of immaturity changed to maturity in nine, to IND in six and to hypoganglionosis in eight patients. In five patients IND was seen before hypoganglionosis occurred later (Figs. 17.7–17.12).

17.13 Association Between IND and HD

Neonatal obstruction or fecal retention since birth and severe chronic constipation that responds only transiently or not at all to conservative measures were the leading symptoms in Schärli's series of 75 children with aganglionosis and/or other intestinal neuronal malformations. In

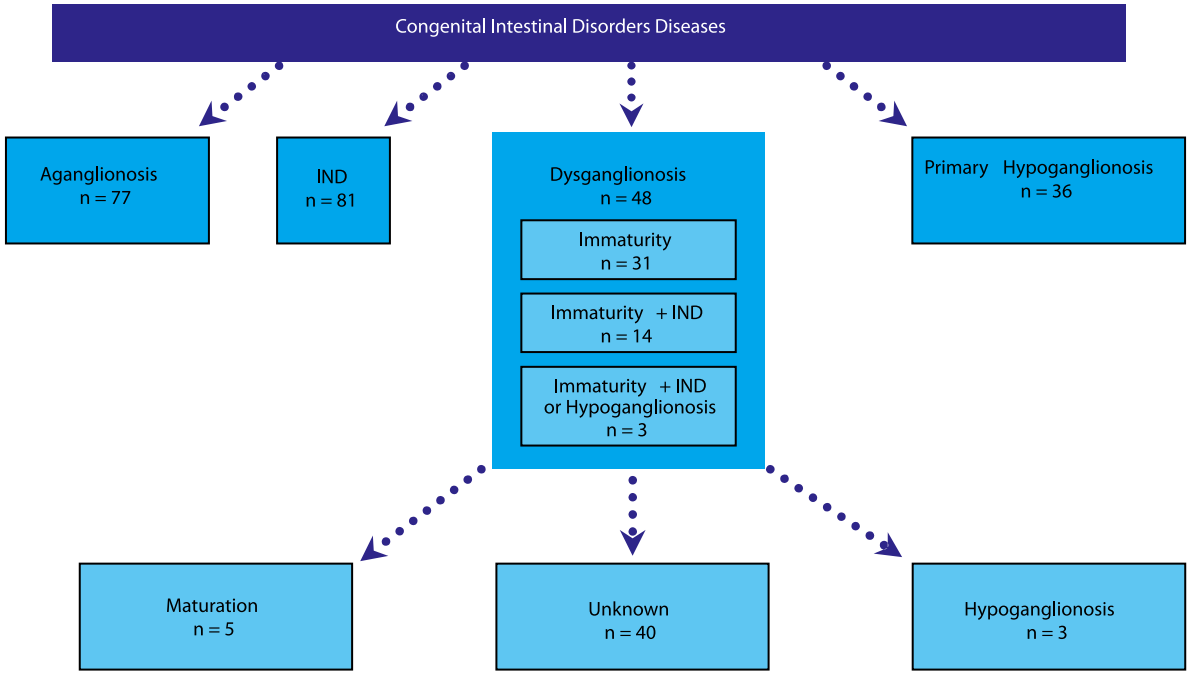

Fig. 17.6 Possible pathways of maturation and apoptosis in 48 children with Dysganglionosis (1993–2004, Children's Hospital, City of Cologne)

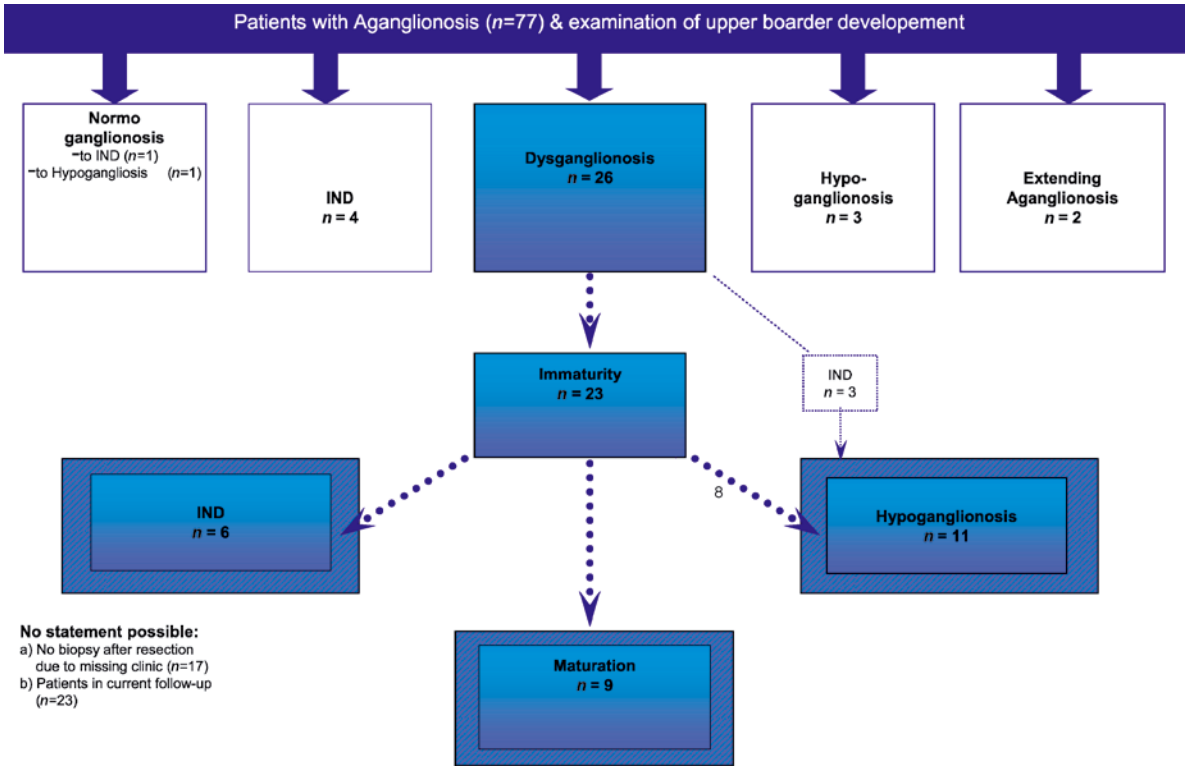

Fig. 17.7 Summary of the examination of the upper border area in patients with HD detailing the changes in 26 patients with dysganglionosis on the first histopathological examination after resection

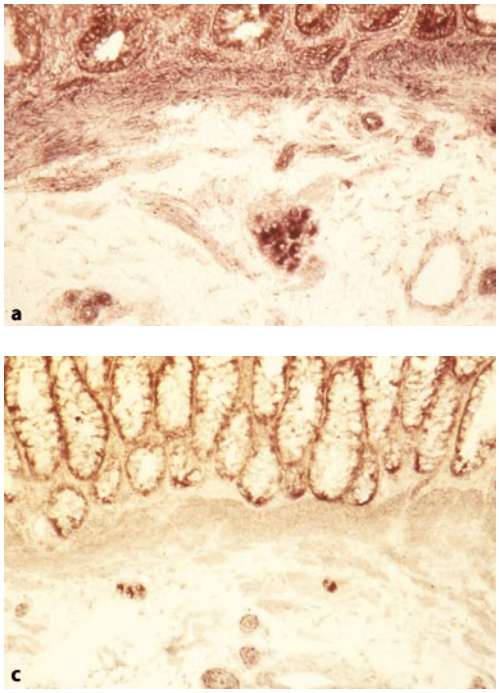

Fig. 17.8a–c Maturation of ganglion cells. Histological findings at (**a**) 2.5 years of age showing immaturity/IND, (**b**) 4.5 years showing increasing maturation, and (**c**) 6 years showing normalization

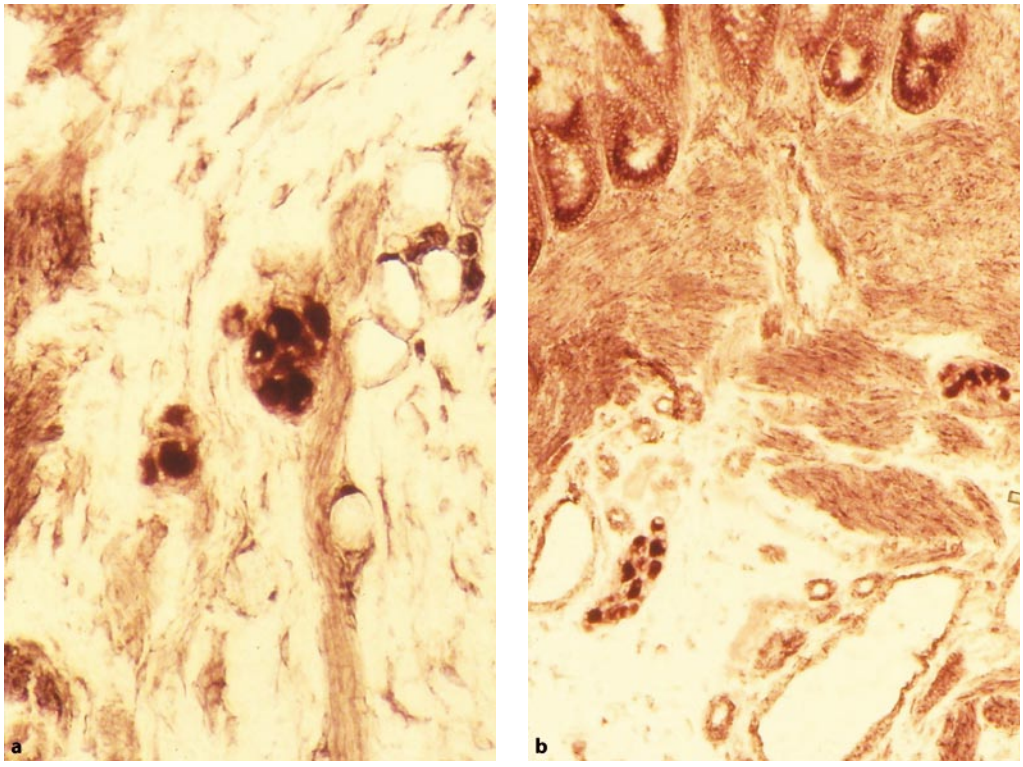

Fig. 17.9a–c Development to mild IND. Histological findings at (**a**) 2 years of age showing immaturity/IND, (**b**) 3 years showing IND, and (**c**) *see next page*

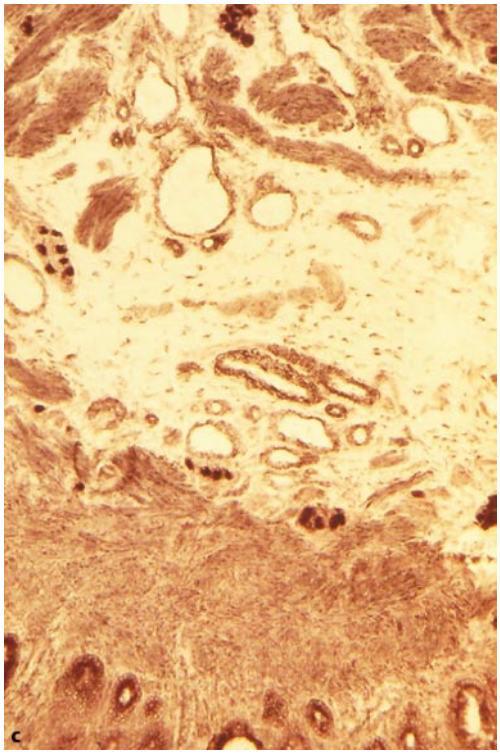

Fig. 17.9a–c (*continued*) Development to mild IND. Histological findings at (**c**) 5 years showing mild IND

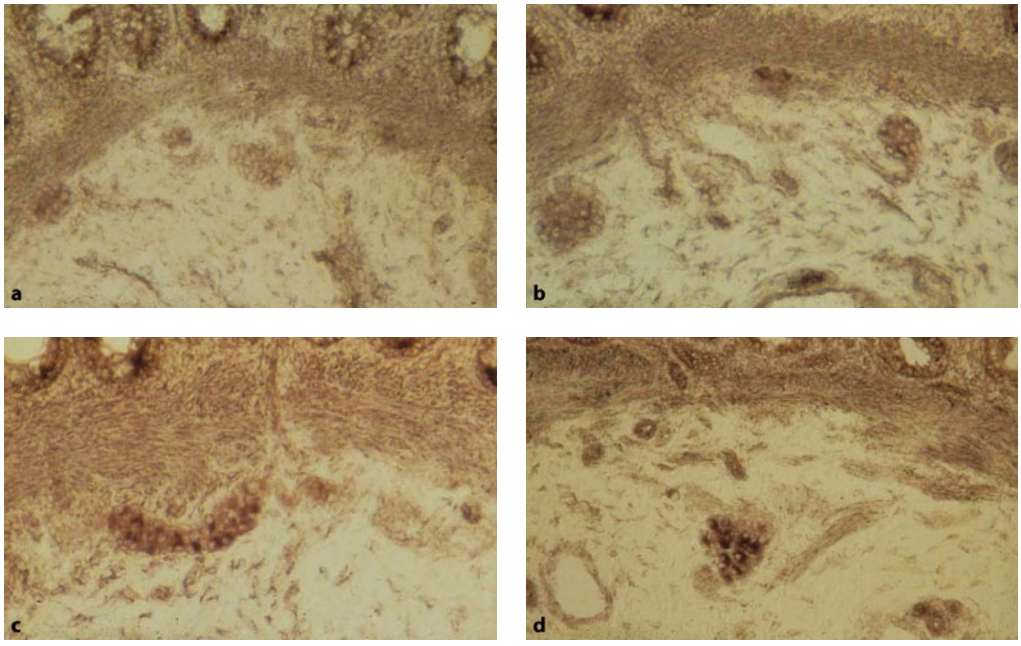

Fig. 17.10a–d Development to severe IND. Histological findings at (**a**) 2 months of age, (**b**) 12 months, (**c**) 4 years showing IND, (**d**) 12 years showing severe IND

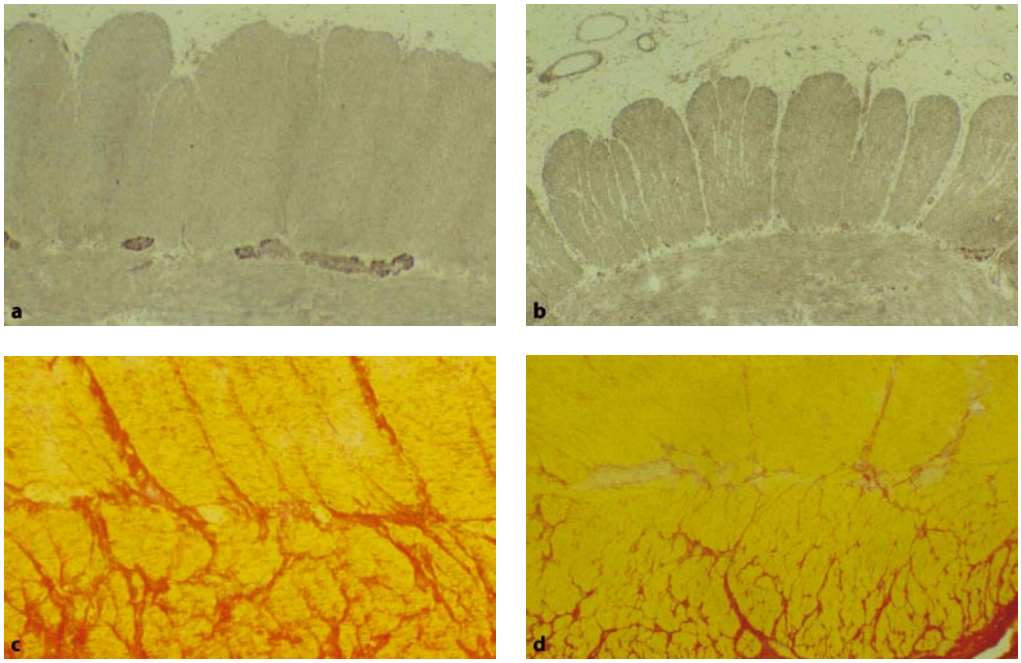

Fig. 17.11a–d Simultaneous development of severe hypoganglionosis and desmosis in a male patient. **a** at 2 years of age, almost normal myenteric plexus (LDH staining) right colon.; **b** at 12 years of age, severe hypoganglionosis, right colon. **c** at 2 years of age, normal connective tissue network between the muscle layers (Sinus red staining); **d** at 12 years of age, atrophy of connective tissue

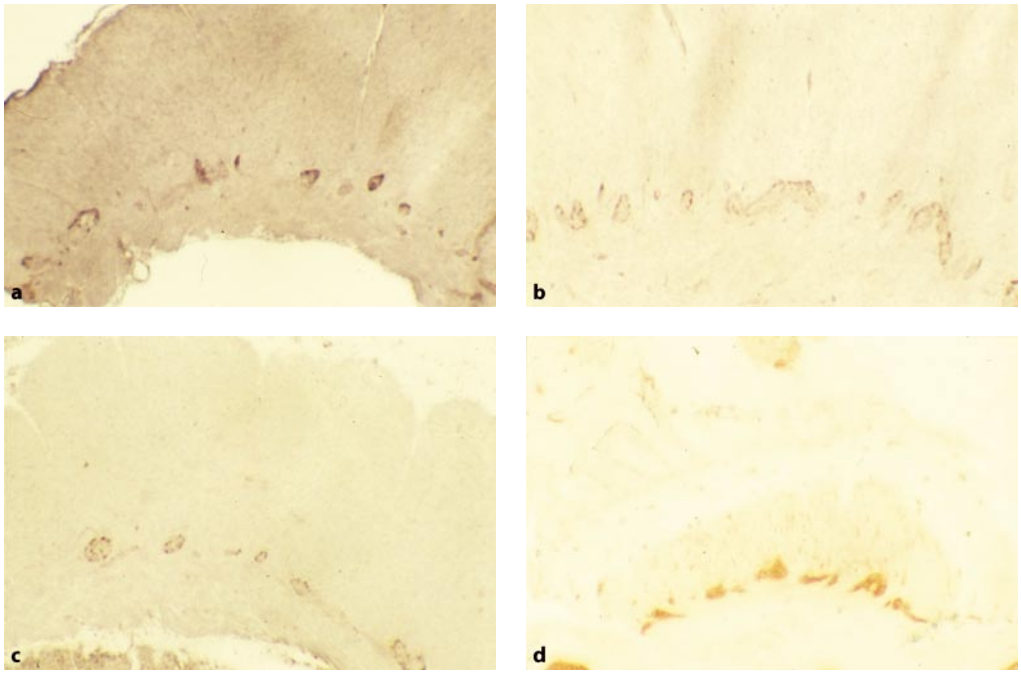

Fig. 17.12a–f Simultaneous development of severe hypoganglionosis and desmosis in the same patient. **a** at 1 month of age (LDH staining); **b** at 3 years; **c** at 5 years. **d–f** Desmosis: **d** at 1 month of age (ACh staining); **e, f** *see next page*

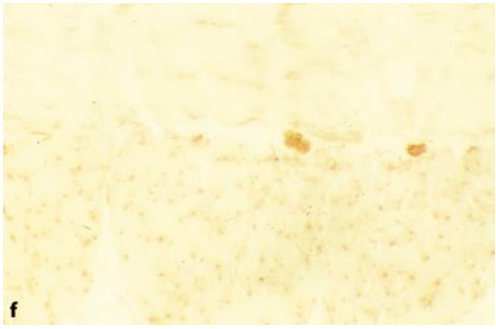

Fig. 17.12a–f *(continued)* Simultaneous development of severe hypoganglionosis and desmosis in a female patient. **e** Demosis at 3 years of age (ACh staining); **f** at 5 years

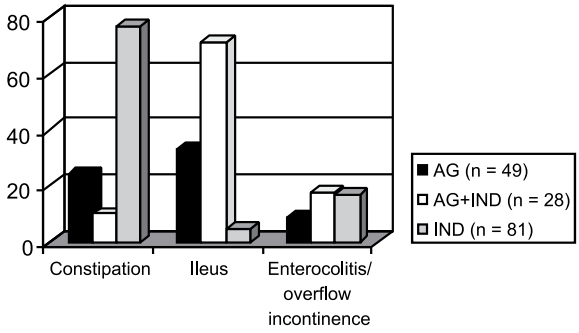

Fig. 17.13 Leading symptoms in 158 patients with isolated aganglionosis (n = 49, *black bars*), isolated IND (n = 81, *gray bars*) and aganglionosis combined with IND (n = 28, *stippled bars*) (Children's Hospital of Cologne 1993–2004)

accordance with these findings, constipation was the main symptom in 89 patients (56%) in our own analysis. From among 242 children, mentioned in Fig. 17.2 49 patients showed aganglionosis, 81 showed IND and 28 showed aganglionosis combined with IND (see Fig. 17.2).

Of these 158 HD/IND patients, 38 (24%) suffered from ileus including 21 out of the 28 patients with aganglionosis associated with IND (75%) and 16 of the 49 patients with isolated aganglionosis (32%) (Fig. 17.13).

This acute onset of illness in patients with IND-associated HD indicates an additive effect of both lesions and corresponds with the reports of Briner et al. [74] and Fadda et al. [42].

In contrast, Hanimann et al. [75] found no significant difference in the pattern of symptoms in 11 patients with HD-associated IND compared to 36 with isolated aganglionosis. Distension was the most frequent complaint in both groups. These findings are in accordance with investigations on the histology of the proximal border of resected bowel after resection of the aganglionic segment in HD [76]. IND proximal to an aganglionic segment had no adverse effect on the functional outcome. The same was shown by Banani et al. [77] and others [73, 78].

HD-associated enterocolitis was identified by Elhalaby et al. [79] in 12% of 168 patients, which is significantly lower than the incidence of up to 29% reported by other authors [69, 80–82]. Nevertheless, in the present analysis 15% of children with aganglionosis suffered from enterocolitis, but the symptom was not significantly more frequent when aganglionosis was associated with IND or with other intestinal malformations.

All patients with aganglionosis in our series underwent anterior resection and subsequent closure of an eventual colostomy. Seven of these children (9%) needed a second resection because of untreatable constipation (in four of these patients the primary resection had not been performed in our institute). Six of the seven re-resected children had aganglionosis combined with IND type B; only one had isolated aganglionosis.

Moore et al. [43] investigated 16 children with constipation or obstructive symptoms after HD of whom 4 had an aganglionic segment left in situ and 9 IND. Kobayashi et al. [78] investigated 31 consecutive patients resected for aganglionosis. Ten who had associated IND all suffered from enterocolitis, soiling or constipation, compared to only 4 out of 21 with isolated aganglionosis. The authors had performed Swenson's or Duhamel's procedure.

The patients In our series underwent anterior resection according to Rehbein, but since 2001 we have switched back to Soave's procedure which we gave up many years ago due to some severe septic complications. The numbers in the literature of children with aganglionosis and IND are too small to compare techniques of resection, but it may be concluded that children with aganglionosis associated with IND type B primarily should undergo a more extended resection in order to avoid persisting symptoms or re-resection.

At follow-up at a mean of 2.4 years (SD 1.4 years) after initial therapy, 49 of 71 patients (69%) with aganglionosis including re-resected cases had normal bowel movements and were free of symptoms. There was no significant difference in the percentage of patients who were asymptomatic after classical aganglionosis compared to those with aganglionosis combined with IND type B or specific malformations. This corresponds with the report of Hanimann et al. who found no significant difference in late complications and in the results at 5 years' follow-up after Duhamel's procedure in 36 patients with isolated aganglionosis and in 11 with associated IND type B. However none of these patients had the neuronal dysplastic segment been resected.

Reports on the association of aganglionosis with other intestinal malformations are rare. Yamataka et al. found an abnormal distribution of nerve terminals in specimen from two patients with disturbed postoperative defecation. Spares neuromuscular junctions in the longitudinal or circular muscle layer or spares synapses were seen. However another patient with these findings had normal bowel movements. In the present series, children with aganglionosis associated with hypoganglionosis (n = 47) or heterotopia of the myenteric plexus (n = 15), which are known to cause severe disturbances by themselves, did not suffer significantly more frequently from postoperative symptoms compared to patients with classical aganglionosis.

17.13.1 Interstitial Cells of Cajal

Another possible part in the understanding of disorders allied to HD might be that played by the interstitial cells of Cajal [83]. These are considered to be the pacemaker cells of the gut generating physiological slow waves in the gastrointestinal tract. They form a complete network in the inner border of the circular muscle layer and intermuscular zone and are interposed between enteric nerve endings and smooth muscle cells [84]. The proto-oncogene c-kit encodes a transmembrane tyrosine kinase receptor that is essential to control growth, differentiation and other effects.

Sandgren et al. described a change in neuropeptides and neuronal nitric oxide and in the number of enteric neurons and interstitial cells of Cajal in regions above aganglionic parts of the gut. They suggest that this might be a reason for dysmotility after surgical correction of aganglionosis [85]. Rolle et al. found reduced Cajal cells in resected bowel specimens of eight patients in comparison to a control group. In the normal bowel Cajal cells formed the described dense network, but in HD the expression of myenteric Cajal cells was reduced [86]. Similar findings were shown by Taguchi et al. who examined 15 full-thickness bowel specimens from 15 Japanese children with HD and found a normal distribution in the normoganglionic segment of 13 of 15 patients within the circular and longitudinal muscle layers and in the intermuscular zone around Auerbach's plexus and occasionally in the submucosa around Schabadasch's plexus. In the remaining two patients a decrease in the number of c-kit-immunoreactive cells was found. These patients were those with unsatisfactory postoperative results: one with severe and persistent constipation and manifest colonic dilatation who needed re-resection, and one dying due to severe sepsis from enterocolitis 3 months after surgery [87].

It seems that the decrease in interstitial cells of Cajal may also play a role in the pathogenesis of intestinal dysmotility disorders.

17.14 Management

Since a definite correlation between histochemical findings and clinical symptoms has not been demonstrated, the need for surgery cannot be determined by the histochemical picture alone. However, in 1992, Schärli [15] performed operations in 59% of his patients with IND type B, in 57% of his patients with immaturity of ganglion cells and in 92% of those with dysgenetic heterotopia. Although IND was thought to be responsible for markedly reduced propulsive activity leading to chronic constipation, sphincteromyotomy rather than resection was the treatment of choice in two-thirds of his patients with dysganglionosis. Fadda et al. [42] recommended further observation up to age 3 years because colon motility tends to normalize during this period. If no maturation takes place an extensive resection is recommended. Among our own earlier series of 67 patients with dysganglionosis [52], 70.1% were treated conservatively. Only 12 children (17.9%) underwent resection, and of these 12 children, 7 had hypoganglionosis, 1 IND, 2 reduced parasympathetic tone, and 2 heterotopia of the myenteric plexus. Three further patients with IND underwent a colostomy which was subsequently closed without further resection. Therefore, we recommend strict conservative treatment for patients with dysganglionosis at least for 2 years and resection only in elderly patients when conservative treatment has failed, the anorectal resting pressure profile is high, no internal anal sphincter relaxation can be obtained and transit time is highly delayed. For the different types of neuronal intestinal disorders, however, the following therapeutic recommendations can be made.

17.14.1 Isolated IND Type A

IND type A is extremely rare. The dramatic clinical course is characterized by bloody stools combined with the symptomatology of ileus and bowel perforation [3, 15, 42]. Therefore, there is unanimous agreement that children with IND type A should undergo rectosigmoidectomy or even more extended resection of the colon [15, 37, 42, 44]. At the time of writing there had been no patient with IND type A in our recent series. Probably some patients considered to have necrotizing enterocolitis in reality have IND type A.

17.14.2 Isolated IND Type B

Multiple types of treatment have been suggested for patients with IND type B including management with laxatives and enemas, total parenteral nutrition, various types of partial or total resection with or without leaving IND-affected bowel in place, techniques of sphincteromyotomy, and creation of colostomies [18]. As varying degrees of histochemical involvement and severity of symptoms have been reported, the results with these treatments have been very discordant.

As usual, constipation was the leading symptom in the 81 patients of our recent series (1993-2004). 73 patients (90%) suffered from constipation: 42 (52%) with constipation as single leading symptom, 27 (33%) with constipation and secondary symptoms (encopresis, anal fissura or rectal bleeding) and 4 (5%) with a more severe development which led to subileus. In 5 patients (6%) the parents described encopresis as the leading symptom and in 3 patients (4%) enterocolitis occurred. These three were premature and underwent surgery with temporary enterostomy. (Fig. 17.14)

Duration of stool-retention ranged from 2 days to more than 14 days, average 4.8 days for spontaneous defecation without laxans or mechanical help. In 24 patients (29.5%) laxans or purgative messures were initated by the parents before our treatment started.

All of our patients first underwent conservative treatment with stool softening diet, laxantives like Movicol, Lactulose or Bifiteral, enema at stool retention for longer than 2-3 days and toilet training. In 65 patients (80%) conservative treatment was successful. 13 out of those 81 patients (16%) underwent repeated anal dilatation in general anaesthesia as supplement to conservative treatment. Surgery to cure IND problems was only performed in three (4%) patients: 2 boys underwent Rehbein's procedure due to persistent and progredient symptoms. Histopathology showed IND together with heterotopia of the plexus submucosus and hypoganglionois in one of them, the other suffered from Wardenburg syndrome. One child underwent sphinctermyotomy in our clinic after surgery due to sigma elongatum in another hospital. She suffered from persistent, massive constipation (Fig. 17.15).

Munakata et al. [17] found severe constipation requiring resection in five of nine patients with IND; three patients died. Csury and Pena [18] were discouraged to see in their review of 25 publications and 322 patients, that information on operative treatment and outcome was available for only 7 patients with partial resection of IND, of whom 2 were asymptomatic at follow-up. Schärli [15] reported a high rate of failure after rectosigmoidec-

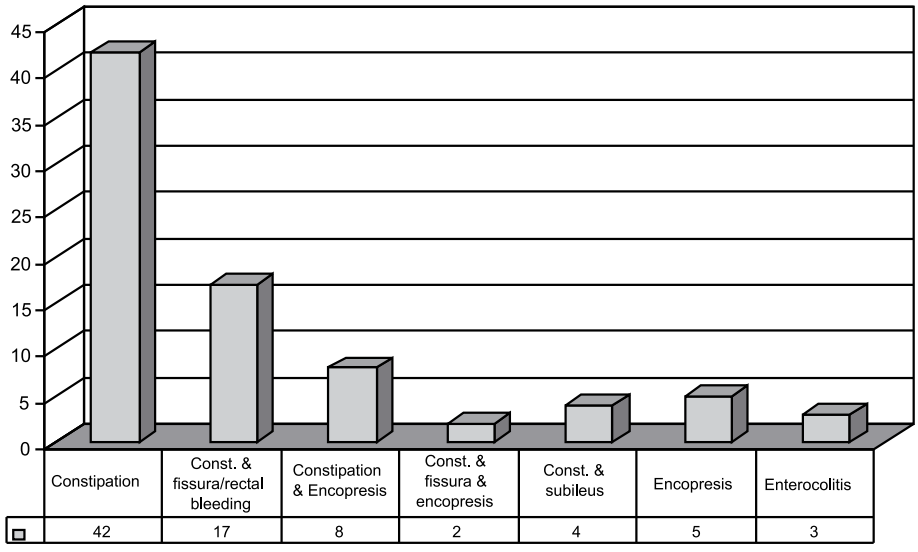

Fig. 17.14 Leading symptoms in patients with isolated IND B (n = 81, Children's Hospital of Cologne 1993–2004)

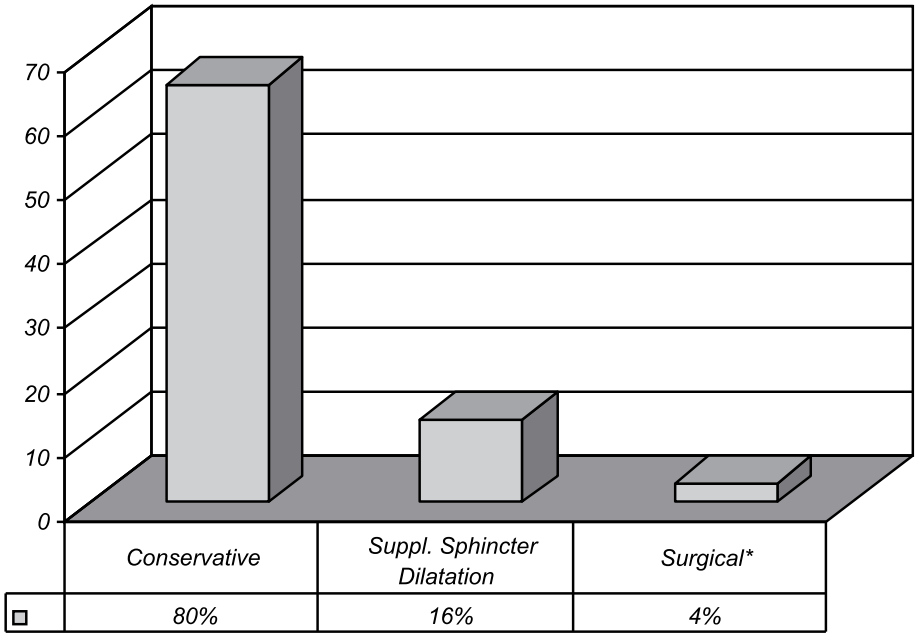

Fig. 17.15 Quantitative overview of the treatment of 81 patients with isolated IND B

tomy in adolescents in whom bowel dilatation and loss of function are advanced and recommended ascendorectostomy in these patients. However, he performed posterior sphincteromyotomy in 59% of 22 children with isolated IND and achieved a cure of 90% within 3 months; astonishingly, no child required resection in his series.

Temporary colostomy has been suggested in patients with severe symptoms, but the clinical course after closure of enterostomies has not been investigated in a large series. Rintala et al. [88] reported on two children with bowel obstruction who remained symptom-free, and Briner et al. [74] on another. Pistor et al. [89] achieved amelioration of symptoms by conservative treatment in only three out of seven children; in two a temporary colostomy was necessary. The concept of temporary colostomies which are closed after normalization of colonic transit time is also supported by the findings of Simpser et al. [66] who documented one child with normalization of biopsy findings after 5 years' follow-up. A patient of Kaiser et al. [90] with IND of the colon, small bowel and stomach had improvement of symptoms but no change in histological patterns 3 years after temporary enterostomy. In our earlier and present series, five children had normal bowel movements after closure of a temporary colostomy, but long-term results more than 5 years are lacking. This phenomenon seems to be the result of apoptosis of ganglion cells inside a ganglion. The clinical course of IND type B often shows a spontaneous recovery in the motility of the colon with an increase over a period of several years [13, 15, 42, 37] (see Sect. 17.12 Maturation and Apoptosis).

Therefore, there is agreement that medical treatment with laxatives and enemas remains the method of first choice in constipated children with IND type B without obstruction [15, 18, 88, 91]. In our recent series 80% of patients with isolated IND type B were successfully managed without surgery and had improvement of symptoms during medical treatment. These findings correspond with those of a study by Schimpl et al., who investigated 105 patients treated for constipation with histopathological confirmed IND B between 1993 and 1996. All patients underwent conservative treatment involving diet, and 70% were treated with cisapride, 52% with laxatives and 12% with repeated anal dilatations. The mean duration of treatment was 6 months and in the clinical follow-up 5–9 years later, 80% of the patients reported daily defecation and 14% defecation every second day. The remaining 6% of these patients suffered from recurrent constipation but responded well to diet and laxatives [92].

However, reports on the long-term results of conservative treatment of children with IND type B are still rare, and the number of patients investigated is limited. Out of 19 children with isolated IND type B reported by Heimig and Glück [91], 58% were successfully managed over several years by medical treatment, but larger patient numbers and longer follow-up periods are mandatory to elucidate whether clinical regression correlates with histological improvement and "maturation" of ganglia.

17.14.3 Hypoganglionosis

No large series of patients with hypoganglionosis of the myenteric plexus as an isolated entity have been reported. Seven out of nine of our patients with hypogan-

glionosis required resection, and two were re-resected [52]. Munakata et al. [93] reported on 12 children with hypoganglionosis, out of whom 11 were operated on for presumed HD. The authors gave no information on the clinical course. At follow-up, four of the nine children with hypoganglionosis in our series were suffering from significant constipation, and one from overflow encopresis; all were managed conservatively. These unfavorable results are due to the fact that we preferred to leave a short hypoganglionic colon segment in place to avoid ileorectostomy. Nevertheless, symptoms in hypoganglionosis do not improve with time and mostly resection of the affected bowel segment is required.

Meier-Ruge et al. [3, 13, 14] emphasized that only full-thickness biopsies are reliable for establishing the diagnosis of hypoganglionosis, as analysis of the intramural plexus is essential. It may be assumed that the diagnosis is missed in patients with a less severe clinical course and conservative treatment, in whom exclusively suction biopsies are investigated.

17.14.4 Heterotopia of Ganglia in the Myenteric Plexus

Heterotopia of the myenteric plexus is extremely rare and is known to cause severe symptoms [3, 13, 14, 44]. The heterotopic neurons of the myenteric plexus in the circular and longitudinal muscles contain practically no plexus in the space between the two layers of muscle and therefore surgical therapy is very common. In our experience both children with heterotopia of the myenteric plexus underwent resection of parts of the colon and both were suffering from persisting constipation at follow-up after 10 months and 2 years, respectively.

Meier-Ruge [13] stated that heterotopia of the submucous plexus is very common and seems to be a normal variant. In our series, only 1 out of 12 children with this finding underwent sphincteromyotomy; in 11 conservative treatment was successful. However, in the series of Schärli [15], out of 13 patients with heterotopia of the submucous plexus and associated IND, 12 were operated on compared to 59% of the children with isolated IND who underwent sphincteromyotomy. Therefore, heterotopia of the submucous plexus in the muscularis mucosa may rarely aggravate symptoms in children with IND type B, but more frequently seems to be a normal finding.

17.14.5 Reduced Parasymphaticotonus

In contrast, a reduced parasympathicotonus seems to be a more severe finding. Out of 12 children in whom a reduced parasympathicotonus was the main histochemical finding, 9 underwent medical treatment with laxatives and enemas. However, one required sphincteromyotomy and two underwent resection for untreatable constipation. It may be concluded that a reduced parasympathicotonus may lead to severe symptoms in some patients.

17.14.6 Ganglion Cell Hypogenesis

Ganglion cell hypogenesis was found in 7 out of 73 patients by Schärli [15], and 4 of these needed surgery. In our series hypogenesis was identified in numerous specimens but was never the main histochemical diagnosis. Therefore the clinical significance of this diagnosis remains controversial.

No patient in the present series with immaturity or mild dysganglionosis underwent resection. All children with immaturity or dysganglionosis were treated conservatively and were free of symptoms at follow-up.

17.15 Conclusion: Is IND a Real Disease?

IND is a distinct histopathological entity but shows great clinical variability. We do not yet know which of the observed histopathological criteria are primary – probably inherited – and which secondary phenomena.

Many authors have reported findings similar to those in IND as a secondary phenomenon after fetal bowel obstruction such as meconium peritonitis, mucoviscidosis, volvulus, rectal stenosis, small-bowel atresia, small-bowel intussusception [18, 45], ileal and colonic atresia, imperforate anus [4, 5, 37], small left-colon syndrome, gastroschisis, pyloric stenosis, diaphragmatic hernia, necrotizing enterocolitis and others [94]. Besides, IND changes have been reported in a few patients with congenital hypothyroidism, bilateral inguinal hernias, GERD, perinatal anoxic insult, formula protein-sensitive enteropathy [93], and multiple endocrine neoplasia type II B syndrome [95]. IND has also been observed in addition to primary obstructing aganglionosis. Therefore it seems that fetal obstruction could introduce histomorphological changes similar to those in IND. In 1981, Pickard et al. [96] demonstrated histochemical findings similar to those in IND in experimental intestinal atresia in fetal lambs. Moore et al. [97] did not succeed in reproducing these changes in a rat model. However, these experiments were performed after weaning and not in fetal life.

However, the recent application of new histochemical and immunohistochemical techniques indicates that IND has characteristic histological features such as hyperganglionosis of the submucous and myenteric plexus, giant ganglia, increased AChE-positive nerve fibers around submucosal blood vessels and in the lamina propria, a deficient innervation of the neuromuscular junction and abnormal internal sphincter enervation. Moreover, animal studies have shown that a genetic defect could also be responsible for IND type alterations of the submucous plexus in mice and rats.

The lack of correlation between clinical symptoms and histology is not surprising. Even in HD the clinical symptoms vary, but to a lesser degree. The same length of aganglionic segment of the rectosigmoid can lead to different clinical manifestations: to an ileus in the neonatal period and to chronic constipation from birth with late admission to hospital in adulthood. It is not surprising that in IND the symptoms vary over a much wider range. The motility of the gut is not only based on different neuronal elements in the intrinsic enteric nerve system but also on the effect of the interstitial Cajal cells, the input of the extramural nerve system and the remaining function of the dysganglionic neuronal structures. A comparative analysis of the concepts of treatment and outcome in different series of children with intestinal neuronal disorders remains therefore questionable. Therefore, from the histopathological findings alone no conclusion concerning therapy can be drawn.

To conclude, the pathological changes described for IND may be genetically determined, may be secondarily induced, may represent part of normal development, or may reflect a distinct histopathological variation of their normal histological pattern without clinical correlation [7]. From the therapeutic point of view, however, the following recommendations can be made:

- In patients with aganglionosis and an acute onset of disease the search for associated IND and other associated intestinal malformations should be emphasized.
- In patients with associated IND in the proximal segment resection should be extended a little more. However, it is not necessary to resect the whole involved segment.
- Almost all children with isolated hypoganglionosis or heterotopia of the myenteric plexus severe symptoms indicate the need for resection. A hypoganglionic segment in addition to aganglionosis should be resected completely.
- Heterotopia of the submucous plexus and mild dysganglionosis represent normal variants which indicate that the development of the gut innervation was slightly altered. In these children surgical treatment is rarely needed. Heterotopia or hyperganglionosis of the myenteric plexus, however, needs resection.
- A reduced parasympathicotonus in the mucosal biopsy represents a functional indicator which usually disappears with maturation of the vegetative nervous system. It can be a sign of hypoganglionosis and lead to severe symptoms. Especially in patients in whom another anomaly of the plexus is suspected, full-thickness biopsies are mandatory to establish the diagnoses.
- Immaturity of the submucous plexus is a developmental retardation of the submucous plexus and may be monitored over time. Surgical therapy is only indicated if immaturity develops in the direction of IND with severe symptoms.
- In all patients with IND surgical treatment should only be considered after at least 2 years of conservative treatment.

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Neurocristopathies and Particular Associations with Hirschsprung's Disease

S. W. Moore

18

18.1 Introduction

Primitive neural cells migrate from the neural crest during embryogenesis to reach their target organs. They then undergo differentiation into melanocytes, adrenal medulla cells, C cells of the thyroid, sympathetic ganglia and the enteric nervous system (ENS) of visceral ganglia, sensory tracts of cranial and spinal nerves, as well as the membranous bones of the face and palate.

Neurocristopathies (a unifying concept of conditions which arise from a common site of neural crest development [13]) arise from disturbance of cellular development and tissue of neural crest origin and result in a number of clinical phenotypes, which include a variety of tumors. These tumors occur largely from disturbances in the oncogenes and tumor suppressor genes involved in cellular development. The combination of Hirschsprung's disease (HSCR) with a neurocristopathy strongly indicates the need to investigate the sympathetic amine precursor uptake decarboxylase system for associated lesions.

18.2 Neurocristopathies Associated with HSCR

Because the etiology of HSCR is thought to be largely genetic in nature, the observed genetic variation in HSCR has become an emerging resource for studying the complex pathophysiology of this multifaceted condition as well as understanding reported clinical associations. At a molecular level, HSCR appears to arise as the result of a disruption of normal developmental molecular signaling. Major susceptibility genes known to be involved include the RET (REarranged during Transfection) protooncogene, at 10q11.2, the recessive EDNRB gene, located at 13q22 and its ligand endothelin 3 (EDN3), and the glial cell line-derived neurotrophic factor (GDNF) [1, 129].

The resulting neurocristopathies include the following:

- Neurocristopathies associated with the RET protooncogene
- Neurocristopathies associated with the endothelin system (EDNRB, EDN3) and SOX10
- Congenital central hypoventilation syndrome (CCHS)
- Other rarer neurocristopathies

18.2.1 Neurocristopathies Associated with the RET Protooncogene

The relationship to the major susceptibility gene RET protooncogene, at 10q11.2 and multiple endocrine neoplasia (MEN) syndromes appears to be highly significant. The association is now clearly understood to be genetic in nature and early identification may have implications for preventative and early intervention strategies.

18.2.1.1 The Role of the RET Protooncogene in HSCR and MEN Syndromes

The RET protooncogene appears to be the most significant susceptibility gene in HSCR where it appears to result from loss of function. Although major mutations appear to account for up to 50% of familial and 30% of nonfamilial cases [145], a number of lesser genetic variations have been identified in RET which also appear to play a significant role. These include specific alleles at RET-1VS, certain single nucleotide polymorphisms (SNPs; e.g. A45A) as well as specific haplotypes (haplotype 0) [1, 17, 18, 98, 131]. It is not yet clear whether these variations can give rise to HSCR by haploinsufficiency per se or whether lesser mutations require the multiplicative effects of other disturbed signaling pathways.

The pathophysiology involved in the MEN and related syndromes appears to be reliant upon completely different sites on the RET protooncogene and results in a RET-activating rather than a RET loss of function action. RET protooncogene mutations have now been associated with MEN 2A and 2B syndromes, familial medullary thyroid carcinoma (MTC), and (partly) papillary thyroid carcinoma. The position of mutations seems to be important in terms of the phenotypic expression. For example, those RET variations associated with the six cysteine positions in the extracellular region of the RET protooncogene [20, 66] plus exon 14 SNP S826S [126] and the 918 mutation in exon 16 [79, 132] have been strongly associated with MTC.

18.2.1.2 The Multiple Endocrine Neoplasia Syndromes

The association between HSCR and MEN-related syndromes concerns mainly the MEN2 syndromes (A and B) and HSCR. MEN1 is a clinical syndrome consisting of pituitary, parathyroid, pancreatic neuroendocrine tumors not genetically related to RET mutations, its current association being with chromosome 11q13, and it is usually not associated with HSCR.

MEN2 Syndromes

MEN2A is an autosomal dominant genetic condition characterized by the development of a number of tumors including pheochromocytoma, MTC, thyroid C cell hyperplasia and parathyroid tumors. There are distinct genotype–phenotype correlations in MEN2. The most common subtype is MEN2A (Sipple syndrome), which includes two known variants: associations with HSCR, and associations with cutaneous lichen amyloidosis.

The association of aganglionic megacolon with megaloureter, pheochromocytoma and neuromatosis [139] actually preceded the landmark report by Sipple of a 14 times higher association between pheochromocytoma and thyroid tumors [86, 142]. A diffuse ganglioneuromatosis (GN) within the wall of the stomach, and small and large intestine was then identified at autopsy in two patients with MTC and pheochromocytoma [166], thus expanding the concept of a neurocristopathy. Steiner et al. [147] introduced the term multiple endocrine neoplasia syndrome which is transmitted in an autosomal dominant manner [69]. The association of MEN2 syndromes with the RET protooncogene subsequently appeared in the literature and is discussed later in this chapter.

MEN2B, on the other hand, is an association of pheochromocytoma, MTC, C cell hyperplasia, and ocular and gastrointestinal ganglioneuromata in patients with marfanoid features. Isolated cases of GN probably represent incomplete gene penetrance.

Familial MTC represents the familial transmission of MTC without the full features of MEN 2, and is sometimes referred to as MEN3 (Froboese syndrome)

Clinical Features of the MEN Syndromes

MEN2 syndromes are defined by the presence or absence of pheochromocytomas, hyperparathyroidism, MTC and other characteristic clinical features. It has not yet been possible to differentiate between the thyroid carcinomas or pheochromocytomas of the MEN2A and MEN2B syndromes on histological grounds, and patients with MEN2A are generally completely asymptomatic in the preclinical phase. Similar to other genetically determined premalignant conditions (e.g. familial polyposis coli and the APC gene), the prevention of the resulting neoplasms depends entirely on familial pedigree, genetic analysis and timely removal of the target organ. It is important to identify patients with MEN2 early as 52–75% of MTC have lymph node metastases at the time of clinical diagnosis. The high morbidity and associated mortality in these radio- and chemoresistant tumors makes surgical preventative removal of the target organ an essential goal of treatment.

The clinical presentation of pheochromocytoma is well described. It may be asymptomatic or missed where patients present early. Because of the association with MEN2, patients with pheochromocytoma should be screened for blood chemistry and calcitonin levels and, if necessary, referred for genetic evaluation

MEN2B on the other hand presents with marfanoid features as well as the classical ganglioneuromas of the oral cavity and gastrointestinal system. The gastrointestinal involvement associated with the MEN2B syndrome means that patients not uncommonly present with intractable chronic constipation and megacolon. Intestinal obstruction resulting from a colonic mucosal neuroma has also been described [119].

Genetic Aspects of the MEN2 Syndromes

The MEN2 syndromes result from gene upregulation as a result of germline activating mutations in the RET protooncogene. In general, HSCR and MTC affect different parts of the RET gene but a certain amount of genetic overlap leads to therapeutic dilemmas in apportioning risk (see 18.2.1.4 HSCR and MEN-related RET Mutations).

Activating mutations of RET appear to be of the order of 1:500,000 in the general population [127]. Many are de novo genetic variations which involve germline mutations in exons 10, 11, 13, 14, 15, and 16 of the RET protooncogene in at least 92% of patients with MEN2 presenting with MTC [21]. MEN2B is a less common subtype, but is mostly associated with exon 16 (M918T) RET mutations [24].

Recent advances have resulted in a clearer understanding of RET function and the effect of RET mutations on RET signaling and activation (e.g. MTC) or inactivation (e.g. HSCR) by means of a number of different mechanisms. As the resulting mutant proteins appear to determine the phenotypic expression, the higher the penetrance of the MEN2 phenotype, the earlier and more aggressive the cancer. The method by which RET mutations produce cancer is less clear, as mutations are mostly de novo and the cause unknown. Radiation exposure is the only clear factor associated with thyroid carcinoma and can actually be capable of inducing RET mutations [32], but is usually absent from the patient's history.

MEN is caused by "gain of function" variations in the cysteine-rich extracellular domain of RET and is associated with variations at one of the six cysteine residues (viz. 609, 611, 618, 620, 630 and 634 positions). The MEN2A mutations probably activate RET by inducing disulfide-linked homodimerization [8, 133]. In addition, RET extracellular domain mutations may result in the unfolding of RET by affecting polarity (e.g. C620S). The RET2B mutation (significantly more than the RET2A mutation) results in an increase in Ret-MEN specific potentiated phosphorylation of tyro 1062 (Y1062; a RET multiple effector docking site that mediates the recruitment of the Shc adaptor and of phosphatidinylinositol-3 kinase, P13K, at the Y1062 docking site) [27, 130]. RET MEN2B has been shown to be more active in associating Shc and in causing constitutive activation of the Ras/mitogen-activated protein and P13K/Akt cascades [27].

In the light of the apparent genotype–phenotype correlation between RET and MEN2 [23, 168] and the identification of specific sites on chromosome 10q11.2 associated with MEN2A [106] and MEN2B [76, 104], predictive DNA testing for MEN2 is now possible. Genetic screening for RET has been shown to be an extremely sensitive marker in MEN2 syndromes [106] with the majority of mutations relating to the cysteine radicals in exons 10, 11 and 16. As a result, the diagnosis of MEN2 is currently mostly confirmed on the basis of the genetic features, although the clinical phenotype remains important. Effective management therefore depends on early diagnosis and the gene carriers can now be identified before any clinical or biochemical abnormalities are present. These children can therefore be offered a prophylactic thyroidectomy which is successful in preventing the development of MTC with its associated high rate of metastatic disease (Fig. 18.1).

It is therefore clear that genetic screening should occur prior to the onset of any clinical symptoms to allow adequate early risk assessment and prophylactic management. It has been established that RET testing is vastly superior to calcitonin in identifying preclinical cases with specificity approaching 95–100% [106]. Mutations of codons 634 and 618 have been found in the youngest patients (3 and 7 years, respectively) making this a high-risk age group [91]. On the other hand, codons 790, 620 and 611 appear to be associated with an intermediate risk, and codons 768 and 804 with a relatively low risk of developing MTC. Nevertheless, a 12-year-old patient in an intermediate risk group has been reported with MTC, stressing its relevance in the prepubertal age group [91].

In most patients with MEN2B a methionine to threonine substitution occurs at position 918 (M918T) of the

Fig. 18.1 Familial MEN2A and C634S RET. Mother had pheochromocytoma plus MTC. Two affected children were treated by total thyroidectomy

RET-kinase domain. This currently appears to be the most significant alteration in oncogenesis, and may be of prognostic significance. The tumors display aggressive behavior and distant metastatic spread [10, 58–60, 76, 104].

In addition to the known sites, there is also over-representation of the variant S836S in patients with MTC [71, 126]. Associations with RET polymorphisms L769L, V804M and S904S have also been reported [93], although not consistently [165]. The role of these other RET variations is unclear as many authors of the various studies fail to state whether all 21 exons of the RET gene were investigated in a systematic manner or whether only the specific exons known to be associated were probed.

Patients with phenotypic features resembling MEN2B require genetic testing in spite of a negative family history because of the high incidence of spontaneous mutations (approximately 50%) [29, 153].

Treatment of MEN Syndromes

The multiple neoplasias encountered in MEN are treated on their own merits. Prophylactic total thyroidectomy is performed on gene carriers in accordance with their risk stratification. Screening should at least include the cysteine-containing codons 10, 11 and 16, but should also include exons 13 and 14. It is now established that the risk groups are determined by the genotype and should be used to dictate timing of prophylactic surgery [92]. In MEN2B it is recommended that testing should be done before 1 year of age (particularly in 883/918 codon mutations) and before 5 years in MEN2A (especially in the presence of mutations of codons 611, 618, 620 and 634). The assessment of risk in patients with isolated intestinal GN with the same genetic background without other features of MEN2B then remains problematic, and is addressed in section 18.2.1.4 Intestinal Ganglioneuromatosis.

As it is difficult to entirely predict tumor risk in affected individuals, it has been recommended that children with HSCR plus RET abnormalities undergo prophylactic thyroidectomy in accordance with their risk profile [141]. A high incidence of early aggressive tumors in MEN2B warrants an aggressive surgical approach with early prophylactic thyroidectomy in gene carriers (less than 1 year of age). Colonic disease in MEN2B is generally managed conservatively where possible. A localized segment of affected colon may be resected, but more commonly, especially where the small bowel is affected, there is little therapeutic benefit to be gained from such surgery.

18.2.1.3 HSCR and MEN-Related RET Mutations

The uncommon association between HSCR and MEN2 in the same patient is extremely interesting, as opposite effects have to occur in the RET protooncogene for this to take place. "Gain of function" variations result in MEN syndromes, and "loss of function" mutations result in HSCR [151], and these would have to take place simultaneously. Mulligan et al. [105] suggested that mutations at RET codons 618 and 620 not only give rise to MEN2A and familial MTC but also may predispose to a low penetrance way to HSCR.

Although cosegregation of these two conditions is uncommon, there are reports in at least 24 families of documented RET mutations associated with HSCR and MEN2A [12, 19, 22, 31, 47, 78, 105, 114, 115, 125, 134, 141, 159]. Recorded RET mutations in patients with cosegregation of HSCR with MEN include C609Y (n=2) [9, 109], C611S (n=1) [109], C618R (n=5), C618S (n=3) [22, 31, 115], C620R (n=8), C620S (n=4) [16, 78, 109, 125, 134] and C620W (n=1). We have reported a further case of a C620W mutation occurring in a patient with long-segment HSCR but without yet developing MTC.

The 620 mutation has been named the Janus mutation and is of interest as it accounts for approximately half of the reported cases of cosegregating MTC and HSCR, although it makes up only 12% of genetic variations associated with MTC itself [65]. The importance of this mutation is demonstrated by the reported case of familial MTC occurring in a patient with a C620S mutation 12 years after surgical correction of HSCR [134], the mother having developed MTC 7 years after the child's birth. On the other hand, Fernandes et al. [63] reported a kindred with a C620S mutation MTC but without HSCR . They suggested that the observed RET mutation had little to do with the development of HSCR in these patients and hypothesized that another area of RET is responsible for the HSCR phenotype. Our patient had total colonic aganglionosis and other genetic variations apart from the C620W in exon 10, namely a further RET SNP in exon 13 plus an exon 4 (831 G/A) SNP in EDNRB (which was probably neutral).

The hypothesis that the 620 mutation has a dual function is supported by the report of Arighi et al. [7] who have provided an theoretical explanation for the dual phenotypic Janus mutation at cys 620 of RET. Working with Madin-Darby canine kidney cells (MDCK) with a transfected C620S mutation, they demonstrated that although the mutation impairs the GDNF-induced effects on cell migration, differentiation and cell survival, it also simultaneously results in increased rapid cell proliferation. This dual action may also be true of certain other RET genetic variations. Borst et al. [22] suggested that the 618 RET codon could also predispose patients with MEN to HSCR in a similar manner. More information is required before this picture becomes clear, but based on current knowledge it does appear as if the 620 codon mutation has a dual or Janus potential.

18.2.1.4 Intestinal Ganglioneuromatosis

GN is an uncommon condition affecting peripheral nerves in the intestinal wall. It is important to note the transmural nature of the hypertrophied nerves (Fig. 18.2) to distinguish it from the thickened peripheral nerves seen in association with HSCR and the thickened nerves sometimes visible on low rectal biopsy. Although it displays similarities to the circumscribed or diffuse neuromatosis encountered in certain patients with neurofibromatosis, GN usually presents as an isolated condition with pseudoobstruction (presumably related to incomplete penetrance of the genetic defect [61].

GN is known to occur in association with MEN2B where there are also GN of the lips and tongue. This association with the MEN syndromes links it to the RET protooncogene and as a result, it potentially carries the risk of MTC and pheochromocytoma. Further ganglioneuromas (GN) of the ENS are also a possibility. In addition, GN may also be associated with abnormal neuropeptide secretion (e.g. VIP) [49, 123, 140] and diarrhea especially when it involves the small bowel and pancreas [140]. Although it has been described in animals [39], as part of intestinal neuronal dysplasia [43, 62] or part of intermuscular plexus hyperplasia [123], it must be seen as a separate entity, preferably with its identity being confirmed genetically.

Although often asymptomatic, initially patients may present with constipation or diarrhea [30] or in a similar manner to those with HSCR [94]. Other reported clinical features include failure to thrive, chronic diarrhea and abdominal distension. Radiological features include abnormal haustral patterns of the colon with thick mucosal folds, defective peristaltic movements and possible colonic diverticulae [5, 52]. In addition, areas of spasm and dilatation of the colon are often present [94], and it may even mimic Crohn's disease on radiological assessment [35]. Esophageal dysmotility has also been reported [48].

Rectal biopsy may show the massive transmural hypertrophy of nerve fibers among autonomic ganglia of the ENS. Ganglion cells are usually present in normal numbers and in our own studies PGP9.5 staining was within normal limits [83]. On the other hand, neural markers neurofilament protein and S100 protein demonstrated some variation with a marked increase in S100 staining being observed in the muscularis propria (but not in the lamina propria) as well as a mild reduction in neurofilament protein staining in both layers.

Patients with MEN2B often present with symptoms related to the ganglioneuromas of the intestinal wall [30]. Gastrointestinal symptoms may precede the clinical presentation and may lead to the diagnosis. There is a clear association with diarrhea (possibly on the basis of excessive VIP secretion). The relationship to constipation and recurrent episodes of pseudoobstruction and a Hirschsprung-like clinical picture is a definite mode of presentation. Verdy et al. [159] reported a connection with MEN syndromes in 9 out of 92 patients in their series which is the highest on record.

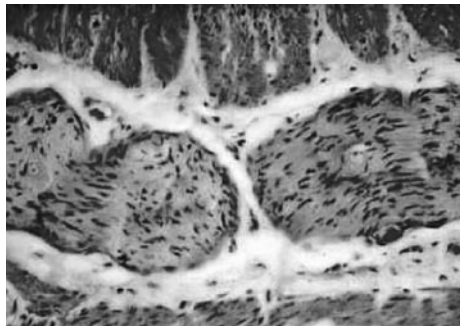

Fig. 18.2 Section of bowel wall demonstrating the massive transmural hypertrophy of nerve fibers typical of ganglioneuromatosis (H&E, ×25) (Photo P Beale, used with permission)

When associated with the MEN syndromes, the risk of thyroid carcinoma is increased but there is little available information as to the risk of developing MEN-related tumors in patients presenting with an isolated intestinal GN during childhood. The answer to this conundrum must surely lie in the genetic abnormalities associated with the condition. There is at least one reported patient, a 27-year-old man, who developed the phenotypic expression afterwards and diagnosis and prevention could have been obtained from earlier genetic investigation [11]. There is also a report of another patient with typical ganglioneuromas in whom the diagnosis was not made until tumors were present [111]. Shekitka et al. [138] concluded that the solitary polypoid ganglioneuroma of the gastrointestinal tract did not carry the same risk of neurofibromatosis or RET as the diffuse type.

The histological features of ganglioneuromatosis and its place in the neurocristopathies are of interest. Ganglioneuromas of the ENS are rare tumors, which consist of ganglion cells, nerve fibers and supporting cells. There are at least two morphological patterns of GN [43], the polypoid and diffuse types. Transmural GN affects all layers of the bowel wall which show neural hyperplasia with predominantly the myenteric plexus being involved, and is the form generally associated with MEN syndromes. The other form, mucosal GN (often as polyps [34, 100]), is more associated with von Recklinghausen's disease, adenocarcinoma of the colon and multiple adenomas with megacolon. The significance of GN is that it may be an indicator of the genetic background which may carry the risk of eventual cancer [111].

A germline RET codon 918 mutation has been reported in apparently isolated GN of the intestine [144]. Many series on intestinal dysplastic conditions contain

similar patients without the phenotypic features of a MEN syndrome, but with GN of the bowel. The risk of MTC is unclear (and hence its prevention by prophylactic thyroidectomy). The question as to whether the risk to patients with GN but without the features of a MEN syndrome can be predicted genetically has not yet been answered. Little is known about the way in which the exon 16 (M918T) germline mutation relates to GN, but it was present in all three patients reported by Smith et al. [144]. What is known is that pheochromocytoma cells transfected with RET M918T mutation are resistant to nerve growth factor inhibition [26], which may well explain the overgrowth of nerve elements. This study supports earlier findings of increased nerve growth stimulating activity acting preferentially on sympathetic nerve terminals [49].

It is well documented that the RET/GFR-alpha-1/GDNF complex is responsible for a signal which is essential for the survival of early crest derived neural precursors which in turn colonize the gut giving rise to the ENS [70, 151]. The RET 2B mutation (significantly more than the RET 2A mutation) results in an increase in Ret-MEN-specific potentiated phosphorylation of tyro 1062 (Y1062). Y1062 is a RET multiple effector docking site that mediates the recruitment of the Shc adaptor and of P13K at the Y1062 docking site. The MEN mutations convert RET into a dominant mutant protein which results in activation of its tyrosine kinase activity and tumor formation via the downstream mediator Shp-2 [42].

18.2.2 Neurocristopathies Associated with Endothelin System (EDNRB, EDN3) and SOX10

18.2.2.1 Waardenburg Syndrome

Waardenburg syndrome (WS) is a human genetic condition characterized by defective melanocyte function (with pigmentation anomalies of the skin, hair and iris; Fig. 18.3), cochlear sensorineural deafness and craniofacial abnormalities [160]. It occurs in association with intestinal aganglionosis as the uncommon Shah-Waardenburg subtype (WS4) [137]. EDNRB-deficient cells have been shown not to develop into differentiated pigmented melanocytes [77] and mutations of the EDN3 gene also appear to be important in WS [33, 64].

The Waardenburg-HSCR association is uncommon in most series and we have encountered only 1 patient out of more than 500 patients with HSCR. It does appear in kindreds, however [120, 121], where no increasing penetrance of aganglionosis was observed between generations in 25 unrelated kindreds (i.e. almost identical aganglionic length) [15, 85, 113].

This Waardenburg-HSCR association is transmitted in an autosomal recessive manner and appears to be related to genes at 13q22 (EDNRB) [121] and other related genes required for the normal development of the neural crest cells migrating to the eye, inner ear and colon. Experiments with Sp (Splotch) mutation on chromosome 1 in mouse models have provided a link to a PAX3 deletion (a transcription factor with two highly conserved DNA motifs) [54]. It has since been shown that PAX3 functions with SOX10 to activate c-ret transcription and that interruption of these pathways at various stages will result in intestinal aganglionosis [87]. To emphasize the role of EDNRB, a mouse model with exon 2 and 3 mutations has been reported to demonstrate the features of WS4 [97].

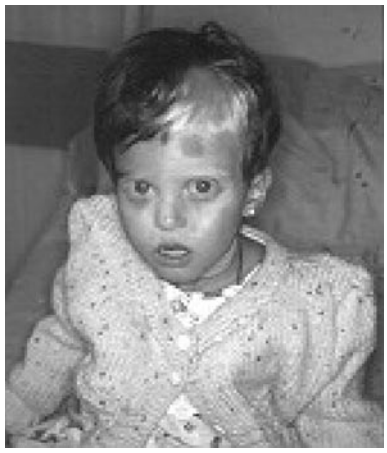

Fig. 18.3 Waardenburg-Shah association of HSCR with WS4. Note the white forelock

Thus, at least four genetic links are currently associated with the Waardenburg-Shah phenotype (viz. a transcription factor from SOX10, EDN3, the EDNRB gene [98], and a possible link to the MITF gene) [161].

18.2.2.2 The Role of SOX10

It is now understood that SOX10 activity (corresponding to the animal dominant megacolon DOM trait) modulates a number of critical signaling pathways controlling the differentiation of neural crest-derived nerve cells and melanocytes [101]. In addition to the known PAX3–SOX10–c-ret functions, interaction between SOX10 and the severity of aganglionosis has been shown in an animal model [28]. The presence of certain EDNRB mutations was shown to increase penetrance. In addition to EDNRB, further links have been demonstrated between SOX10 and RET (MOLa) binding sites in the RET promoter region where SOX10 has been shown to regulate transcription from the RET M promoter [161]. Lack of the normal SOX10-mediated RET activation may therefore also lead to intestinal aganglionosis. In addition,

overexpression of other genes coding for myelin proteins may result in some of the syndromic neurological associations of HSCR. A report of a patient with pseudoobstruction and SOX10 (without EBNRB and EDN3) mutations, and no pigmentation disorder [118], demonstrates its importance in intestinal neuronal development.

It is clear therefore that dosage-sensitive heterozygosity with incomplete penetrance of SOX10 could predispose to HSCR, whereas homozygosity would result in more complex neurocristopathies associated features of HSCR and WS [2]. WS has also been associated with mutations of the MITF (microphthalmia-associated transcription factor) gene [88, 161] which encodes a transcription factor with the basic helix-loop-helix leucine zipper (bHLH-zip) motif, which has been shown to be involved in melanocyte differentiation [110].

18.2.3 Congenital Central Hypoventilation Syndrome

Congenital central hypoventilation (CCHS, Ondine's curse) is an uncommon syndrome occasionally associated with HSCR (14–20% of cases), as well as with tumors of neural origin and autonomic dysfunction HSCR-CCHS (Haddad's syndrome). It is mostly associated with long-segment aganglionosis. CCHS involves a loss of autonomic control and is often associated with other autonomic nervous system abnormalities such as tonic pupil and other ophthalmic anomalies, especially when it occurs in association with HSCR [40]. It is a life-threatening condition as it results in an impaired ventilatory response to hypercarbia and hypoxemia, and patients often spend long periods on mechanical ventilatory support.

It has been reported to occur in 1 in every 200,000 live births in France [157]. It affects boys and girls equally and may be familial [73], the recurrence risk to sibs being 4%. These sib pairs together with identified genetic links with HSCR and associated tumors suggest a genetic basis for this syndrome. The pathogenesis of CCHS is most likely multigenic, although novel mutations of the RET and EDN3 genes have been reported [14]. A novel RET mutation (R114H) has been described [81, 128] as well as a corresponding GDNF variation [2]. Variations in brain-derived neurotrophic factor gene have been reported [162]. The CCHS-like picture resulting from a disrupted RNX gene (HOX11) in an animal model, in embryonic stem cells [96], has not been replicated in humans. Other workers have reported PHOX2B as a candidate [3], and more recently, heterozygous mutations of the paired-like homeobox gene PHOX2B have been identified in 91% of patients. It is not infrequently associated with tumors such as neuroblastomas [124], ganglioneuromas and ganglioneuroblastomas. Because of the known genetic associations, it is reasonable to speculate that the latter two arise in situations of lower gene penetrance.

18.2.4 Other Rarer Neurocristopathies

18.2.4.1 Extended Plasticity of the Enteric Nervous System

This group of conditions incorporates those variants of HSCR in which plasticity of the ENS appears to not follow the usual course and the plasticity of the ENS is prolonged. These conditions include prolonged or delayed maturity of ganglion cells, segmental aganglionosis and acquired postoperative aganglionosis.

Immaturity of Ganglion Cells

A wide spectrum of dysplastic features occur in the bowel in HSCR, one of which is immaturity of cells. This has been seen mostly in neonates and premature infants, the so-called "immaturity of prematurity". The ENS function in these patients appears to improve with maturation and is mostly managed conservatively. It may, however, persist giving rise to clinical problems.

It has been observed that, although differentiation of ENS neurons occurs early, a significant pool of precursor cells persists in the ENS, and the numbers of enteric neurons continue to increase until well after birth or hatching [67]. Immaturity of ganglion cells has been reported to influence the function of the intestine [25, 57]. Immaturity must be interpreted in the light of the gestational age, postnatal age and knowledge of the variations in normal postnatal development. In addition, the recognition of immature cells is not always easy as other cells such as hypertrophied glial cells and fibroblasts may lead to misinterpretation [6]. These immature ganglion cells have a smaller, darker nucleus without a recognizable nucleolus [6]. Special staining methods may be necessary to clarify the ganglion cell morphology and identify immature cells [108, 135].

To a certain extent, ENS immaturity may also explain the relatively low levels of acetylcholinesterase (AChE) not infrequently observed in neonatal ganglion cells [45], and the increase in staining patterns over time. The immature or developing cells would express AChE as they attempt to differentiate, and the timing of this would depend on the proportion of immature cells present.

18.2.4.2 Segmental Aganglionosis (Zonal Aganglionosis or Skip Lesions)

HSCR is normally defined as a functional obstruction resulting from congenital absence of ganglion cells in the myenteric plexuses of the distal segment of the gastrointestinal tract. A single distal aganglionic region therefore extends from the anal margin to the level of the proxi-

mal ganglionated bowel. Segmental aganglionosis, on the other hand, involves only a limited segment of bowel interposed between segments of normally innervated bowel. Understanding this phenomenon poses considerable theoretical and practical challenges.

Despite it being reported very early on in HSCR [75, 85, 146, 152], the existence of zonal aganglionosis is often questioned on theoretical grounds [170]. It has. however, been described in both children [4, 46, 72, 74, 89, 95, 116, 136, 149, 170] and adults [68], as well as in a number of animals [148]. It has been reported as including both the small bowel and the large bowel, and occasionally the appendix [4].

Munakata and Holschneider [107] classified the reported cases into:

- Single zonal aganglionosis or hypoganglionosis with distal normal innervation (ten patients)
- Double zonal analysis with distal normal innervation (four patients)
- Zonal normoganglionic or hypoganglionic colon within aganglionic intestine (eight patients)

The generally held view that all enteric neuroblasts arise from the vagal crest [117] and populate the bowel in a craniocaudal wave gives rise to certain theoretical difficulties in understanding how zonal aganglionosis could come about. Possible etiologic causes include the following:

- Anoxic damage to the myenteric plexus
- Migratory theory: a meeting point of the craniocaudal neuroblast migration as well as the neuroblasts arising from the sacral outflow
- Unfavorable microenvironment hypothesis
- Intrauterine inflammation or viral infection
- A primary abnormality of the developing gastrointestinal anlage

The hypoxic theory is discussed in the next section (18.2.4.3 Acquired Aganglionosis). The migratory hypothesis lacks support and there is little evidence that the sacral outflow produces a significant contribution to the ganglionation of the terminal bowel. In fact, the contrary appears to be the case [117]. In contrast to the migratory theory, a localized defect in the microenvironment of the specific segment of bowel resulting in a failure of enteric neurons to differentiate and undergo normal development and undergo apoptosis appears a distinct possibility. The pathogenesis of this condition would then depend upon developing and migrating neural crest cells confronting a segmental abnormal and hostile and microenvironment as a result of deranged intracellular signaling systems relating to the specific genes and gene protein.

The plasticity of the ENS after birth has long been the subject of debate. Current concepts include the idea that average neuronal activity levels are maintained by a set of homeostatic plasticity mechanisms, which adjust levels to achieve stability [158]. Recent findings demonstrate the important role of Hox genes (e.g. SOX10) in promoting the survival of neural crest precursors prior to differentiation [101]. Mutations may lead to apoptosis, thus offering a further explanation of ENS plasticity.

A primary abnormality of the developing gastrointestinal anlage appears to be a real possibility. It is currently supported by recent animal experiments on embryos of ls/ls minus mice (a model of classic short-segment aganglionosis) [82] in which a transient phase in the migratory pattern has been demonstrated. It would seem that ganglion cells appear in the middle colon of these mice as a result of an extramural phase of neuroblast migration at a stage when they are still absent from the ascending colon and distal large intestine. This unique observation suggests some sort of theoretical understanding of zonal aganglionosis. Should ENS development be arrested and persist after birth, it would give rise to the same clinical picture as reported by Martin et al. [95], where the ascending and descending colon were aganglionic with ganglion cells present in the middle colon. There are also similarities to one of the cases reported by Yunis et al. [170] and Taguchi et al. [149], and the zonal hypoganglionosis reported by Kadair et al. [80] could probably also be explained in this way.

18.2.4.3 Acquired Aganglionosis

Secondary aganglionosis following pull-through procedures for HSCR is a rare event. We previously reported an incidence of 1.5% in our series (5 patients out of 324 HSCR patients with pull-through operations) [38]. All the patients had a satisfactory initial postoperative course, but developed recurrent symptoms such as abdominal distension, pain and constipation, and in some cases soiling, several months later. Carefully controlled rectal biopsies above the level of the original anastomosis in these patients indicated that the previously histologically proven ganglionic pulled-through segment had become aganglionic.

Previous studies have been criticized because of possible sampling errors whereby the biopsy may have been taken from the level of residual aganglionic bowel inadvertently or deliberately retained at the original procedure (e.g. Rehbein's procedure or the anterior rectal wall following a Duhamel procedure). Nevertheless, acquired aganglionosis has been reported following the Swenson [44, 55, 56], Duhamel [90, 122, 164] and Soave procedures [37, 41]. As in our patients, the aganglionosis in all these patients seems to have been acquired postoperatively, the pulled-through bowel being ganglionated at the time of surgery. The pathogenetic mechanism by which aganglionosis may be acquired following pull-through procedures remains uncertain, but a number of possibilities exist. These include vascular insufficiency as well as a number of other possible mechanisms.

Since the first description of this condition by Ehrenpreis in 1965 [55], vascular impairment of the pulled-through segment with consequent neuronal hypoxia has been postulated. The evidence attributing a vascular cause to HSCR still seems to be largely based on circumstantial evidence, however [103]. The fact that hyaline fibrosis was observed in certain vascular walls together with an increase in fibrous tissue in the submucosa in two of our patients [38] and in one reported by Ehrenpreis in 1965 [55, 56] would appear to support this hypothesis. On the other hand, fibrosis has not been a feature of other studies [37]. There is some experimental data supporting a vascular accident in the pathogenesis of HSCR [53], and abnormal arteries have been found in aganglionic areas and in the transitional zone of resected bowel [90, 150]. Although it is a stated view that marked regional differences in the sensitivity of the neuromuscular system to hypoxia in experiments on the large intestine of piebald mice [167] could possibly account for the divergent experimental results [37, 51, 53, 55, 164], the possibility still exists that hypoxia of the pulled-through segment could lead to degeneration or a failure of differentiation of developing or immature ganglion cells. On the other hand, in other animal experiments [50, 99], selective ischemia failed to cause aganglionosis and ganglion cells were still clearly identifiable after the hypoxic event in spite of other features of hypoxia in the mucosa and muscle. Meijers et al. [99] concluded that the induction of such ischemia at an early stage of development results in stenosis or intestinal atresia without selective loss of enteric neurons. It is also possible that other pathogenetic mechanisms such as environmental toxins play a part in acquired aganglionosis. Degeneration and destruction of colonic ganglia have been experimentally produced in animals by injection or administration of various toxins [55, 112, 169], but the hypothesis appears to lack clinical support. Acquired intestinal aganglionosis has also been reported in association with circulating immunoglobulin G class enteric neuronal antibodies in high titer [143]. This is of particular interest due to other observations of increased immunoglobulins in congenital aganglionosis [102], and raises the question as to the role played by the immune system in the pathophysiology of aganglionosis.

In addition to acquired aganglionosis following pull-through procedures, there are a number of reports of acquired aganglionosis occurring without surgery [155, 156, 163]. In all these patients the diagnosis of HSCR, although clinically suspected, was eliminated by the presence of distinctive ganglion cells on rectal biopsy. Following several months of clinical intestinal obstruction, repeat rectal biopsies revealed hypertrophic nerves and an absence of ganglion cells typical of HSCR. Touloukian and Duncan [154], reporting acquired aganglionosis in a stressed premature baby with enterocolitis, attributed it to ischemia generated by the redistribution of the capillary circulation away from the gut during a state of shock. Chow et al., reporting a patient with degenerated ganglion cells and a mononuclear infiltrate in the submucosa of the rectum at the age of 5 days and subsequent aganglionosis at 7 months [36], speculated that a viral infection, probably acquired in utero, could be the cause of HSCR in some patients. Smith et al. [143] reported two patients with enteric ganglionitis with a loss of neurons together with vacuolated nerve cells surrounded by $CD3^+$ and $CD4^+$ T lymphocytes.

We emphasize the need for repeated sequential biopsies in patients with recurrent symptoms and features of HSCR following pull-through procedures. The specific etiology and pathogenesis of this entity needs to be elucidated.

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Megacystis-Microcolon-Intestinal Hypoperistalsis Syndrome

P. Puri

19

19.1 Introduction

Megacystis-microcolon-intestinal hypoperistalsis syndrome (MMIHS) is a rare congenital and generally fatal cause of functional intestinal obstruction in the newborn. This syndrome is characterized by abdominal distension caused by a distended nonobstructed urinary bladder, microcolon and decreased or absent intestinal peristalsis [1]. Usually incomplete intestinal rotation and shortened small bowel are associated.

19.2 Pathogenesis

MMIHS was first described in 1976 by Berdon et al. and to date 182 cases have been reported in the literature [1–87]. The etiology of this syndrome remains unclear. Several hypotheses have been proposed to explain the pathogenesis of MMIHS: genetic [20, 28, 36, 37, 42, 44, 52, 61, 63, 75], neurogenic [5, 8, 12, 15, 20, 21, 35, 39, 40, 53, 63], myogenic [2, 57, 80, 81], and hormonal [11].

Histological studies of the myenteric and submucosal plexuses of the bowel of MMIHS patients have found normal ganglion cells in the majority of patients, decreased in some, and hyperganglionosis and giant ganglia in others [63]. An imbalance between several kinds of intestinal peptides has been suggested as one of the possible causes of hypoperistalsis in MMIHS patients [39, 60]. Recently, Piotrowska et al. [81, 87] reported absence of interstitial cells of Cajal (ICCs) in the bowel and urinary bladder of patients with MMIHS. ICCs are pacemaker cells which facilitate active propagation of electrical events and neurotransmission, and their absence may result in hypoperistalsis and voiding dysfunction in MMIHS. Puri et al. [2] showed, in 1983, vacuolar degenerative changes in the smooth muscle cells (SMCs) with abundant connective tissue between muscle cells in the bowel and bladder of patients with MMIHS and suggested that a degenerative disease of SMCs could be the cause of this syndrome. Several subsequent reports have confirmed evidence of intestinal myopathy in MMIHS [57, 80, 81]. Ciftci et al. [57] reported a patient without vacuolar degeneration but with excessive smooth muscle glycogen storage. They postulated that the pathogenesis involves a defect of glycogen-energy utilization. Other investigators have reported the absence or a marked reduction in α-smooth muscle actin and other contractile and cytoskeletal proteins in the smooth muscle layers of MMIHS bowel [80, 81]. Contractile and cytoskeletal proteins are important structural and functional components of SMCs and play a vital role in the interaction of the filaments in smooth muscle contraction.

Recent work with transgenic mice lacking certain nicotinic acetylcholine receptor (ηAChR) subunits, which show some of the phenotypic features of MMIHS suggests a basis for this condition. Xu et al. [88, 89] produced a MMIHS phenotype in beta 4/alpha3 (two of the seven neuronal nicotinic acetylcholine receptor subunits) knockout mice. The alpha 3 and beta 4 subunits have been localized to human chromosome 15. Recently, Richardson et al. [74] carried out in situ hybridization and immunocytochemistry studies to determine whether alpha 3 mRNA or alpha 3 subunit protein was expressed in the resected specimens of small bowel from patients with MMIHS. They found lack of α3 ηAChR staining in most MMIHS tissues, thus suggesting that the absence of functional α3 subunit containing ηAChR may provide a

possible explanation for the underlying pathogenesis of MMIHS.

19.3 Prenatal Diagnosis

There are 54 previous reports describing fetal sonography findings associated with MMIHS. The most frequent finding was enlarged bladder (88%), with hydronephrosis seen in 31 fetuses (57%) [63, 72, 84]. Normal amniotic fluid volume was revealed in 32 fetuses (59%), increased volume in 18 (33%) and decreased volume in 4 (7%). In three fetuses (5%) [19, 36, 52] abdominal distension caused by a dilated stomach was detected. Three cases of oligohydramnios during the second and early third trimesters were reported [13, 23, 46], probably related to the functional bladder obstruction. In one fetus [46], oligohydramnios changed in polyhydramnios at the end of the third trimester.

Serial obstetric ultrasonography showed that the earliest finding in MMIHS is an enlarged bladder, detectable from 16 weeks of gestational age (Fig. 19.1). A later finding is hydronephrosis, caused by the functional obstruction of the bladder. Usually polyhydramnios develops late, appearing during the third trimester.

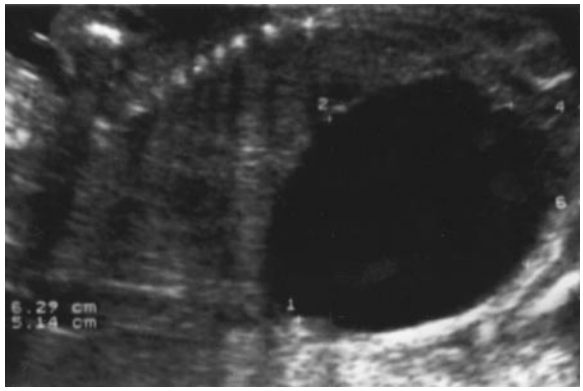

Fig. 19.1 Large fetal bladder seen on a longitudinal abdominal ultrasound image at 22 weeks of gestation. The fetus is in the prone position

19.4 Clinical Presentation

Of the 182 patients reported in the literature, the sex of 141 patients was mentioned: 98 were female and 43 were male. Four pregnancies were terminated after ultrasonography had detected MMIHS, which was confirmed at autopsy in all cases. The duration of 98 pregnancies was reported: 58 patients (59%) were born at term, 25 (25.5%) at 36 to 39 weeks of gestation, 12 (12%) at 32 to 35 weeks, and 3 (3%) at 31 weeks or less. Dystocia caused by abdominal distension was reported in eight cases. In four cases cesarean section was required [14, 33, 36, 45] and in four cases the bladder was so distended that the baby could only be delivered vaginally after removal of 250, 500, 650 and 500 ml of urine, respectively, from the fetal bladder by paracentesis [2, 39, 43, 56]. The mean birth weight was normal (3 kg) for gestational age.

The clinical symptoms of MMIHS are similar to those of other neonatal intestinal obstructions. Abdominal distension is a constant and early finding; other symptoms include bile-stained vomiting and absent or decreased bowel sounds. A distended, nonobstructed urinary bladder can be relieved by catheterization. Of 182 infants, 61 had bilious vomiting and 23 failed to pass meconium. The majority of patients were not able to void spontaneously.

A total of 19 sets of siblings affected with MMIHS have been reported—18 families had two affected siblings and 1 had three. Four sets of affected siblings were born to consanguineous parents [20, 29, 36, 37]. Consanguinity was also present in the parents of an affected child [52] born to a member of the family reported by Penman and Lilford [36]. In three further reports an older sibling of an affected child died just after birth because of intestinal obstruction [5] or multiple abnormalities [34, 54]; another sibling of an affected child was affected by prune-belly syndrome [16]. The occurrence of MMIHS in 19 sets of affected siblings together with consanguinity in four sets of parents suggest an autosomal recessive pattern of inheritance [29, 36, 52].

19.5 Radiological Findings

Radiological evaluation usually suggests the diagnosis of MMIHS. Plain abdominal films showed either dilated small-bowel loops or a gasless abdomen with evident gastric bubble. An enlarged urinary bladder was present in all patients who had cystography or ultrasonography (Fig. 19.2). Cystography showed vesicoureteral reflux in eight patients [6, 10, 19, 62, 63] and a urachal remnant in one patient [16]. Intravenous urography or ultrasonography detected unilateral or bilateral hydronephrosis in 84 patients [62, 63]. In one patient ultrasonography detected a dysplastic right kidney [44]. One patient had bilateral duplex kidneys [82]. Among 44 patients who had an upper gastrointestinal series both before and after laparotomy, hypo- or aperistalsis in the stomach, duodenum and small bowel was a constantly detected symptom. In three patients reverse peristalsis from the small bowel into the stomach was also observed [1–11]. In two patients hypoperistalsis was associated with gastroesophageal reflux [7, 28] and in one patient the esophagus was aperistaltic [46]. Barium enema showed microcolon in all 71 patients in whom this study was performed (Fig. 19.3); in 39 patients malrotation was associated.

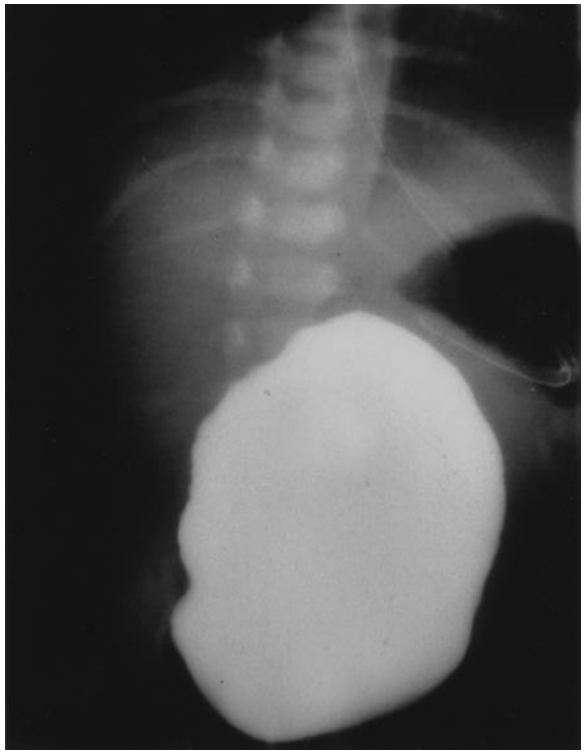

Fig. 19.2 Voiding cystourethrogram showing a massively enlarged bladder in an MMIHS patient

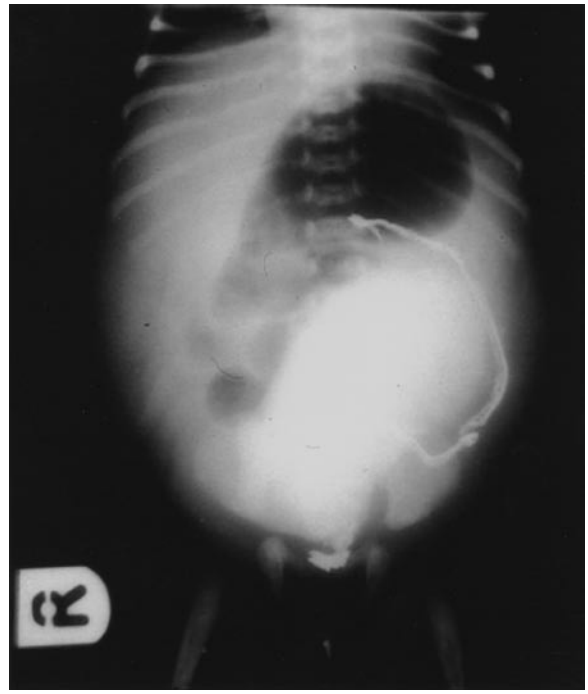

Fig. 19.3 A contrast enema showing microcolon in an MMIHS patient

19.6 Surgical or Autopsy Findings

Megacystis (Fig. 19.4) and microcolon were the two most frequent findings at surgery or autopsy and were present in all patients. Short-bowel syndrome was found in 37 patients, dilated proximal small bowel in 19, segmental stenosis of the small bowel in 3, duodenal web in 1, and Meckel's diverticulum in 1. Malrotation was found in a total of 81 patients. Although surgical management was not mentioned in several reports, 93 patients (70%) underwent one or more surgical procedures. Different kinds of interventions were performed: gastrostomy, jejunostomy, ileostomy, cecostomy, segmental resection of the jejunum and ileum, lysis of adhesions, and internal sphincter myectomy. Surgical manipulation of the gastrointestinal tract generally has been unsuccessful, and in most patients total parenteral nutrition was required. In 37 patients vesicostomy was performed to decompress the urinary tract and to preserve renal function.

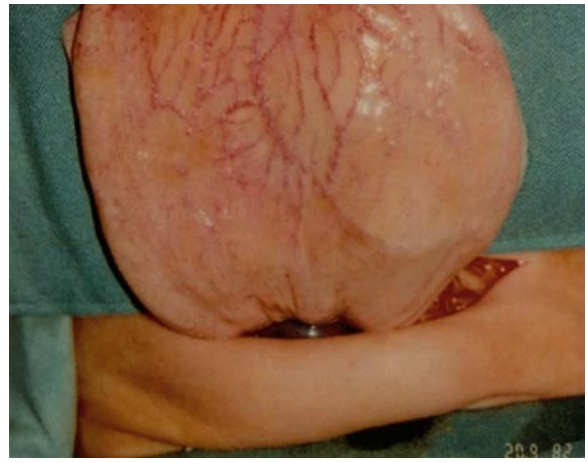

Fig. 19.4 Operative photograph of a massively dilated urinary bladder in MMIHS

19.7 Histological Findings

Histological studies of the myenteric and submucous plexuses were reported for 93 out of 182 patients. In 72

patients the ganglion cells were normal in appearance and number. Young et al. [12] found one patient with diffuse hypoganglionosis, and Vezina et al. [5] found aganglionic zones together with hyperganglionic zones in another patient. Immature ganglion cells were found by Manco and Osterdahl [21] in one patient. Kirtane et al. [20] found two patients with immature ganglion cells and hypoganglionosis. Krook [8] found both aganglionic zones and immature zones throughout the bowel. In four patients [11, 15, 53], hyperganglionosis was evident. Bindl et al. [35] reported neuronal intestinal dysplasia type B in one patient. Observations on the nerve fibers in the intestinal plexuses were reported for 26 patients. In 15 the appearance was normal, in 9 the nerve fibers were observed to be increased, and in 2 they were decreased. Taguchi et al. [39] noted an abnormal peptidergic innervation caused by a decrease in vasoactive intestinal polypeptide and peptide histidine methionine fibers and an increase in substance P and leucine-enkephalin fibers. At autopsy, neonatal axonal dystrophy was found in a patient with previous findings of hypertrophic nerve bundles and dystrophic neuritis in the rectal biopsy [48]. Kobayashi et al. [53] observed hyperganglionosis of the submucous and myenteric plexuses, and giant ganglia and ectopic ganglia throughout the entire gastrointestinal tract in two patients. Acetylcholinesterase staining and neural cell adhesion molecule (NCAM) staining of the uterus in one patient demonstrated a large number of ganglioneuromas [53]. Recently Piotrowska et al. [81, 87] reported absence of ICCs in the bowel and bladder of patients with MMIHS.

The majority of reports do not mention the histological findings in the muscle layers of bowel and bladder wall. Nevertheless, some authors found significant abnormalities in SMCs. In nine patients [2, 19, 33, 34, 42, 53] thinning of the longitudinal muscle was found on light microscopy. Electron microscopy showed vacuolar degeneration in the center of the smooth muscle of the bowel in 11 patients [2, 33, 34, 44, 80, 81] and of the bladder in eight patients [2, 33, 34, 53]. Connective tissue proliferation was found in the bowel in nine patients [15, 53, 80] and in the bladder in eight patients [34, 42, 50, 80]. In three more patients the bladder showed elastosis [12, 19]. In two patients electron microscopy revealed vacuolar degeneration of smooth cells in the muscle layers of the bowel and the bladder in addition to neuronal abnormalities (Fig. 19.5) [53]. Ciftci et al. [57] reported a patient without vacuolar degeneration but with excessive smooth muscle glycogen storage. They postulated that the pathogenesis involves a defect of glycogen-energy utilization. Other investigators have reported absence or marked reduction in α-smooth muscle actin and other contractile and cytoskeletal proteins in the smooth muscle layers of MMIHS bowel [80, 81].

19.8 Outcome

The management of patients with MMIHS is frustrating. A number of prokinetic drugs and gastrointestinal hormones have been tried without success. Surgical manipulation of the gastrointestinal tract has generally been unsuccessful. The outcome of this condition is generally fatal: only 23 of the 182 reported patients were alive, the oldest being 18 years old. Of the 23 surviving patients, 21 were being maintained by total or partial parenteral nutrition. The need for surgical intervention should be carefully evaluated, and the intervention individualized, since most explorations have not been helpful and probably were not necessary.

19.9 Conclusion

MMIHS is a rare and the most severe form of functional intestinal obstruction in the newborn. The major features of this congenital and usually lethal anomaly are abdominal distension, bile-stained vomiting, and absent or decreased bowel peristalsis. Abdominal distension is a consequence of the distended, unobstructed urinary bladder with or without upper urinary tract dilatation. Most patients with MMIHS are not able to void spontaneously. Surgical intervention of the gastrointestinal tract has generally been unsuccessful [90].

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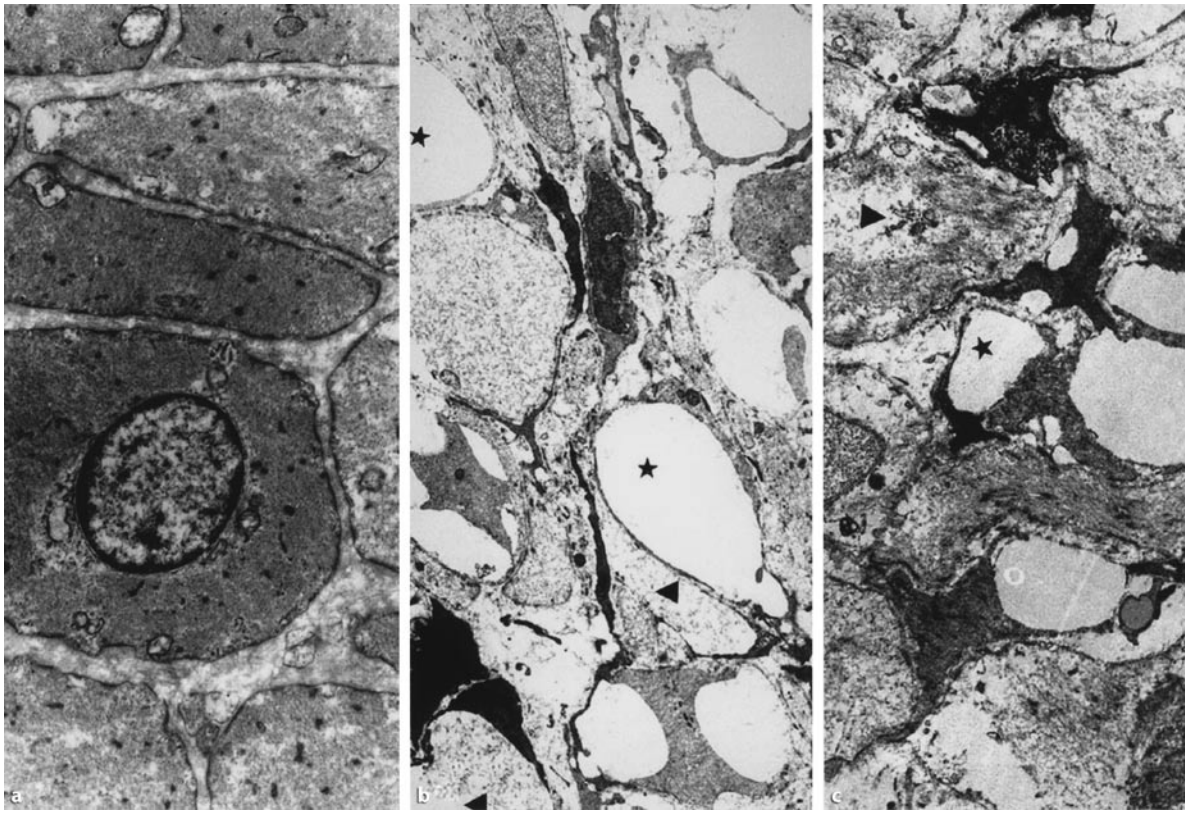

Fig. 19.5a–c Electron microscopy. **a** Smooth muscle cells from normal ileum. **b** Ileum from a patient with MMIHS showing vacuolar changes in the center of smooth muscle cells. **c** Vacuolar degeneration of smooth muscle cells in the urinary bladder from the same patient (*asterisk*s vacuolated cells, *arrowheads* excessive collagen between smooth muscles) (×6800)

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Degenerative Hollow Visceral Myopathy Mimicking Hirschsprung's Disease

H. Rode, R.A. Brown and A. Numanoglu

20

20.1 Introduction

Intestinal motility is a highly coordinated process and depends on smooth muscle contractility, the pacemaker activity evoked by the intestinal cells of Cajal, and the summation of the effects of the enteric and autonomic nervous systems on gut function. Crippling gastrointestinal dysfunction can result from a variety of abnormalities, involving these elements individually or in combination.

The pathological abnormalities underlying chronic idiopathic intestinal pseudoobstruction (CIIP) can be classified into four major groups: myopathies, neuropathies (inflammatory, degenerative or immune-mediated), mesenchymopathies (changes in the intestinal cells of Cajal network), and idiopathic. Alternatively, they may be secondary to systemic diseases involving the intestinal smooth muscle, endocrine disorders, drugs and toxins or other miscellaneous causes. Secondary pseudoobstruction syndromes in pediatric patients are uncommon compared to their occurrence in adults. Refinements in morphological techniques have improved our understanding of them.

CIIP is a clinicopathological syndrome characterized by ineffective prograde intestinal propulsion and recurrent symptoms of bowel obstruction in the absence of mechanical occlusion [1–6]. These disorders can lead to malnutrition and a protracted debilitating illness with impaired life expectancy. The disorders may differ in genetic transmission as well as pattern and distribution of involvement within the gastrointestinal tract and there may be concomitant extraintestinal manifestations. They may cause functional abnormalities without a discernible morphological diagnosis or, alternatively, may cause mechanical obstruction with changes easily recognizable on routine light microscopy of biopsied tissue.

Hollow visceral myopathy (HVM) constitutes part of CIIP. These disorders usually manifest during adolescence or early adulthood, although infants and children may be similarly affected with significant morbidity and mortality [2, 4, 7–17]. Symptoms tend to be more severe and prognosis worse in the primary myopathies [6, 7].

It is probable that many of these disorders were previously reported in the pediatric literature under a variety of different titles, viz. pseudo-Hirschsprung's disease, segmental small-bowel dilatation, hypo- or adynamic bowel syndrome, megaduodenum, idiopathic megacolon, and megacystis-microcolon-intestinal hypoperistalsis syndrome.

20.2 Classification

The disorders can be classified according to heredity, age at presentation, or morphological characteristics. A modified and practical classification based on the clinical and morphological features is presented in Table 20.1 [1, 2, 8, 9, 16, 17]. Various pathological subtypes of HVM are increasingly being recognized with H&E staining, Smith's silver staining and electron microscopy. This classification reflects our current understanding of these disorders, although a significant number of children with pseudoobstruction have no demonstrable primary disease or identifiable histological changes in the affected viscera and are idiopathic.

20.3 Etiology

A specific etiological factor has not been identified, and no genetic defect is known. The disorders may be caused by genetic aberrations, abnormal protein synthesis, toxins, autoimmune disorder or other factors.

The patterns of inheritance in familial visceral myopathy (FVM) are varied and may be autosomal dominant with high or low penetrance. The absence of demonstrable male to male transmission in some kindreds excludes the possibility of a sex-linked dominant mode of transmission [8, 9, 10, 14, 18, 19, 20]. The clinical expression of the disease amongst families with dominant inheritance usually starts after the first decade of life and asymptomatic but affected members are not uncommon. Symptoms are less severe than amongst those patients with autosomal recessive inheritance. Gastrointestinal lesions in patients within the same family are similar. In 15 reported families, the inheritance pattern was autosomal dominant in 8 and autosomal recessive in 7, although sex-linked dominance could not be excluded in 4 [20, 21]. The genetic aberration is unknown, but has been linked to a defect in synthesis of a contractile protein resulting in the degeneration of smooth muscle fibers [22].

Individuals affected by either FVM or mitochondrial myopathy manifest many common features, including intestinal pseudoobstruction as well as extraintestinal neurological manifestations, ophthalmoplegia, leukoencephalopathy, polyneuropathy, dementia and seizures, suggesting that a mitochondrial DNA mutation could be the molecular lesion in FVM [23].

In HVM a deficiency of smooth muscle alpha-actin within the circular muscle coat has also been implicated as an etiological factor [24]. There is additional evidence that the pronounced fibrosis has its origin in the transformation of smooth muscle fibers from a purely contractile to a myofibroblast collagen synthetic phenotype [25]. The etiology of sporadic cases is uncertain. Spontaneous mutations, pre- or postnatal acquired diseases, or exposure to a common environmental agent cannot be excluded.

Although the etiology of degenerative leiomyopathy (DL) remains obscure, the morphological and functional defects are considered postnatal events. It appears to be

Table 20.1 Hollow visceral myopathy

1. Familial visceral myopathy	**Autosomal dominant**
	Autosomal recessive with ophthalmoplegia
	Autosomal recessive with total bowel involvement
2. Sporadic visceral myopathy	**Infantile**
	Childhood
3. Degenerative leiomyopathy	
4. Muscle disease	**Myotonic dystrophy**
	Progressive muscular dystrophy
	Progressive systemic sclerosis
	Miscellaneous
5. Idiopathic	

Adapted from Krishnamurthy and Schuffler [2]

region-specific and presents usually after a number of years, suggesting that the disease is acquired rather than congenital. A smooth muscle toxin is the most likely pathogen, supported by the geographical distribution in ethnic groups from rural areas in southern, central and eastern Africa [26]. Alternatively, it may present as a "burn-out" autoimmune disorder [27]. There is no association with other congenital abnormalities and the predilection for specific geographical regions suggests cultural–environmental causes [28].

20.4 Diagnosis

The diagnosis of HVM should be contemplated in children with the typical clinical presentation, aided by radiological findings and supplemented by other special investigations including manometry, scintigraphy and histology [5]. An underlying mechanical obstruction must be excluded. Histological confirmation is mandatory for the diagnosis.

20.4.1 Clinical

Although varied, the predominant feature of HVM is that of intestinal obstruction which may present at any age [3, 4, 5, 8, 11, 13, 17]. No sign or symptom is pathognomonic of pseudoobstruction. The location of the affected bowel and the fact that it may be diffusely involved, is more important than the underlying cause [6]. A family history must always be sought, as it supports the diagnosis of pseudoobstruction and may determine the pattern of inheritance for genetic counseling. An intrauterine diagnosis can be suspected if the fetus is noted to have megacystis in conjunction with dilated loops of bowel [8]. The longitudinal muscle layer is predominantly involved in HVM, and this could reflect an insult at a specific time within the first trimester of pregnancy. Symptoms may fluctuate markedly in frequency and severity and may be present for years before pseudoobstruction is established, with myopathy usually presenting earlier than a neuropathy [8, 9, 29].

Nonspecific symptoms of gastrointestinal involvement include dysphagia, nausea, vomiting, colicky abdominal pain, and constipation or diarrhea [8]. Unfortunately, these overlap with many other conditions which may obscure the true nature of the disease. On examination, malnutrition and weight loss are evident, together with abdominal distension, present in 85% of the series of Vargas et al. (Fig. 20.1) [8]. Loops of bowel may be visible or palpable, and bowel sounds may be absent or even hyperactive. A succussion splash may be elicited [3, 11]. Anal sphincter tone is normal on rectal examination. Progressive symptomatic episodes of intestinal obstruction occur with increasing frequency and severity, necessitating further investigations to establish a diagnosis for treatment and counseling.

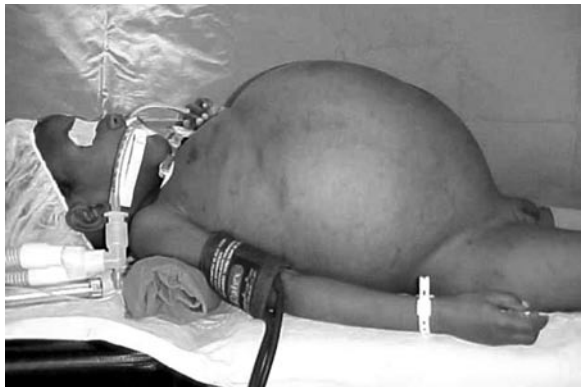

Fig. 20.1 Marked abdominal distension in a 3-year-old girl presenting with degenerative leiomyopathy

20.4.2 Radiology

20.4.2.1 Abdominal Radiographs

These are essential to exclude a mechanical cause of intestinal obstruction [3, 8, 30]. They reveal dilated loops of small and/or large bowel, which may be gross, featureless and contain air-fluid levels. Colonic dilatation is often misdiagnosed as cecal or sigmoid volvulus (Figs. 20.2 and 20.3). Fecal loading is present in 50% of patients; however, the findings are nonspecific and may be absent in up to 20% of patients, especially if there has been preceding gaseous evacuation of the colon and an empirical trial of pharmacological management [18].

20.4.2.2 Contrast Radiology

Barium rather than Gastrografin should be used for this investigation, as the hygroscopic action of large volumes of intraluminal Gastrografin in a small child can result in hypovolemia. Generalized dilatation of the entire intestine favors the diagnosis of HVM (or neuropathy) [30]. The dilatation may be associated with abnormal peristalsis and hypocontractility, delay in gastric emptying with or without gastroesophageal reflux in the presence of a normal lower esophageal sphincter, megaesophagus and megaduodenum, valvular "packing" in the small intestine because of circular muscle fibrosis and an enlarged redundant colon with loss of haustral patterns, and retention of barium for more than 24 hours (Fig. 20.4). Bowel dilatation is an absolute requirement for diagnosis and radiological deterioration can be demonstrated (Fig. 20.5). Malrotation of the bowel must be excluded [8].

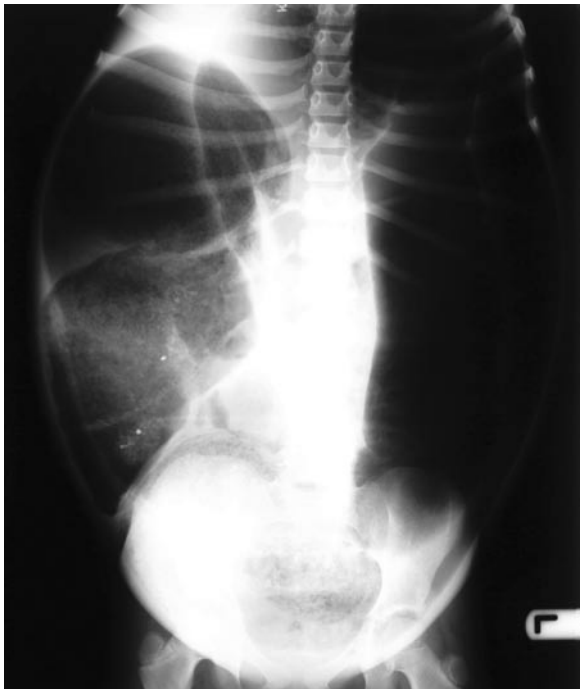

Fig. 20.2 Plain abdominal radiograph showing gross and featureless distension of bowel, air-fluid levels and fecal loading

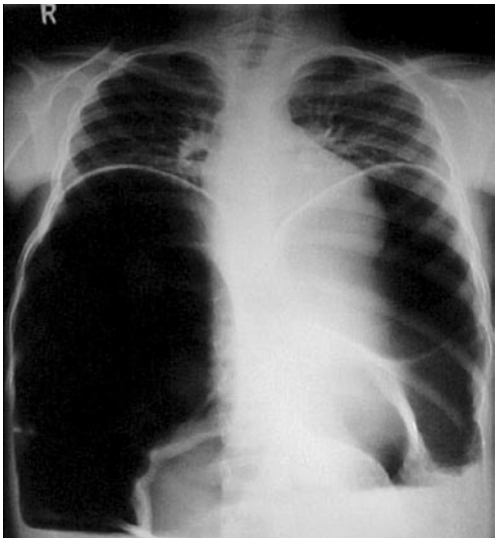

Fig. 20.3 Chest radiograph showing gross bowel distension, diaphragmatic elevation and decreased lung volumes

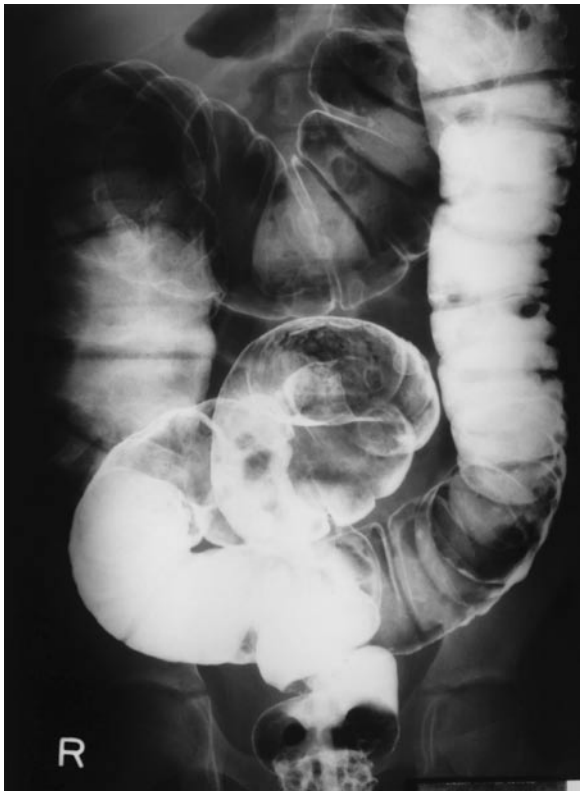

Fig. 20.4 Barium enema showing distension of the colon, deficient haustral markings and redundancy

20.4.2.3 Transit Studies

These will confirm delay in gastric emptying and intestinal transit as well as a decrease in motility. In addition to radioopaque markers, the breath hydrogen technique and sulfasalazine absorption and organic dyes have been used [31].

20.4.3 Manometry

Prolonged (>6 hours) manometric readings of the various anatomical regions of the intestine offer a valuable means of diagnosing HVM as well as differentiating it from other forms of pseudoobstruction [4, 5, 19, 29, 32]. It shows hypomotility with low amplitude coordinated contractions in myopathy, compared to normal amplitude uncoordinated contractions in neuropathy [31]. It was 95–100% accurate in the diagnosis of pseudoobstruction in the series of Vargas et al. and Boige et al., supporting its use as a screening procedure for pseudoobstruction [8, 33]. Manometric abnormalities correlate with both the extent of the pathological process and the prognosis [5, 34].

Esophageal peristalsis is uncoordinated with low-amplitude waves or absent contractions, and with normal or high lower esophageal sphincter pressures. Antroduodenal motility is coordinated with poor propagation and the phasic waves of the migrating motor complex (MMC) are infrequent, with low amplitude and coordinated or

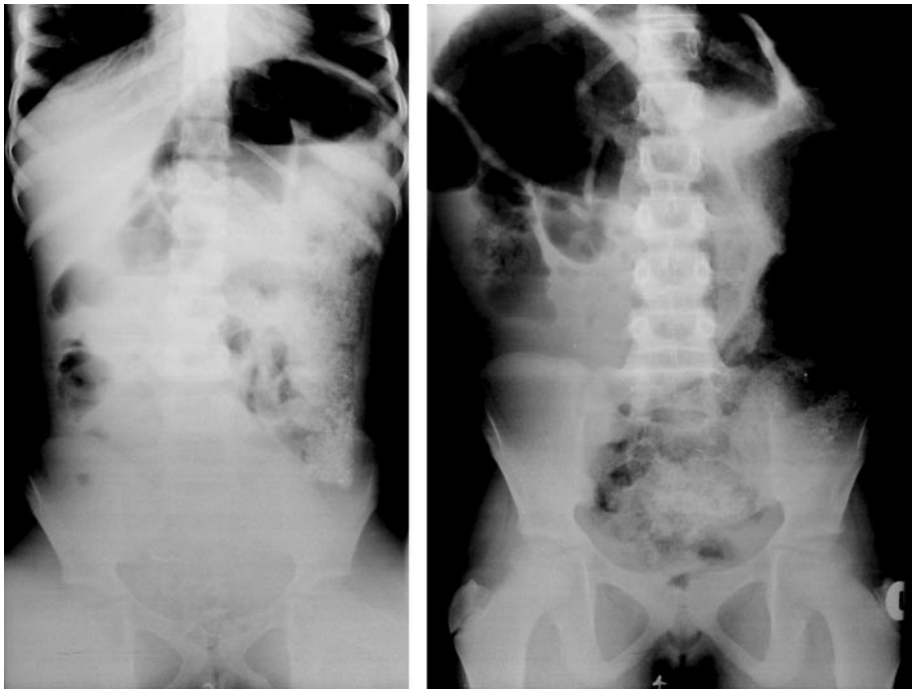

Fig. 20.5 Plain abdominal radiographs taken at presentation and 3 years later showing progressive bowel dilatation associated with clinical deterioration

absent contractions [19, 29, 32, 34]. This distinguishes it from a visceral neuropathy and can be explained on the basis of bowel damage, weak contractions, and grossly dilated intestine [29]. The presence of a normal migrating complex may predict successful enteral feeding. Disordered foregut motility can be detected in relatives of patients with HVM, and may precede other manifestations of the disease by months or even years [18]. Colonic contractions are absent in the decompensation phase of the disease. The biliary tree has low-amplitude phasic contractions with low basal sphincter of Oddi pressures [29]. Anorectal manometry shows a normal rectosphincteric inhibitory reflex. The rectum may be so dilated that the rectal balloon volume is insufficient to elicit a relaxation reflex response.

20.4.4 Scintigraphy

Radioisotopes allow accurate quantification of the pattern and efficacy of propulsion of intestinal contents along the bowel lumen. Technetium-99 is the most widely used radioisotope, as it is easily obtained and cheap, and the radiation dosage is low. For longer studies indium-111 and iodine-131 have been used. Prolonged small-bowel and colonic transit times are commonly seen in visceral myopathy and accumulation or "clumping" of radioactivity may identify the functionally most impaired segments of the dilated bowel [5, 35].

20.4.5 Electrogastrography

This technique is similar to electrocardiography and measures gastrointestinal electrical activity via surface electrodes attached to the abdominal wall [36]. The surface electrogastrography (EGG) can assess gastric emptying and shows a low-amplitude trace in a myopathy, compared to a tachygastria in children with idiopathic pseudoobstruction and a neuropathy. The severity of the dysmotility cannot be assessed with the present technique, but ongoing investigations may lead to advances in this noninvasive method [37].

20.4.6 Histology

It is essential to confirm the diagnosis of HVM [38]. Endoscopic mucosal biopsies are inadequate for histological assessment. DL should be diagnosed on full-thickness rectal biopsies performed after careful preparation of the distal bowel. At exploratory laparotomy full-thickness biopsies of an adequate size (2×2 cm), taken from the stomach, small bowel, and large bowel, are necessary. A laparotomy is especially indicated in children in whom a congenital or mechanical cause for the obstruction has to be excluded. This was necessary in two-thirds of the patients in the series of Vargas et al. [8]. Histological methods should include a large variety of techniques including H&E, Smith's silver and Meier-Ruge staining, immuno-

cytochemical staining, and electron microscopy [2, 3, 4, 28, 38]. Normal histology may not exclude HVM. In 9 of 20 patients in the series of Smith and Milla from Great Ormond Street Hospital, London, abnormalities were not detected on routine paraffin sections, but required further special studies, i.e. electron microscopy, immunohistochemistry and histochemistry [27]. Unfortunately, laparotomy for biopsies predisposes the child to potential repeated laparotomies for adhesive obstruction, as the symptoms and signs may be difficult to differentiate from the underlying chronic pseudoobstruction. Noninvasive techniques, e.g. laparoscopic biopsy, may decrease the incidence of postoperative adhesions [39].

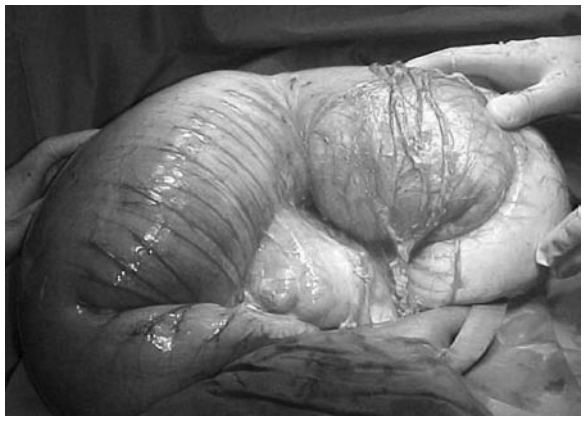

Fig. 20.6 Operative picture of massively distended colon

20.5 Pathology

The pathology can be localized to a segment of bowel or it may be more extensive, affecting the entire gastrointestinal tract. The most important histological features—smooth muscle cell vacuolar degeneration and fibrosis—are easily recognizable on routine light microscopy. Although the target area is predominantly the smooth muscle layer, nonspecific changes may be observed in all layers of the intestinal wall [21, 27, 28].

20.5.1 Macroscopy

At surgery the bowel may be distended, thin-walled, redundant and lack haustrations (Fig. 20.6). The colon is predominantly involved and the dilatation usually extends proximally into the small bowel, duodenum, stomach and esophagus to varying degrees. The bladder may also become megacystic. The presence of early esophageal and duodenal involvement favors a diagnosis of chronic intestinal pseudoobstruction syndrome over DL.

20.5.2 Microscopy

20.5.2.1 Muscular Layer

Visceral myopathy is characterized by specific alterations in the muscularis propria (Figs. 20.7 and 20.8) [2, 28, 38]. The pathology varies from mild to severe, with the most extensive changes affecting the clinically diseased bowel. Muscle layers are thin and attenuated with muscle cell degeneration, muscle cell loss, amorphous debris and extracellular edema. There is an increase in fibrous tissue with replacement of muscle fibers by collagen fibers surrounding both residual muscle cell fragments and areas of drop-out, imparting a vacuolated appearance. These fibrotic changes are most prominent in the longitudinal muscle layer, whereas extracellular edema is most obvious within the circular muscle layer. Enlargement, irregularity and hyperchromia of the smooth muscle nuclei have been reported in two adult patients with FVM in association with hypertrophy of the muscularis mucosa [21]. Severity of involvement is not uniform, with clusters of apparently well-preserved muscle fibers interspersed amongst degenerated muscle fibers [38]. Inflammatory foci are occasionally seen within the muscle layers.

20.5.2.2 Neuronal Plexus

The myenteric plexus in HVM remains morphologically intact and no damage is evident on histology.

20.5.2.3 Mucosal Lesions

Mild to severe damage and inflammation of the mucosal architecture may be present and probably reflect mucosal insult from the underlying stasis syndrome [18, 28, 38]. These changes may be similar to those in celiac disease or progressive systemic sclerosis.

20.5.2.4 Ultrastructure

The muscularis propria is predominantly affected with loss of internal structure. The earliest changes consist of smooth muscle cells appearing more electrolucent with disorganization, loss of myofilaments and mitochondrial swelling (Fig. 20.9). In established disease damaged cells have discontinuous plasma membranes with loss of alignment of contractile elements, vacuolated mitochondria and clear cytoplasm. In advanced disease muscle cells show degeneration with replacement by fibrosis and

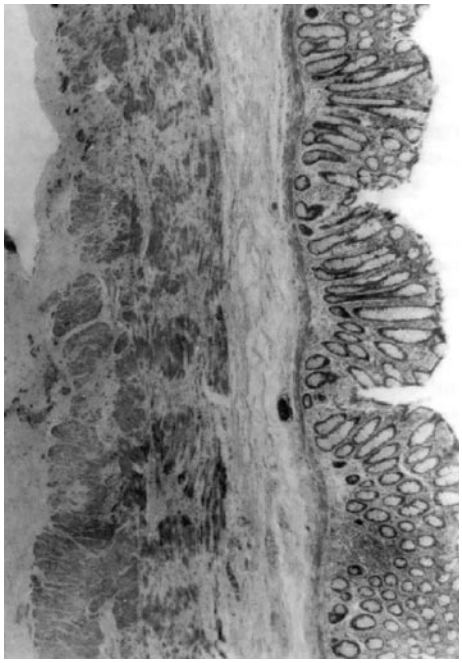

Fig. 20.7 Full-thickness colonic wall in degenerative leiomyopathy showing thickened and partly fibrosed muscularis mucosa, with extensive degeneration and loss of smooth muscle in circular and longitudinal layers associated with fibrous replacement together with subserosal fibrosis (H&E, ×16)

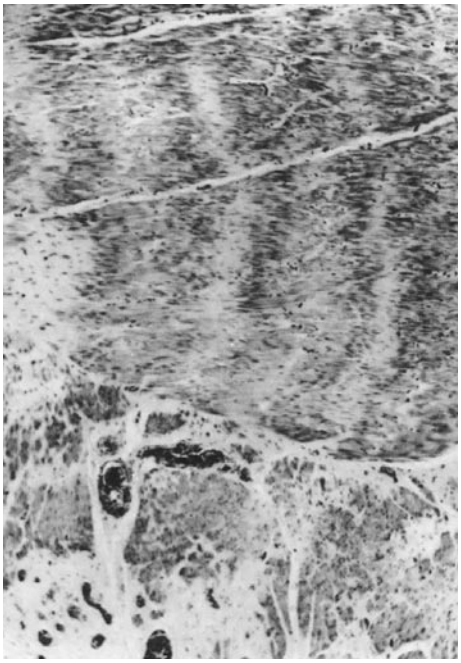

Fig. 20.8 Degenerative leiomyopathy showing circular and longitudinal layers of muscularis propria. There is regular alignment of nuclei and cytoplasm of smooth muscle cells with intervening fibrosis in the circular layer. In the longitudinal layer the smooth muscle is degenerate and being replaced by connective tissue (H&E, ×40)

collagen. The intracellular spaces are filled with edema, muscle debris and collagen, and there is no evidence of vasculitis or inflammation [2, 28, 38].

20.6 Extraintestinal Lesions

Megacystis is present in 33–86% of patients with HVM and is easily demonstrated by sonography or cystography [9, 10, 14, 40]. Histologically the bladder wall is either normal or thickened and partly replaced with mature collagen. Extraintestinal lesions may include external ophthalmoplegia [16, 19]. Autonomic and peripheral neuropathy are seen in neuropathic and not myopathic diseases.

20.7 Specific Disorders of Smooth Muscle

20.7.1 Familial Visceral Myopathy

Three types of FVM have been identified (Table 20.2) with two modes of transmission and variable expression

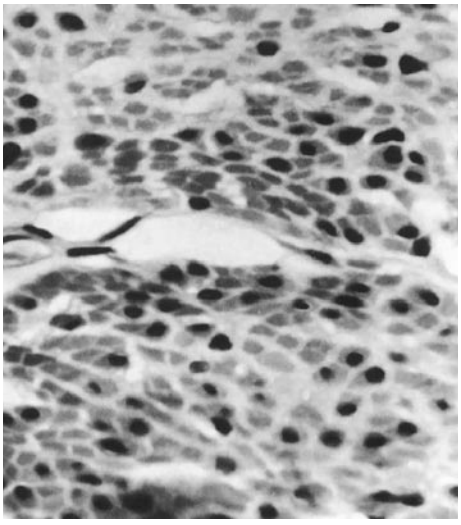

Fig. 20.9 Degenerative leiomyopathy showing vacuolation of smooth muscle cells with pyknotic nuclei in smooth muscle of circular layer (H&E, ×400)

of gastrointestinal involvement, symptoms, response to treatment and associated extraintestinal manifestations [2, 9]. Histologically intestinal smooth muscle degeneration and increased fibrosis are indistinguishable throughout the various types, raising doubt about the specificity of bowel involvement in the subgroups [20].

20.7.2 Sporadic Visceral Myopathy

The pathological features are identical to those of FMV in adolescents and adults, but with more severe symptoms, earlier onset and a worse outcome (Table 20.3). The etiology is unknown and the possibility of genetic transmission unlikely as only 4 of 170 family members were effected in one study [10]. The entire gastrointestinal tract and bladder may be affected, and it has been reported in infants with the megacystis-microcolon-intestinal hypoperistalsis syndrome [41].

20.7.3 Degenerative Leiomyopathy

DL is a distinct entity indigenous to young Africans from southern, central and eastern Africa [17, 26, 28, 42].

In only one instance has a family history with affected siblings been reported. DL is characterized by a long history of increasing abdominal distension with massive megacolon presenting in older children (mean age 9.5 years) [17]. The disease primarily affects the distal bowel but it may extend proximally into the small bowel, stomach and esophagus, and may also affect the urinary tract. The accumulation of intraluminal fluid and bacterial overgrowth probably accounts for malabsorption and progressive clinical deterioration.

Although the etiology of the condition remains unknown the morphological and functional defects are considered to be postnatal events. A smooth muscle toxin or an autoimmune disorder could be implicated in the pathogenesis [26, 27]. Comorbidity with pulmonary tuberculosis is seen in 50% of patients [17].

Histologically the presence of interstitial and intracellular edema of the muscularis propria, the absence of vacuolated mitochondria and pattern of submembrane cytoplasmic translucency on electron microscopy separate DL from other forms of HVM. The degenerative changes tend to be distributed focally or in alternating waves along the longitudinal axis of the muscle. Muscle cell cytoplasm is homogeneous and eosinophilic with shrunken and pyknotic nuclei, and 25% will have a predominant lymphocyte inflammatory cell infiltration in the muscularis propria, which could represent a response to an infective agent.

Although the myenteric plexus appears morphologically normal, ganglion cells are displaced centripetally in more than 50% of patients, with an excess of thick stubby acetylcholinesterase-positive nerve fibers in the muscularis propria. In 29 of 35 children (83%) in the study of Moore et al. there was a raised vasoactive intestinal peptide (VIP) level in the intestine, and in 7 of this group

Table 20.2 Familial visceral myopathy

	Type 1	Type 2	Type 3
Mode of transmission	Autosomal dominant	Autosomal recessive	Autosomal recessive
Pathology	Degeneration and fibrosis of muscularis propria; myenteric plexus normal	Indistinguishable from type 1	Indistinguishable from type 1
Intestinal lesions	Esophageal dilatation; megaduodenum and dilatation of proximal jejunum; redundant colon	Gastric atony and dilatation; dilatation of entire small bowel; multiple diverticula	Dilatation of the entire intestinal tract
Clinical manifestations			
Age of onset	After first decade of life	Teenager	Adult life
Symptomatic	<50%	>75%	Most
Symptoms	Pseudoobstruction; variable expression	Pseudoobstruction; malnutrition	Pseudoobstruction; malnutrition
Treatment	Symptomatic relief with medication and surgery	Recalcitrant	Recalcitrant
Prognosis	Moderate	Poor	Poor
Extraintestinal manifestations	Megacystis; ophthalmoplegia	Ophthalmoplegia; ptosis	None observed

Adapted from references 2, 9 and 14

Table 20.3 Hollow visceral myopathy and degenerative leiomyopathy

	Infantile	Childhood	Degenerative leiomyopathy	Idiopathic (unclassified)
Mode of transmission	Sporadic	Sporadic	Geographic distribution; sporadic	Mixed
Pathology	Visceral myopathy	Visceral myopathy	Visceral myopathy with blood vessel changes	No demonstrable abnormalities
Intestinal lesions	Gastric, small-bowel and colon dilatation	Gastric and small bowel dilatation	Predominantly distal bowel; whole gastrointestinal tract may be involved	Dilatation of gastrointestinal tract distal to esophagus
Clinical manifestations				
Sex distribution	72% female	75% male	Equal	60% female
Age of onset	Within 1–3 weeks	First year of life	Early childhood (9.5 years)	Newborn to 4 months
Symptomatic	100%	100%	100%	100%
Symptoms	Pseudoobstruction; emaciation	Pseudoobstruction; malnutrition	Pseudoobstruction; malnutrition	Pseudoobstruction
Treatment	Recalcitrant	Symptomatic relief	Recalcitrant	Recalcitrant
Prognosis	Fatal	Moderate	Unremitting; progressive deterioration	60% mortality
Extraintestinal manifestations	Urinary tract dilatation 86%	None observed	Urinary tract dilatation small muscular arteries of other viscera 50%; pulmonary tuberculosis	Megacystis
References	2, 9, 16, 41	26, 32	17, 26	13

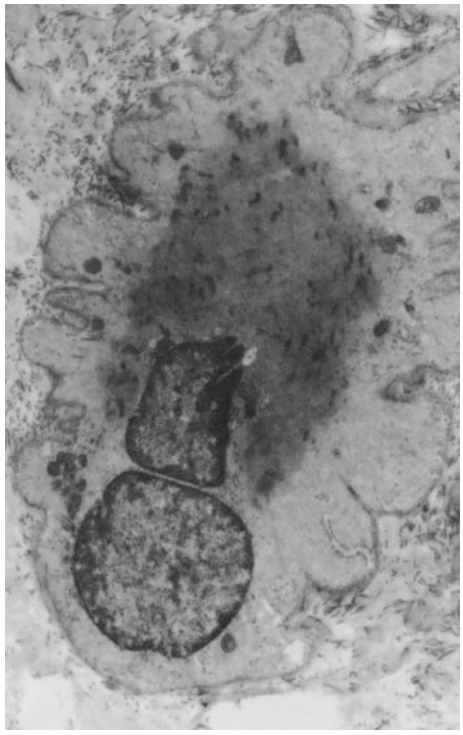

Fig. 20.10 Electron micrograph in degenerative leiomyopathy showing a transverse section of a smooth muscle cell with indentation of sarcolemmal membrane, peripheral rarefaction, central condensation of fibrils with accumulation of spindly densities and an indented nucleus. The cell is surrounded by much glycosylated aminoglycans and collagen fibers (×10,080)

there was hyperplasia of the myenteric plexus [26]. This increased VIP level may cause neurogenic inhibition of the smooth muscle or it may be expressed as a reaction to neurotoxic damage to the bowel. Often encountered at autopsy are diffuse structural lesions affecting the small muscular arteries of the bowel, bronchioli, spleen, liver and kidneys causing fibrotic stenosis of the lumina. This histological pattern separates DL from other muscular lesions occurring in mixed connective tissue diseases of childhood [43].

20.8 Differential Diagnosis

The majority of causes of secondary pseudoobstruction can be excluded by a careful clinical history and appropriate investigations; however, many of these are rarely seen in pediatric patients. These include visceral neuropathies, progressive muscular dystrophy, progressive systemic sclerosis, myotonic dystrophy, generalized leiomyositis, celiac disease, malrotation, toxins, pharmacological agents, and diffuse lymphoid infiltration [2, 3, 8, 41, 44, 45, 46, 47].

20.9 Treatment

Due to the progressive unremitting course of HVM together with the lack of understanding of its etiology, treatment consists at present of dietary manipulation and symptomatic relief with medication, reserving surgery for diagnostic and palliative roles [3, 4, 8, 11, 17]. Because of the erratic response, therapy may be difficult to evaluate, although it tends to be better earlier on in the disease process and in familial types. All forms of therapy confer incomplete and temporary benefit only. As DL may present in theory as a "burnt-out" autoimmune disorder, its progression may be arrested with steroids and immunosuppressive treatment [27].

20.9.1 Medical

20.9.1.1 Gastrointestinal Rest

Nasogastric tube decompression, restricted oral intake, intravenous fluids, colonic lavage, and decompression by rectal tube or colonoscopy are the most effective means of managing the acute attack. This reduces the gas and fluid load, thereby decreasing the diameter of the intestine with symptomatic relief.

20.9.1.2 Dietary Management

Nutritional therapy plays an important role, and initially includes a low residue diet and the avoidance of spicy or gas-producing foods. An elemental or semi-elemental diet may be required and administered as a bolus or continuous drip infusion via a nasogastric or nasoduodenal tube or gastrostomy [48]. This may be supplemented or even replaced by parenteral nutrition. Oral intake should be encouraged despite the presence of obstructive symptoms, but prolonged and even permanent intravenous feeding often becomes necessary. Limited amounts of enteral feeding, however, are desirable to prevent cholestatic jaundice and to maintain bowel mucosal integrity.

20.9.1.3 Pharmacological Agents

The empirical use of broad-spectrum nonabsorbable antibiotics may reduce bacterial overgrowth in the proximal intestine, thereby reducing symptoms such as pain, distension, and diarrhea [3].

Prokinetic drugs are used to encourage prograde peristalsis. Cholinergic drugs increase intestinal activity, and some success has been obtained using Rae's mixture which contains neostigmine bromide and magnesium sulfate. Cisapride, erythromycin and subcutaneous octreotide, a somatostatin analog, which induces propagating phase 3 MMCs during fasting, can be of benefit [49]. Tegaserod is a promotility agent like cisapride, without its cardiac toxicity, and can also be used [49]. Children with an absent MMC, megaduodenum and small-bowel dilatation respond less favorably to prokinetic agents and require more parenteral nutritional support [15].

20.9.2 Surgical

This should be reserved for patients only if there is potential benefit.

20.9.2.1 Management

Because HVM is a generalized alimentary disorder, surgical intervention is confined to a palliative role [8, 17]. Surgery must be tailored as the disease can affect other parts of the digestive or other organ systems. Procedures may include intestinal diversion, decompression gastrostomy, tapering duodenoplasty, duodenojejunostomy, limited small-bowel resection, subtotal or total colectomy, ileostomy, cecostomy or colostomy [18]. In practice, many of these bring only temporary or no relief. Surgery is also unlikely to protect renal function or restore normal micturition.

Symptomatic relief from excessive gaseous distension can be obtained by the placement of a "blow-hole" in the transverse colon in the form of either a catheterizable tubularized colostomy or gastrostomy button device. This allows marked symptomatic relief by intermittent deflation and the administration of antegrade colonic enemas [50].

Mechanical obstruction may subsequently develop due to adhesions, cecal or colonic volvulus, or rarely strictures [17, 51]. Substantial numbers of operations in these patients are therefore for complications of previous surgery. Other postsurgical complications include wound infection, bowel perforation, peritonitis, and the short-bowel syndrome. These are not infrequently the ultimate cause of mortality [3, 10, 17]. Exploratory laparotomy should therefore be avoided except where the patient is incapacitated by symptoms, fails to respond to medical therapy or where unequivocal evidence of mechanical obstruction exists.

20.10 Prognosis

HVM has a poor prognosis with the prospect of increasing obstructive symptoms, malnutrition and deterioration [3, 8, 17]. Emaciation, overwhelming sepsis, parenteral nutrition complications, and surgical complications are the most frequent causes of death. Small-bowel transplantation is only indicated if there are life-threatening complications due to irreversible gut failure. These children may need a multivisceral transplant [52].

20.11 Conclusion

HVM is a long-term illness with a variable natural history. Radiographic examination, intestinal manometry and full-thickness histology will confirm the diagnosis and extent of disease, and allow the rational development of a therapeutic program. This should include pharmacological stimulation of intestinal motor function, preservation of adequate nutritional status, maximum symptom relief, and goal-directed surgical intervention. Unfortunately, the long-term prognosis is guarded, with the disease impacting significantly on the life of the patient with frequent hospital admissions, bowel dysfunction, dietary restrictions, chronic empirical medication and failed surgery.

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Adynamic Bowel Syndrome

P. J. Milla

21

21.1 Introduction

Disorders which mimic the distal colonic obstruction typical of Hirschsprung's disease are described under a number of terms including adynamic bowel, pseudo-Hirschsprung's disease, megacystis-microcolon hypoperistalsis syndrome, and visceral neuropathies and myopathies. They may be caused by a wide variety of different disorders including conditions which are intrinsic to the enteric neuromusculature as well as disorders where the environment in which the neuromusculature operates is abnormal such as in inflammatory conditions. Most of these diseases have their effect by disrupting the normal control mechanisms of the motor apparatus of the gut. The normal patterns of contraction of the muscle coats of the gut are dependent upon the control mechanisms which act upon the smooth muscle cells of the muscularis propria at many different levels to produce the required effects. The control mechanisms consist of properties of the smooth muscle cells themselves, an intrinsic network of nerves, the enteric nervous system with multiple neurotransmitters modulated by extrinsic nerves, paracrine and endocrine hormones, and other neuromuscular active compounds.

If a clinical condition which mimics Hirschsprung's disease is considered using this mechanistic framework then the cause of the patient's symptoms may often be readily understood. In some there may, however, be obstruction of the anus or rectum due to congenital anorectal anomalies, tumors of the anus and rectum, e.g., leiomyoma, hemangioma or external compression of the anorectal area.

21.2 Clinical Presentation

The disorders may present with either the primary or the secondary effects of the underlying condition. These may result in either functional obstruction or severely delayed transit of intestinal contents. A patient may thus complain of severe constipation, acute or chronic urinary retention, a distended painful abdomen or even vomiting. In addition, some further effects of these episodes might include fecal impaction, adhesional obstruction associated with previous surgery, and episodes of bacterial overgrowth. In those conditions in which there is an underlying neuropathy of the gut, the consequences of denervation may not only be on motor activity but also upon intestinal secretion and sensation. Visceral hyperalgesia may be one consequence of a denervation hypersensitivity produced following severe drop-out of enteric neurons. In those conditions in which there are developmental defects of the enteric nerves or muscle layers, children present most commonly either in the neonatal period or under the age of 1 year, whereas children with acquired disease present in later childhood [1, 2].

21.2.1 Antenatal and Neonatal Period

Some may be recognized before birth with dilated loops of bowel or a distended bladder or both on sonography, particularly where the mother suffers from polyhydramnios. In the neonatal period the commonest presentation is failure to pass meconium with constipation dating from within a few days of birth. In others, the abdominal symptoms may be more generalized with, in addition to the failure to pass meconium, abdominal distension and bilious vomiting.

In our experience all those who, in addition to gastrointestinal symptoms, fail to pass urine or have recurrent urinary tract infection secondary to poor bladder contractility, hydroureter or hydronephrosis have disease of both the enteric and urinary musculature [2, 3].

21.2.2 Infancy and Later Childhood

The majority of children presenting in later childhood present because of acquired disease. Some patients with congenital defects of the enteric neuromusculature may present outside the neonatal period but mostly do so with similar symptoms to those presenting earlier [2]. Hypothyroidism may first have its effects during the first year of life, but it is unusual for other acquired disorders to be seen at this time. After the first year of life and in later childhood, whilst children may present as they do in infancy or in the neonatal period, often the initial presentation may simply be for constipation. In some, particularly those who have an inflammatory disorder affecting the myenteric plexus, severe abdominal pain may occur as visceral hyperalgesia consequent upon denervation hypersensitivity responses. In others, symptoms may mimic an acute abdomen. Bowel sounds may be totally absent or markedly reduced, but others will have high-pitched bowel sounds more likely to be due to a mechanical rather than a functional obstruction and clear differentiation is required.

21.3 Disorders Causing Pseudo-Hirschsprung's Disease

Disorders which mimic Hirschsprung's disease cause disturbance of the control mechanisms of the smooth muscle coats. Thus the disorders and disease may be primarily of the intrinsic enteric nerves with or without involvement of the extrinsic autonomic nerves or central nervous system, the smooth muscle cells themselves, and of the humoral and endocrine environment. Primary diseases of the gut motor apparatus are considered under two headings, Enteric Nervous System Disease (Section 21.4) and Disorders Affecting Intestinal and Urinary Smooth Muscle (Section 21.5). Secondary causes of pseudo-Hirschsprung's disease consist of a variety of diseases and drugs, and these are listed in Table 21.1.

21.4 Enteric Nervous System Disease

Disease of the enteric nervous system may be familial and limited to the colon or be part of a more diffuse disorder affecting the whole gut or as part of a familial peripheral and autonomic neuropathy such as familial visceral neuropathy. The commonest disorders mimicking Hirschsprung's disease are those in which there are malformations of intestinal neurons as in intestinal neuronal dysplasia, intestinal ganglioneuromatosis, MEN 2a and 2b, and hypoganglionosis. All of these conditions are considered in Chapters 8, 9 and 10 and are not considered further here.

21.4.1 Intestinal Ganglionitis

21.4.1.1 Idiopathic Lymphocytic Intestinal Ganglionitis

This condition may present with the sudden onset of acute severe constipation usually in late childhood. By the time that investigation takes place the appearance is often that of aganglionosis in the rectum. In two patients studied by the author the process seemed to start in the rectum and gradually ascend the gut [4]. In one patient in whom full-thickness biopsies over a number of years were available for study the condition could be shown to

Table 21.1 Secondary causes of Pseudo-Hirschsprung's disease

Disease of nerves	**Extrinsic enteric nerves**	**Diabetic neuropathy**
		Primary autonomic dysfunction
		Myotonic dystrophy
		Parkinson's disease
		Postviral infection
	Enteric nervous system	**Chagas' disease**
		Paraneoplastic autoimmune "enteric ganglionitis"
Disease of visceral smooth muscle		**Connective tissue disorders**
		Scleroderma
		Dermatomyositis
		Systemic lupus erythematosus
		Muscular dystrophies
		Infiltrative diseases
		Amyloidosis
		Ceroidosis
Metabolic conditions		**Uremia**
		Porphyria
Drugs		**Antidepressants and anxiolytics**
		Opioids
		Anticholinergics
		Cytotoxic

be due to an inflammatory denervating process effecting the intrinsic enteric nerves. In this process, careful examination of the early biopsies showed the myenteric plexus to be attacked by a mononuclear infiltrate largely of T lymphocytes which when resolved resulted in hypoganglionosis or, in severe cases, aganglionosis. Whilst the neurons disappeared with time the glial elements appeared to be spared. In these patients an IgG circulating myenteric antibody was found in the peripheral blood similar to the Hu protein antibody described in the paraneoplastic syndrome associated with oat cell carcinoma of the lung. In neither case has any evidence of carcinoma ever been found.

Histopathologically the key features appear to be the loss of neurons from both the myenteric and submucous plexuses with the preservation of glial cells. The degree of denervation can be assessed using either enzyme histochemical techniques such as acetylcholinesterase activity or immunohistochemical neural markers such as neurofilament protein, neuron-specific enolase or protein gene product 9.5. The preservation of the glial elements can be shown by the use of antibodies to astrocytes (glial fibrillary acidic protein) and Schwann cells (S100 protein). In addition many cells and fibers show immunoreactivity for neural cell adhesion molecule (NCAM) in the myenteric plexus presumably associated with the glial elements after the neurons have been destroyed.

In both the patients studied by the authors the characteristics of the disorder were those of an autoimmune process in which the inflammatory process is directed against enteric neurons. Immunosuppressive treatment with prednisolone and cyclosporin was helpful [4], although eventually in the first patient complete denervation of the gut occurred and she subsequently underwent successful intestinal transplantation.

21.4.1.2 Idiopathic Eosinophilic Intestinal Ganglionitis

Although this condition may present with severe constipation or functional obstruction like a lymphocytic ganglionitis, it does not result in aganglionosis and appears to be caused by a Th2 inflammatory process. In three children aged 1 month to 15 years studied by the author, prolonged functional obstruction occurred with

inflammation of the myenteric plexus and the colonic mucosa [5]. The inflammatory infiltrate was characterized by an excess of eosinophils and T lymphocytes, and had none of the features of the lymphocytic ganglionitis described above. In particular, no autoantibodies were found. The condition had some similarities to a transmural eosinophilic gastroenteropathy. However, the neurons in the myenteric plexus expressed the potent eosinophil chemoattractant interleukin-5, suggesting that the neurons were taking part in the inflammatory process. None responded to dietary exclusion, but all three responded symptomatically to immunosuppression with prednisolone and azathioprine.

Thus both this condition and the lymphocytic ganglionitis described above are examples of conditions causing severe pseudoobstruction syndromes for which there is effective treatment [5].

21.4.1.3 Chagas' Disease

In the older child and adult, aganglionosis in endemic areas may be due to Chagas' disease. It has been known for the last 90 years that this condition is due to a chronic infection with Trypanosoma cruzi. The condition is acquired from bites by large blood-sucking triatomine insects. The acute phase of the infection is commonly mild or asymptomatic with sometimes an inflammatory lesion at the site of entry. Around 30% of infected individuals progress to chronic Chagas' disease in which the heart and gut are primarily involved. In the gut the esophagus and colon are most commonly affected, but occasionally only the colon will be affected giving rise to confusion with Hirschsprung's disease.

The chronic phase of the disease appears to be associated with pseudocyst rupture producing focal inflammatory lesions in response to the pseudocyst in the smooth muscle of the gut. After this acute phase the inflammatory response generally subsides, but in some who go on to develop Chagas' disease a diffuse progressive multifocal lymphocytic inflammatory response develops in which the myenteric and submucous plexus become involved. It was originally thought that the neurons of the enteric nervous system degenerated as a result of a neurotoxin released by the organisms. However, although the precise mechanism of neuronal destruction is not clear, histological features [6, 7] and the presence of circulating muscarinic cholinergic receptor autoantibodies suggest that an autoimmune process may be responsible for the neuronal destruction, and that some of the functional abnormalities found in the condition are caused by the antibodies themselves [8].

Differential diagnosis from Hirschsprung's disease is seldom a problem when the heart and esophagus are involved. However, should the colon only be involved then careful histology of full-thickness biopsies is necessary to differentiate Chagas' disease from Hirschsprung's disease. A history of exposure to T. cruzi transmission and positivity for antibodies to T. cruzi in an indirect immunofluorescence test or ELISA assay are helpful in confirming the diagnosis.

The treatment for severe chagasic megacolon is surgical, and in Brazil the surgical treatment most favored is based on the Duhamel procedure [6, 7].

21.4.2 Proliferation of Glial Cells

Rarely patients who present with this symptom complex may be found on full-thickness biopsy to have proliferation of glial elements in the myenteric plexus [9]. Some have suggested that this is related to neurofibromatosis [10], but at the present time further exploration of this is required.

21.4.3 Immaturity of Myenteric Neurons

In some patients it has been suggested that delay in maturation of myenteric neurons may be associated with symptoms of constipation, and this is discussed fully in Chapter 17 Intestinal Neuronal Dysplasia.

In patients with a syndrome of short small bowel, malrotation and pyloric stenosis who present with complete obstipation and ultimately functional obstruction, it has been thought that the motility disorder was due to immaturity of the neurons and more specifically to the absence of argyrophilic neurons. However, it is now known that the presence of argyrophilic neurons is influenced by the age of the child and up to the age of 1 year the absence of argyrophilic neurons can be normal [11]. Over the age of 1 year argyrophilic neurons are always found. The patients reported by Tanner et al. [12] were all infants, and it is unlikely that the absence of argyrophilic neurons was the primary pathology in these infants.

21.4.4 Degenerative Neuronal Disorders

In adults, degenerative neuronal disorders have been identified in which there is an absence of an inflammatory response in the myenteric plexus. Whether this is an "end result" of a similar process to that that occurs in intestinal ganglionitis is unclear and much work needs to be done in this area.

21.4.4.1 Vitamin B_1 Deficiency

Just as central neurons are damaged in vitamin B_1 deficiency, for example Wernicke's encephalopathy, so damage may occur to neurons of the enteric nervous system.

In addition to gastric dilatation due to atony, peristalsis is also impaired and marked anorexia develops. Hypomotility of the bowel produces a dilated colon with constipation. Where peripheral polyneuritis, Wernicke's encephalopathy or cardiac and other circulatory disorders occurs, the differential diagnosis is easily made, and assay of thiamine levels will readily confirm the diagnosis.

21.4.5 Familial Visceral Neuropathy

Several discrete syndromes which occur familially have been described [13]. Neuronal intranuclear inclusion disease is an autosomal recessive condition with the onset of symptoms in childhood. The condition is characterized by mental deterioration, a range of central neurological abnormalities and functional obstruction which may mimic Hirschsprung's disease. Central, peripheral, autonomic and enteric neurons contain intranuclear inclusions and the number of gut neurons appears decreased in number.

An autosomal dominant visceral neuropathy with variable distribution in the gastrointestinal tract has been reported in a family in which the jejunum and ileum were mostly affected, but in some family members constipation was the presenting symptom [14].

Recently an X-linked enteric neuropathy characterized by malrotation, pyloric stenosis and a short small intestine has been described in which a genetic defect at Xq28 has been mapped by linkage analysis. In this family the affected boys presented as if they had Hirschsprung's disease [15].

21.5 Disorders Affecting Intestinal and Urinary Smooth Muscle

Enteric smooth muscle disease may be a primary condition or may be associated with a number of systemic disorders such as systemic sclerosis, dermatomyositis, systemic lupus erythematosus, and myotonic dystrophy. The majority of patients suffer from two syndromes: the hollow visceral myopathy syndrome or the megacystis-microcolon hypoperistalsis syndrome [16]. The latter is described in more detail in Chapter 19, and is not considered further here.

21.5.1 Hollow Visceral Myopathy Syndrome

In children, the hollow visceral myopathy syndrome mostly commonly affects both the enteric and urinary musculature and may present either before birth, in very early life or later in infancy. In a recent study, 90% of the patients studied had defective enteric and urinary muscle, and 30% presented antenatally, all with urological problems, 60% in infancy and 10% in later childhood [2]. In a few patients the effects of muscle disease may be restricted to a segment of the gut, often the rectum, and are truly present mimicking Hirschsprung's disease.

Very rarely an isolated myositis may occur in which the enteric musculature appears to be involved in an autoimmune-based inflammatory process.

21.5.2 Myopathic and Muscle Morphogenesis Disorders

Gross fibrosis of the muscularis propria and severe vacuolation of myocytes can be detected on routine histology in intestinal myopathies. Often, however, the abnormalities are too subtle for light microscopic detection and in many of these changes can only be detected on electron microscopy [17, 18]. Light microscopy may, however, reveal the presence of abnormality of development of the muscle coats with either an extra coat with no innervation or loss of the coat over large lengths of the gut [19].

21.5.3 Myopathy with Autophagic Activity

In this condition the myocytes are atrophied and widely spaced with an excess of intercellular connective tissue. The muscle cells retain some of their smooth muscle characteristics, but contain within them autophagosomes in which there is an accumulation of degradation products. Lysosomes can be shown to be active within the smooth muscle cells by increased acid phosphatase activity and connective tissue stains show considerable fibrosis. Within the muscle coat abnormal innervation can be detected with numerous nerves ramifying between the myocytes [19].

21.5.4 Disorganization of Myofilaments

On ultrastructural studies in some conditions the myofilamentous content of the smooth muscle cell is disorganized with loss of contractile filaments usually within the central portion of the smooth muscle cells. This results in a rather "moth-eaten" appearance. Some times the areas contain excess glycogen but more often appear as empty holes. The membranous components of the cell, particularly the plasma membrane and the dense bands, appear unremarkable and presumably this is one of the reasons why on routine light microscopy the muscle coat appears normal [19]. Sometimes there appears to be blebbing in the subsarcolemmal region; this is probably artifactual and due to ischemia produced at the time of obtaining the muscle sample [20].

21.5.5 Abnormalities of Contractile Proteins

A recent report provides the first clear description of another cause of visceral myopathy [21]. In a 47-year-old woman who had suffered from constipation and functional obstruction from very early in life an absence of alpha-0actin in the circular muscle layer was found. This was demonstrated by absent immunostaining for alpha smooth muscle actin from full-thickness biopsy material. Normal alpha smooth muscle actin immunoreactivity was present in all other intestinal muscle layers. There were no morphological abnormalities by light or electron microscopy. Embryogenesis of the enteric muscle coats suggests a defect at around 12 to 14 weeks of gestation which results in failure of the alpha isoform to develop later. However, more recent studies suggest that this finding may only be a marker of an unspecified muscle insult, and is a secondary phenomenon [22].

21.6 Disorders of the Endocrine Environment

Disturbance of the environment may occur in a variety of disorders from classical endocrine disorders through tumors secreting polypeptide hormones to inflammatory disorders and disorders of metabolism which create an abnormal humoral environment for the gut motor apparatus to operate in. However, most commonly of all, disorder of the extrinsic innervation, most usually by central nervous system disease or psychosocial disturbance, creates an abnormal modulating influence on the intrinsic enteric neurons.

21.6.1 Endocrine Disorders

Many diseases and drugs may secondarily affect the gut, and these are listed in Table 21.1.

21.6.1.1 Hypothyroidism

Decreased secretion of thyroxine by the thyroid gland may result in constipation. Such decreased secretion of thyroxine may be as a consequence of congenital abnormality of the thyroid gland, defective synthesis of thyroxine or as the end result of an autoimmune disorder causing thyroiditis. The end result of all these conditions is the same, and that is lack of secretion of thyroxine. The clinical presentation of such children is with increasing developmental delay, a rather coarse skin, abdominal bloating and constipation. In those with a congenital onset, the degree of mental retardation may be profound by the time the diagnosis is clear and results in what used to be known as "cretinism". The hypothyroidism may also be part of a more widespread syndrome such as the Johanson-Blizzard syndrome in which, in addition to hypothyroidism, there is pancreatic insufficiency and a sensorineural deafness. The motor disturbance of the gut caused by low levels of thyroxine is due to altered smooth muscle metabolism resulting in decreased frequency of the smooth muscle slow wave and less-effective contractile activity. Simple replacement therapy with exogenous thyroxine is remarkably effective in curing the constipation.

21.6.1.2 VIPoma

Autonomous secretion of vasoactive intestinal polypeptide (VIP) by either a pancreatic adenoma or a neuroblastoma or ganglioneuroblastoma, although usually causing a watery diarrhea with hypokalemic alkalosis, may also result in ileus and constipation [23]. In childhood, VIP-secreting tumors are nearly always neural crest cell tumors such as ganglioneuroblastoma. In later childhood and early adult life VIP-secreting tumors are more likely to be pancreatic adenomata. In both cases whilst VIP may be the predominant hormone secreted, frequently a number of other polypeptide hormones may be secreted by these pluripotential cells. Diagnosis of the condition is suggested by raised circulating levels of VIP.

21.6.2 Inflammatory Disorders

It is now clear that immunomodulation of the enteric nervous system and visceral smooth muscle occurs and that a variety of inflammatory conditions affecting the mucosa of the bowel may present with constipation or a pseudo-Hirschsprung-like condition. The best documented of these are celiac disease small intestinal Crohn's disease, and in children who are atopic and allergic to food proteins [24, 25].

Diagnosis of intestinal inflammatory conditions may require a variety of investigations. Celiac disease may clearly be diagnosed by jejunal biopsy and measurement of appropriate circulating food antibodies such as antigliadin, IgA antibodies or other Ig autoantibodies such as antitissue transglutaminase and antiendomysial antibodies. Atopic individuals will tend to have both raised total and specific IgE antibodies and those who are particularly severely affected may have associated minor immunodeficiency such as IgA or IgG subclass deficiency. Interestingly, atopic individuals have a raised internal anal sphincter tone on anorectal manometry which may result in their motility disorder [25]. In both celiac disease and food allergy, dietary restriction may cure the obstructive and constipating disorder [24, 25].

Crohn's disease will require a variety of imaging techniques together with colonoscopy and biopsy to delineate the disorder. These patients respond to control of the in-

flammatory process, whereas patients with toxic megacolon and ulcerative colitis may not, and may require colectomy as a life-saving measure.

21.6.3 Idiopathic Megarectum

Whilst not truly presenting as a pseudo-Hirschsprung condition nevertheless many children with chronic constipation and a degree of megarectum are referred to a pediatric gastroenterologist for exclusion of Hirschsprung's disease. A detailed history and examination will allow accurate recognition of the clinical features suggesting that the megarectum is as a consequence of the chronic constipation rather than as part of the disease process [26]. It will also provide evidence for the degree of involvement of psychological factors and hopefully exclude pathological causes such as the disorders discussed above. In children, the clinical features are also dependent upon the age of the child [26].

21.6.3.1 Clinical Pattern Changes with Age

In the very young infant the symptoms which parents report are usually difficulty or delay in defecation. Where this is associated with a normal rectal examination and abdominal distension, vomiting and failure to thrive are absent, one can usually be sure that an organic pseudo-Hirschsprung-like condition does not exist. However, the breast-fed infant may be particularly difficult as prolonged periods of time between stool may be normal in such infants.

The next period of time when children's symptoms appear to change are in the early preschool years when the main feature is the distress the delayed stool movement causes the child. It is at this time that many megarectums start to appear as the passage of the hard, large stool is resisted for as long as possible, and the newly gained anal sphincter control is exploited to its utmost by the child in an attempt to avoid the pain of releasing the stool. Following this, presentation may occur at a variety of ages when the social pressures caused by the overflow soiling from the ever-increasing megarectum make themselves felt.

21.6.3.2 Etiology

Severe constipation may be one of the consequences of the large extrinsic innervation of the hindgut. Consequently, children with a neurological disorder such as cerebral palsy and spina bifida have considerable problems with severe constipation where the condition is due to the loss of inhibitory input by the central nervous system to the enteric nervous system. Whilst patients may be referred for exclusion of organic disease such as Hirschsprung's disease, in the vast majority of such infants there is no underlying intrinsic disease of the hindgut.

21.6.3.3 Consequences of the Megarectum

Despite the ancient belief in the toxic effect of retained feces there is no direct evidence of adverse biochemical effects in the absence of liver disease. However, there are some clinical features of children with megarectum which can be explained by the mechanical effects of the loaded megarectum.

Neurological Symptoms

A number of children who have epilepsy and who as a consequence of their central nervous system disease have megarectum, their parents and carers often report that seizures are more likely to occur or be more protracted at the stage of gross fecal retention than at other times. This is an unexplained phenomenon but in view of the intensity of the extrinsic connections between the hindgut and the brain, it is likely that the sensory afferent input from the gross fecal retention results in an altered seizure threshold.

Urinary Tract Symptoms

The bladder is frequently distorted by the loaded rectum and it is therefore not surprising that there is a higher incidence of urinary tract infection in constipated children. Although there appears to be no correlation of the urological symptoms with the degree of palpable loading, little success is achieved in controlling the urinary tract infections until the fecal retention is treated.

Growth and Development

Children with megarectum tend to be lighter and shorter than expected for their families, although they are nearly always within the normal range. In a random sample of 57 children who had completed treatment for their fecal retention, 67% had increased their height standard deviation scores (Clayden, personal communication). Bone age as assessed by radiography of the left hand and wrist shows a delay of 2 to 3 years in most children, in a way somewhat similar to that seen in untreated celiac disease or Crohn's disease. However, for constipated adolescents, if they are able to manage to avoid major fecal retention during their pubertal growth spurt, the ultimate height they attain is usually normal even though their growth spurt may be delayed.

21.6.3.4 Psychological Factors

The recognition of the psychological factors in childhood constipation is essential in both diagnosis and planning effective management [27]. The factors vary in their importance and character according to the age and stage of development of the child. They are closely related to the physical symptoms and are often intricately woven with them in the initiation and persistence of constipation. Figure 21.1 simplifies these relationships diagrammatically. Many vicious cycles develop and aggravate existing difficulties in family dynamics or physiological predisposition to the condition. The clinician's role is often to define and explain the interacting factors and to provide targeted help from the appropriate members of a multidisciplinary team in an attempt to provide effective treatment and to shorten the period of time taken to achieve effective defecation [28].

21.7 Diagnostic Techniques

A number of different diagnostic techniques may be helpful in elucidating the nature of the condition, causing pseudo-Hirschsprung's disease. Again the mechanistic framework outlined above is helpful in planning the investigations, and the author uses a number of screening tests followed by confirmatory tests and definitive diagnostic histopathology.

21.7.1 Screening Tests

21.7.1.2 Radiology

Plain radiographs may show dilated small and large bowel mimicking an obstruction. The dilated area may end abruptly at the level of pathologically affected bowel in much the same way as in Hirschsprung's disease. Contrast radiology may show this effect much more clearly and may delineate stenosis caused by extrinsic compression. In addition, at the time of contrast radiology abnormal motor activity of the gut may be visualized. In cases of visceral myopathy, especially in young children, there is nearly always involvement of the urinary tract with similar smooth muscle pathology. In such cases sonographic examination of the bladder to determine its ability to empty is a useful screening test, followed by intravenous pyelography to determine the presence of hydronephrosis, hydroureter and megacystis.

21.7.1.3 Transit Studies

Three different methods may be used to measure transit in the gastrointestinal tract:

1. Radioopaque markers: Radioopaque markers can be used quite successfully in children to demonstrate delays in intestinal, largely colonic transit. Three sets of radiologically distinguishable markers are ingested on three successive days and a single plain abdominal

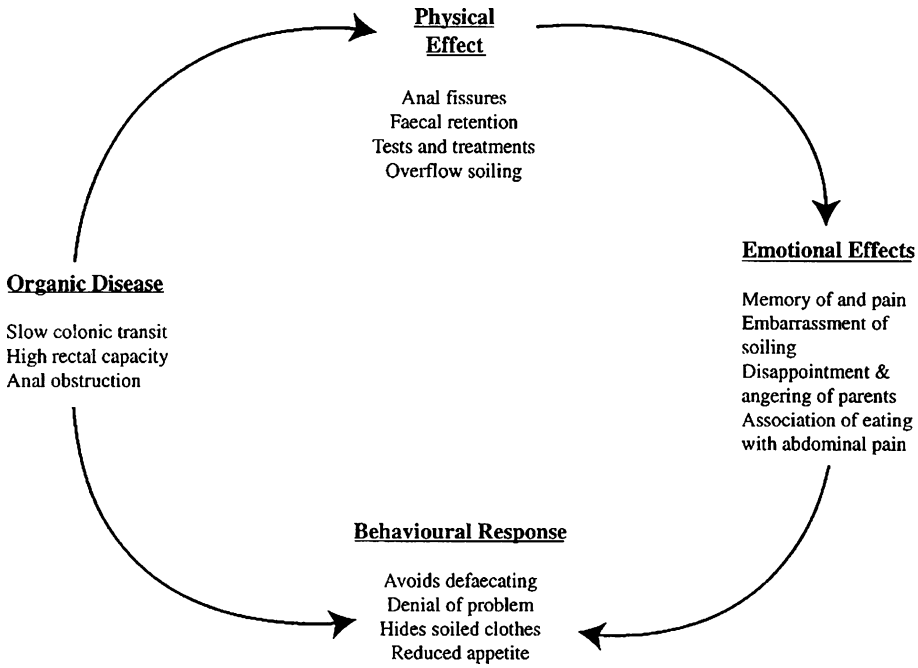

Fig. 21.1 The interaction between psychological and physical factors in idiopathic megacolon

radiograph obtained 120 hours after ingestion of the first set of markers [29].
2. Radioisotope transit studies: The majority of radioisotope studies have been validated for use in adults and there has been little work done other than on gastric emptying in children. However, in adults radioisotope studies have demonstrated different patterns of colonic transit in patients with visceral myopathy and neuropathy [30].
3. Breath hydrogen studies: Small intestinal transit which may reflect disease in the colon can be assessed by breath hydrogen using a disaccharide test meal which is not absorbed in the small bowel but is fermented by bacteria when it reaches the colon. The most commonly used disaccharide is lactulose.

21.7.2 Confirmatory Tests

21.7.2.1 Manometry

Anorectal manometry is a useful diagnostic tool in Hirschsprung's disease and conditions in which there is an intrinsic aganglionosis of the rectum. Its performance and use is described in detail in Chapter 12 and is not considered further here.

Where pseudo-Hirschsprung's disease is part of a diffuse disorder affecting the gastrointestinal tract, small-intestinal manometry may be very informative. The cyclical mature of fasting small-intestinal motor activity is determined by the inherent activity in the enteric nervous system. This intrinsic property can be used to test whether the enteric nervous system is intact or whether extrinsic nervous modulation is present. Observation of the disruption of fasting activity and the establishment of postprandial activity provides information regarding the humorally mediated response to food and clarifies whether enteroenteric responses are intact. In addition myopathic processes produce low amplitude, poorly propagated contractions, whereas neuropathic processes are associated with contractions of normal amplitude which are often bizarre in wave form and abnormally propagated with ill-formed phase 3 activity [31–34].

Colonic manometry has been evaluated in the diagnosis of pseudo-Hirschsprung-like conditions and may be particularly useful where there is slow transit in the colon [35]. It does, however, require a large investment of time and effort, but this may be amply repaid in carefully selected patients.

21.7.2.2 Electrogastrography

Electrogastrography (EGG) can be defined as the recording of myoelectric activity of the smooth muscle of the stomach and duodenum by means of electrodes attached to the abdominal skin. This method has the great advantage of allowing the study of myoelectric activity of the upper gastrointestinal tract totally noninvasively, and readily detects disturbance of muscle cell activity. We have recently used this method in patients with diffuse pseudo-Hirschsprung-like conditions to determine whether it is possible to detect abnormal myoelectric activity [34]. Our results indicate that persistent antral dysrhythmia in the fasting state with no dominant slow wave frequency is found in myopathic disorders and tachyarrhythmia in neuropathic disorders.

21.7.3 Histopathology

Definitive diagnosis of pseudo-Hirschsprung-like conditions in which the intrinsic innervation or smooth muscle of the gut are affected must be made histopathologically. Whilst suction rectal biopsy is satisfactory for determining the presence of aganglionosis, other neuropathic and myopathic disorders require full-thickness biopsy of the affected gut. Methods useful in the diagnosis of neuropathic disorder are discussed in detail in Chapter 12. The diagnosis of muscle disorder requires a variety of different techniques in addition to routinely processed paraffin wax-embedded tissue. These include both ultrastructural studies by electron microscopy and immunocytochemical studies of functional components of the muscle cells. It is beyond the scope of this chapter to discuss these methodologies in detail and the interested reader is referred to a review of the pathology of these conditions by Smith and Lake [19, 20].

21.8 Conclusions

Severe constipation presenting in a pseudo-Hirschsprung-like manner may have a variety of different causes which are best considered using a mechanistic approach and an understanding of the control mechanisms which result in ordered motor activity of the hindgut. Thus intrinsic disease of the enteric nervous system and intestinal smooth muscle must be considered. The hindgut may, however, operate within an abnormal humoral and endocrine environment which modifies its contractile activity. Other disordered modulatory influences such as that from the central nervous system via the extrinsic innervation or from psychosocial disorder are probably by far the commonest causes of a pseudo-Hirschsprung-like state.

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Anal Sphincter Achalasia and Ultrashort Hirschsprung's Disease

A. M. Holschneider and M. Kunst

22

22.1 Anal Sphincter Achalasia

Internal anal sphincter achalasia is defined as the inability of the internal anal sphincter to relax. The term achalasia is derived from the Greek word χαλααξ (loose, relaxed), in its negative form αχαλασξ (rigid, firm). The concept of anal sphincter achalasia originated with Fenwick [1], who believed a spasm of the internal anal sphincter muscle to be responsible for the development of the so-called idiopathic megarectum. Hurst [2] also recognized that the pathological substrate for the megarectum is situated in the internal anal sphincter, but at the same time demonstrated that the cause of the achalasia is not a spasm, but rather—similar to the situation in the lower esophageal segment—an inability of the sphincter to open. Several histology studies [3–6], electromanometric investigations [7, 8], roentgenological examinations [9], and immunocytochemical analyses [10–12] have shown that various causes can underlie the obstructive behavior of the internal anal sphincter.

Internal anal sphincter achalasia can be a disease restricted to the anal sphincter with normal innervation of the adjacent rectum, or may be part of Hirschsprung's disease (HD) and allied disorders. Internal anal sphincter achalasia has to be separated from HD with ultrashort segment aganglionosis from which it is sometimes difficult to distinguish as it is physiological to have some aganglionosis at the junction of anal canal and rectum. Aldridge and Campbell [13] demonstrated in 22 newborns and children without anorectal problems, a very short aganglionic zone extending cranial from the pectinate line. Its length measured in premature infants was 2 mm with regard to the plexus myentericus and 5 mm concerning the plexus submucosus. According to Aldridge and Campbell the aganglionic segment increased with age and extended to 3 and 6 mm in 2-year-old children. From earlier work of Müntefering et al. [5] and Fadda et al. [6], however, we know that the internal anal sphincter is not completely aganglionic, but that the density of ganglion cells diminishes from the proximal part of the sphincter to its distal end and varies between different age groups.

In addition, Tafazzoli et al. showed in their morphometric analysis [14] that neither ganglia nor ganglion cells of the anal canal show a uniform distribution pattern, but decrease continuously towards the anus. However, the lowest segments also contained nerve cells and were not aganglionic. These findings support the concept of a physiological hypoganglionosis of the anal canal and demonstrate segment-specific quantitative differences of the anorectal submucous plexus. In addition, the length of this physiological transition zone is genetically determined [15].

Etiology

The cause of neuronal intestinal malformations in general may be a disturbed neurotrophic influence on the intestinal enteric nerve system (ENS), especially its development and survival, with potential importance in functional differentiation [16]. According to Fujimoto et al. [17], laminin and collagen type IV appear to promote outgrowth of neurites from settled neural crest-derived cells and their maturation. Kapur et al. [18] postulated that a defect in non-neuroblastic mesenchyme impairs regionally neuroblast migration, whereas Parikh et al. [19] suggest that abnormal distribution of extracellular matrix proteins, including laminin in the smooth muscle layer of the muscularis externa, is responsible for the aganglionosis. Components of the extracellular matrix such as laminin may play an important role in enteric neural and glial development [20, 21]. The overabundance of laminin enables the crest-derived cells to colonize the bowel and to develop as enteric neurons. Vaos [22] also suggested, following quantitative assessment of the stage of neuronal maturation in the developing human gut, that any alteration in the fetal gut microenvironment may seriously affect the normal development of a multipotential precursor cell population, resulting in various congenital anomalies of the myenteric plexus. As the internal sphincter is scarcely evident before 12 weeks of gestation and becomes well-formed after 28–30 weeks of gestation, neuronal malformations of the sphincter and adjacent rectum do not occur before this stage of development [23].

22.2 Ultrashort Hirschsprung's Disease

Ultrashort HD is rare and was first described by Davidson and Bauer in 1958 [24]. Meier-Ruge collected 75 cases in 14 years [25–27]. The first symptom of ultrashort HD to develop is chronic constipation in the second half of the first year of life. The aganglionic segment has an extension of 1–3 mm and is characterized by an increase in acetylcholinesterase (AChE) activity in parasympathetic nerve fibers of the muscularis mucosae and the musculus corrugator cutis ani (MCCA) [27]. A similar increase in ACHE in the nerve fibers of the lamina propria mucosae is, however, absent. Strip biopsies including mucosa from the dentate line to the rectum often show the transition from the aganglionic to the innervated mucosa. The average incidence of ultrashort HD has been estimated as 11–14% in relation to all aganglionoses and 6–8% of all inborn innervation failures of the colon. The sex ratio is five males to one female (Figs. 22.1 and 22.2).

Nissan and Bar-Moar [28] were only able to find 38 patients in the literature who had a histologically confirmed ultrashort segment. Duhamel and Duhamel [29] and Madsen [30], in contrast, found ultrashort aganglio-

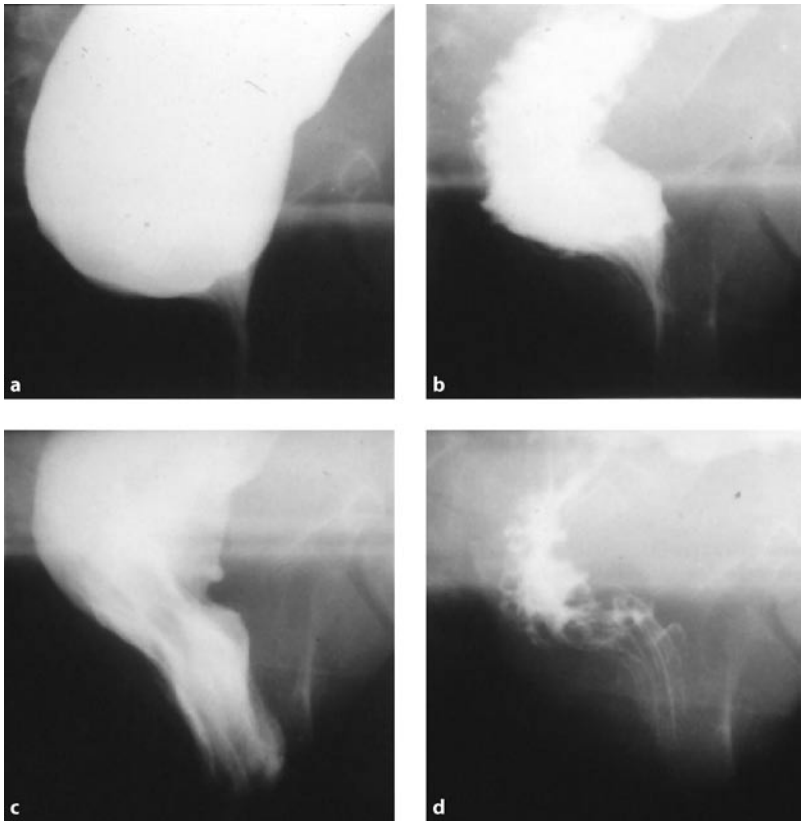

Fig. 22.1a–d Normal defecography. Note the stretching and re-establishment of the anorectal angle (**c**) and the internal sphincter relaxation (*IR*) during defecation

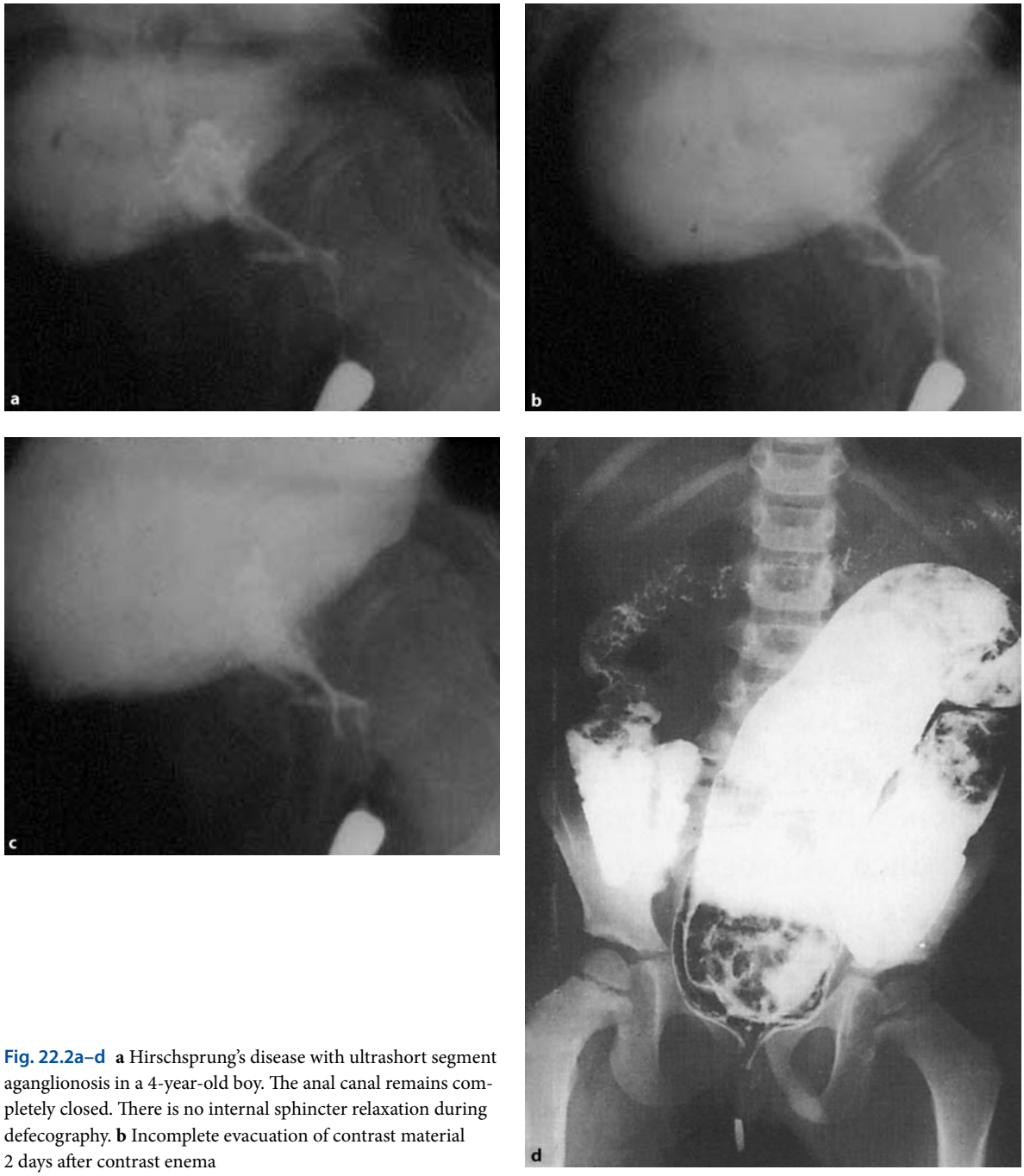

Fig. 22.2a–d **a** Hirschsprung's disease with ultrashort segment aganglionosis in a 4-year-old boy. The anal canal remains completely closed. There is no internal sphincter relaxation during defecography. **b** Incomplete evacuation of contrast material 2 days after contrast enema

nosis in 25% of their patients with so-called idiopathic megacolon. In 1965, Duhamel found megacolon with an ultrashort segment in 10 of 22 patients in whom he had performed sphincteromyectomy for anal sphincter achalasia [3]. Rehbein, in 1969, described aganglionosis which simply extended to the sphincteric region and led to megarectum in 14.3% of 265 patients [31]. Freeman [32] gives a similar assessment, while Clayden and Lawson [33] found an ultrashort segment in only 10 (9%) of 106 children with chronic constipation. These variations in data are due to heterogeneous patient populations. When chronic constipation was used as the basic disease, we found megacolon with an ultrashort segment in only 2.6% of our patients [34, 35]. Taking the presence of HD and allied disorders as a basis, however, the disorder was more common and was present in 10% to 15% of our patients.

However, due to the different length of the transition zone, the extension of the involved segment is difficult to define. Recent studies by Shimotake et al. [15] have shown that the length of the transition zone is genetically determined in RET or SOX10 mutation.

The length of the aganglionic segment for it to be called ultrashort varies from 2 to 3 cm [36] to 10 cm [28]. We believe that the term ultrashort should be restricted to the lowermost 2–4 cm of the anal channel and rectum below the pelvic floor. But there might be rare cases with the possible presence of both ultrashort HD and proximal intestinal neuronal dysplasia (IND) [37].

22.3 Classification of Anal Sphincter Achalasia

The diagnostic problems of anal sphincter achalasia are discussed in Chapter 12, Section 12.2 Physiology of the Internal Anal Sphincter.

22.3.1 Functional (Neurovegetative–Psychogenic) Anal Sphincter Achalasia

The most frequent type of anal sphincter achalasia (95% of cases) is functional or psychogenic (Figs. 22.3–22.6).

22.3.2 Myogenic Anal Sphincter Achalasia

Myogenic anal sphincter achalasia is generally a result of fibrosis of the internal and/or external anal sphincter or desmosis (Figs. 22.4, 22.7–22.10)

22.3.3 Neurogenic Anal Sphincter Achalasia

Neurogenic internal anal sphincter achalasia (Figs. 22.11 and 22.12) can be defined as the neurogenic inability of the internal anal sphincter to relax. This occurs

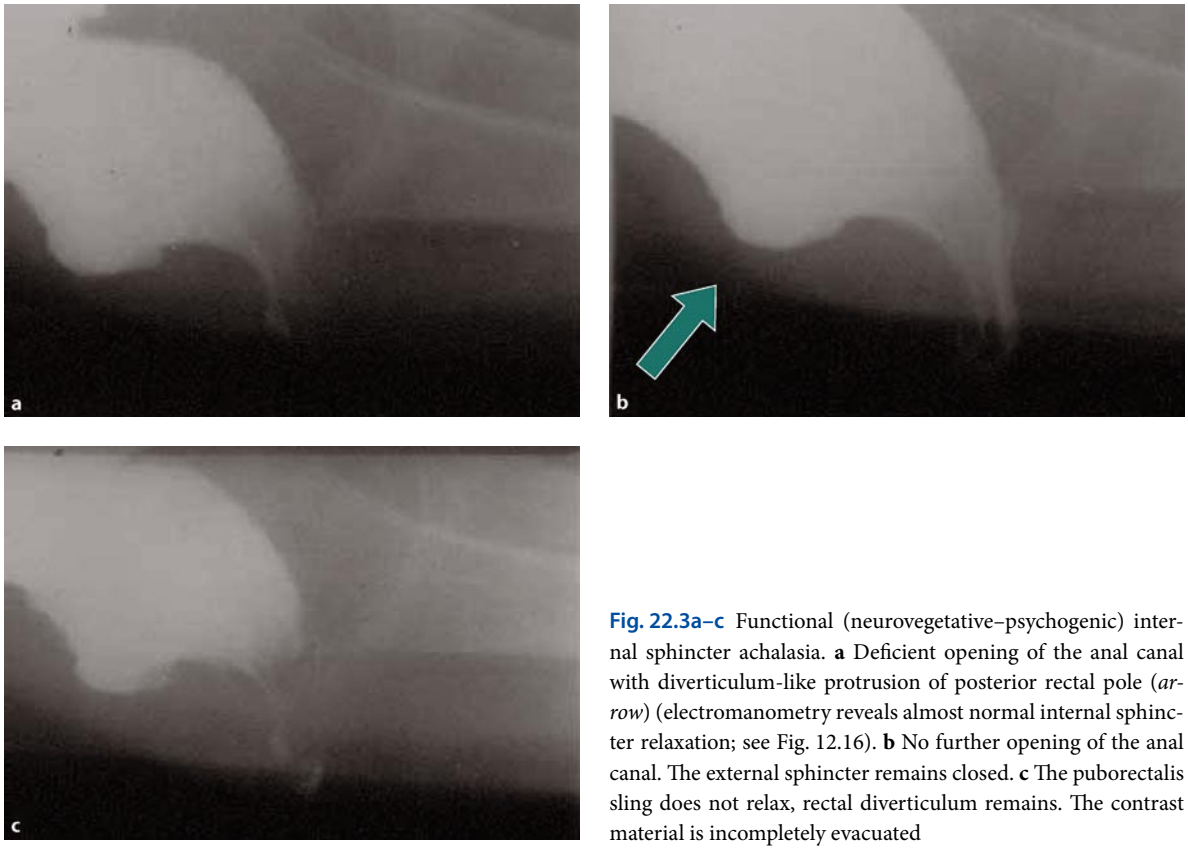

Fig. 22.3a–c Functional (neurovegetative–psychogenic) internal sphincter achalasia. **a** Deficient opening of the anal canal with diverticulum-like protrusion of posterior rectal pole (*arrow*) (electromanometry reveals almost normal internal sphincter relaxation; see Fig. 12.16). **b** No further opening of the anal canal. The external sphincter remains closed. **c** The puborectalis sling does not relax, rectal diverticulum remains. The contrast material is incompletely evacuated

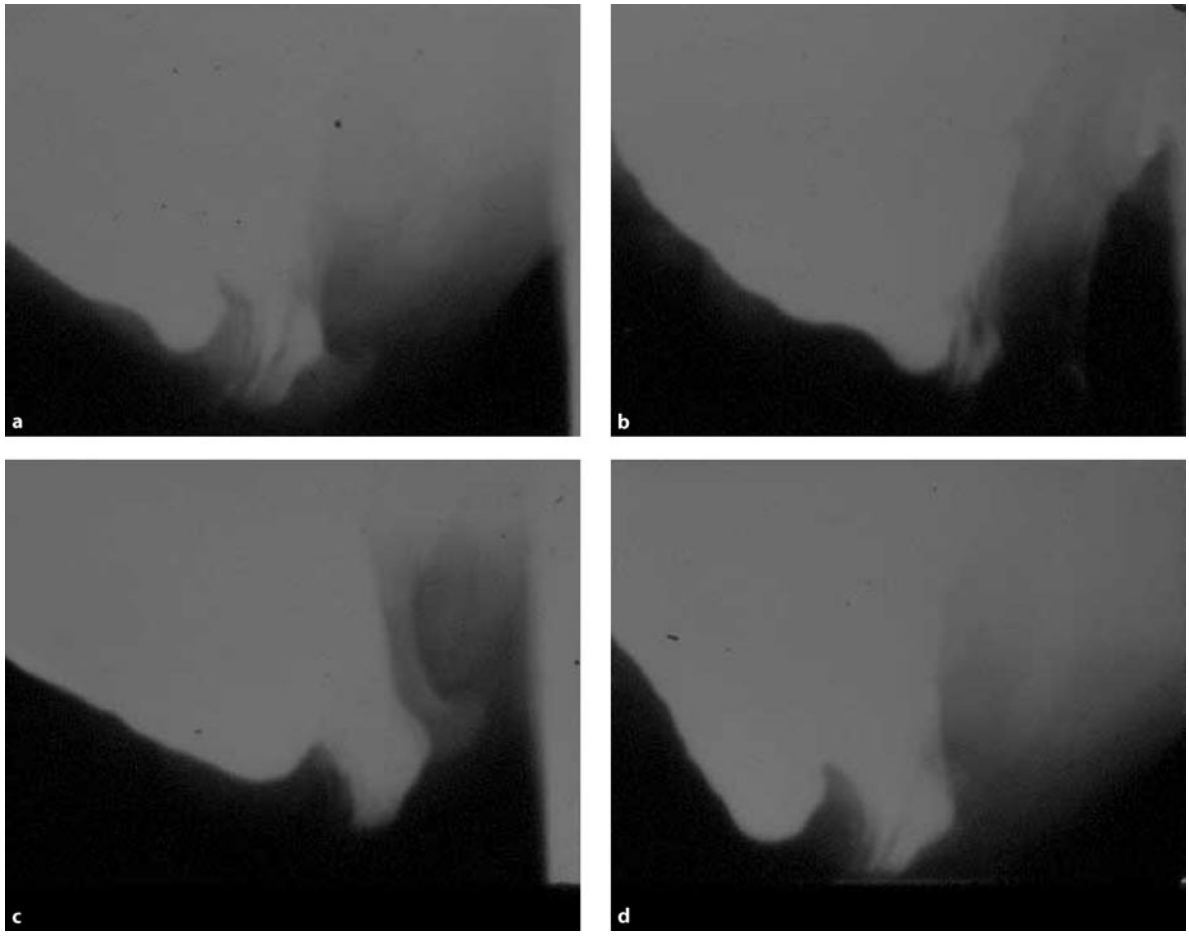

Fig. 22.4a–d Functional (neurovegetative–psychogenic) anal sphincter achalasia. Wide opening of the internal anal sphincter during relaxation, but the external anal sphincter and the pelvic floor muscles remain contracted. Typical case of rectum sphincter dyssynergia with huge rectal diverticulum

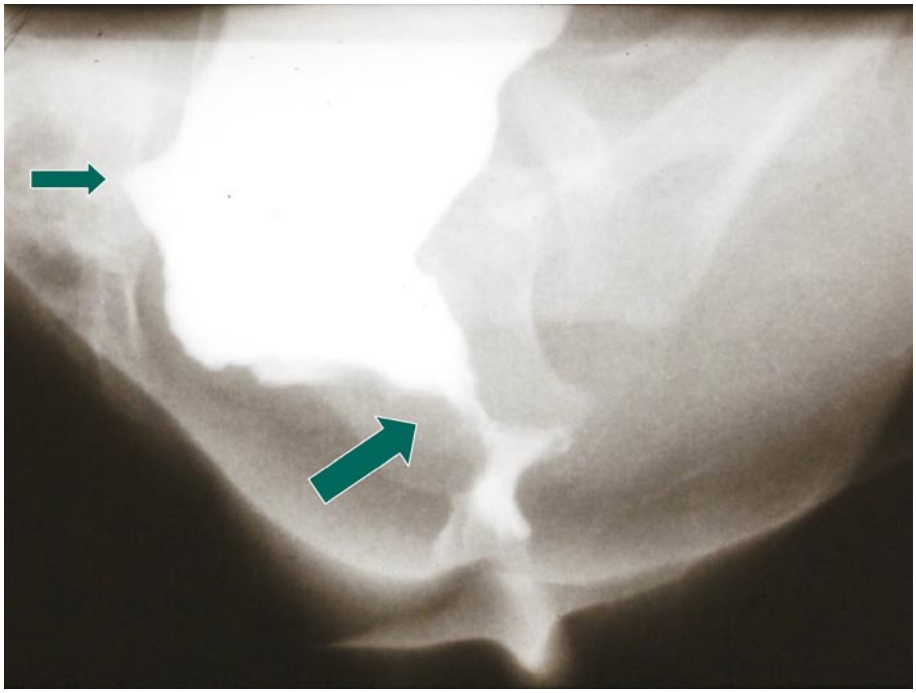

Fig. 22.5 Radiograph of external sphincter contraction with internal sphincter relaxation (*large arrow*) and rectal diverticulum (*small arrow*) in functional anal sphincter achalasia

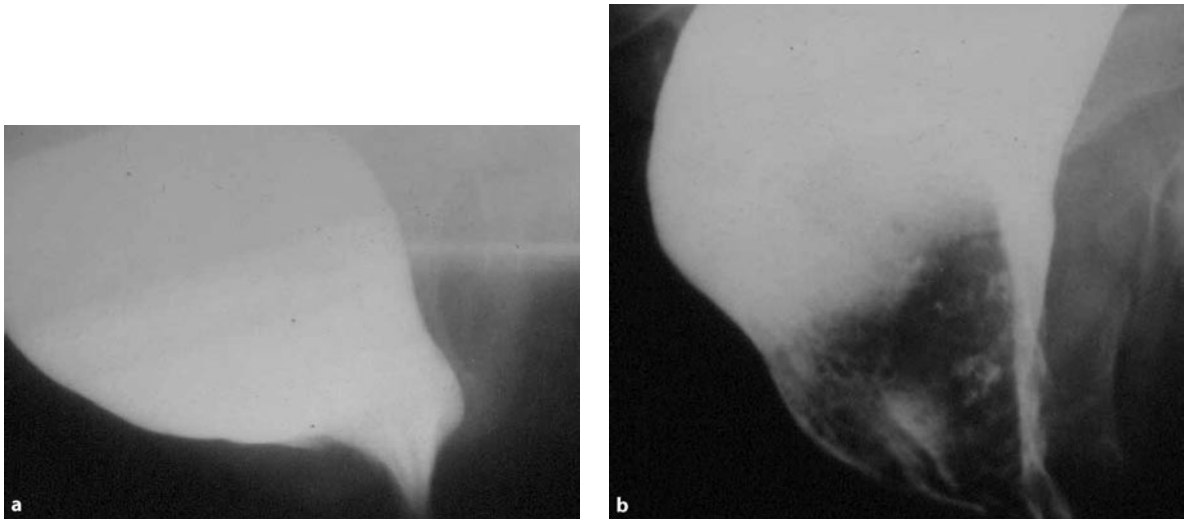

Fig. 22.6a, b Two different types of defecography in two children with chronic constipation and IND: **a** moderate internal sphincter achalasia; **b** achalasia with fecolith

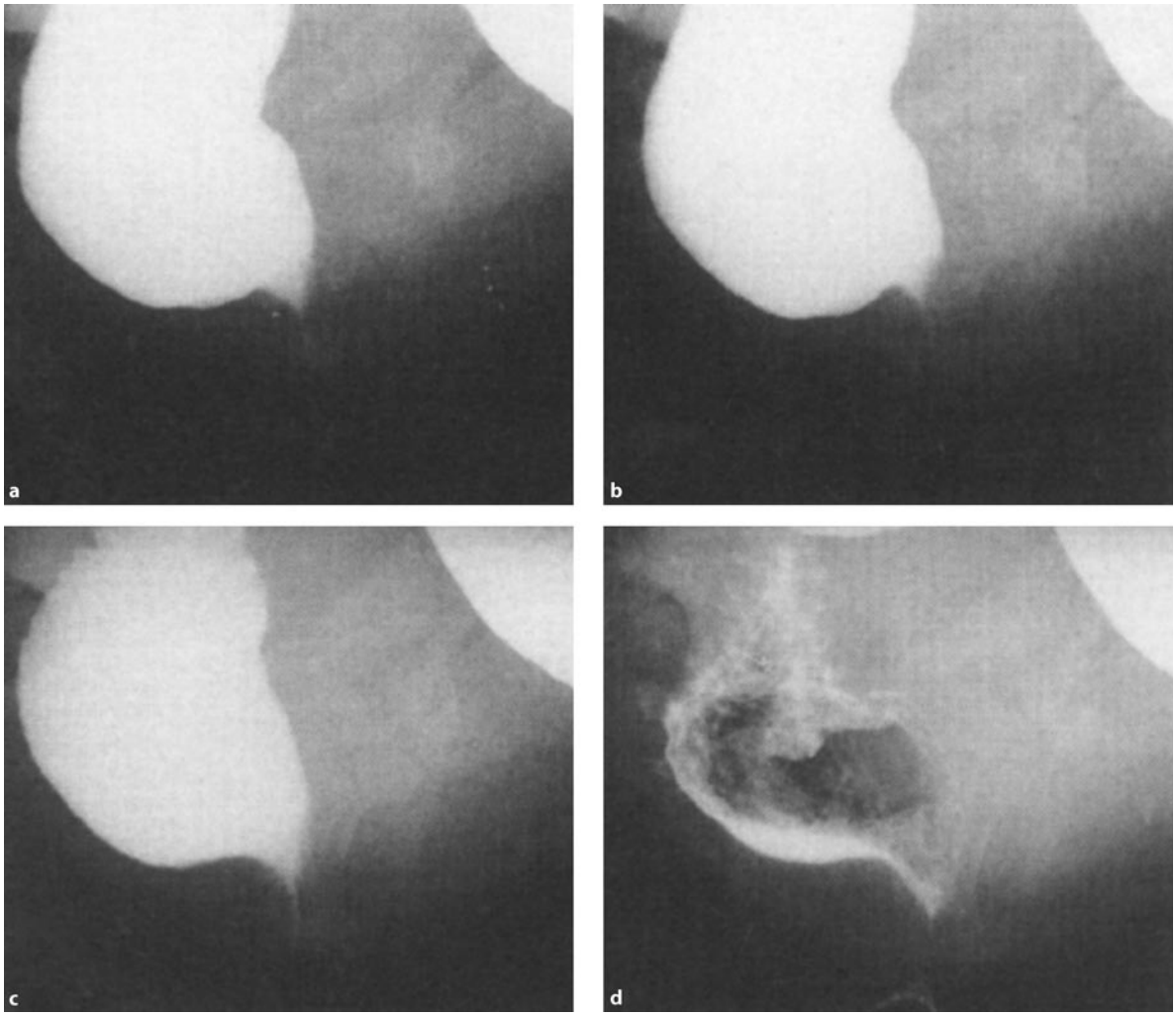

Fig. 22.7a–d Myogenic anal sphincter achalasia. **a–c** During defecography there is only minimal opening of the internal anal sphincter. **d** No evacuation of fecolith but of some contrast material 6 hours later. (electromanometry shows only minimal internal sphincter relaxation; see Fig. 12.17)

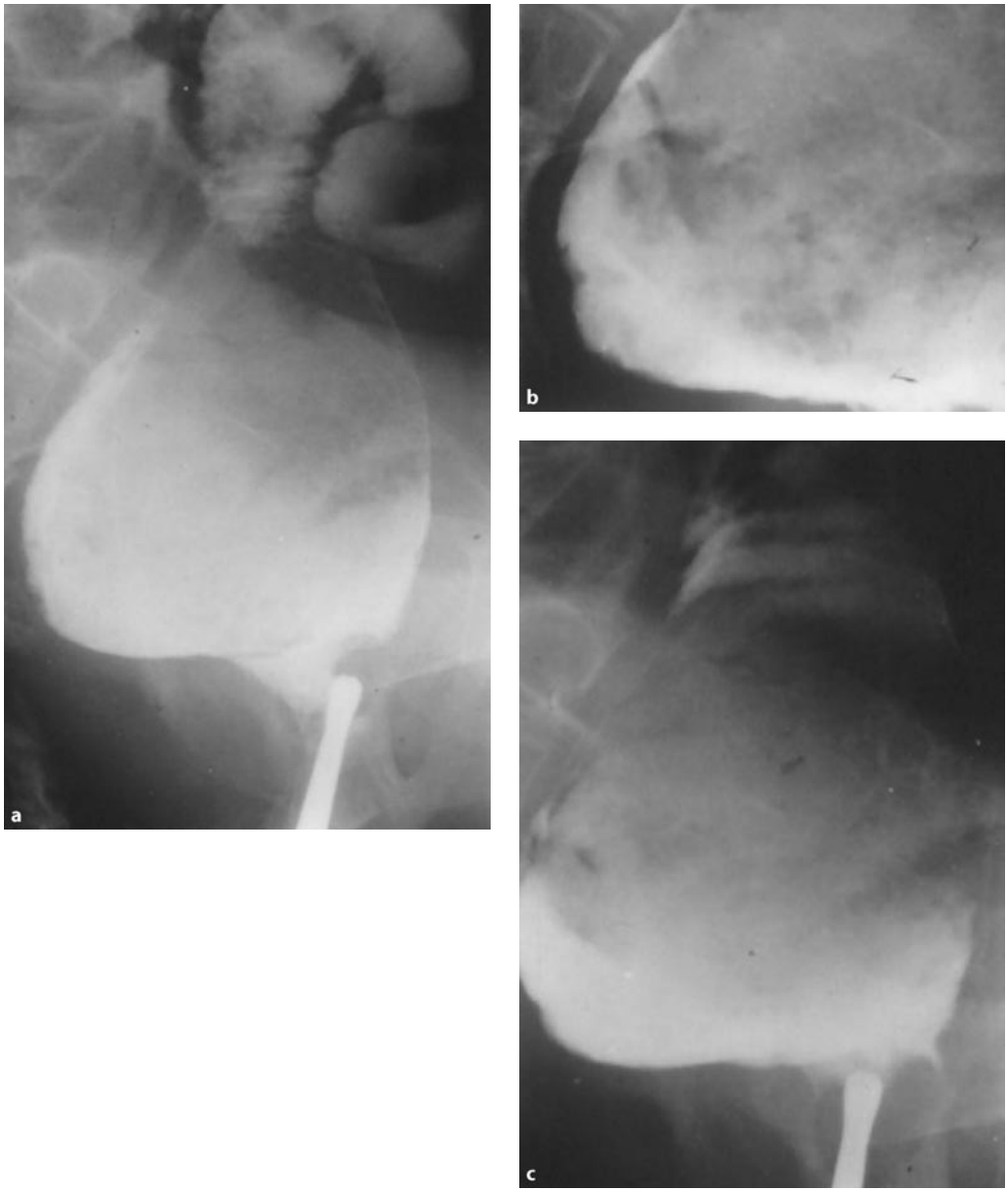

Fig. 22.8a–c Myogenic internal anal sphincter achalasia with megarectum and dilatation of the dorsal rectum. **a–c** There is only minimal opening of the anal canal during defecation

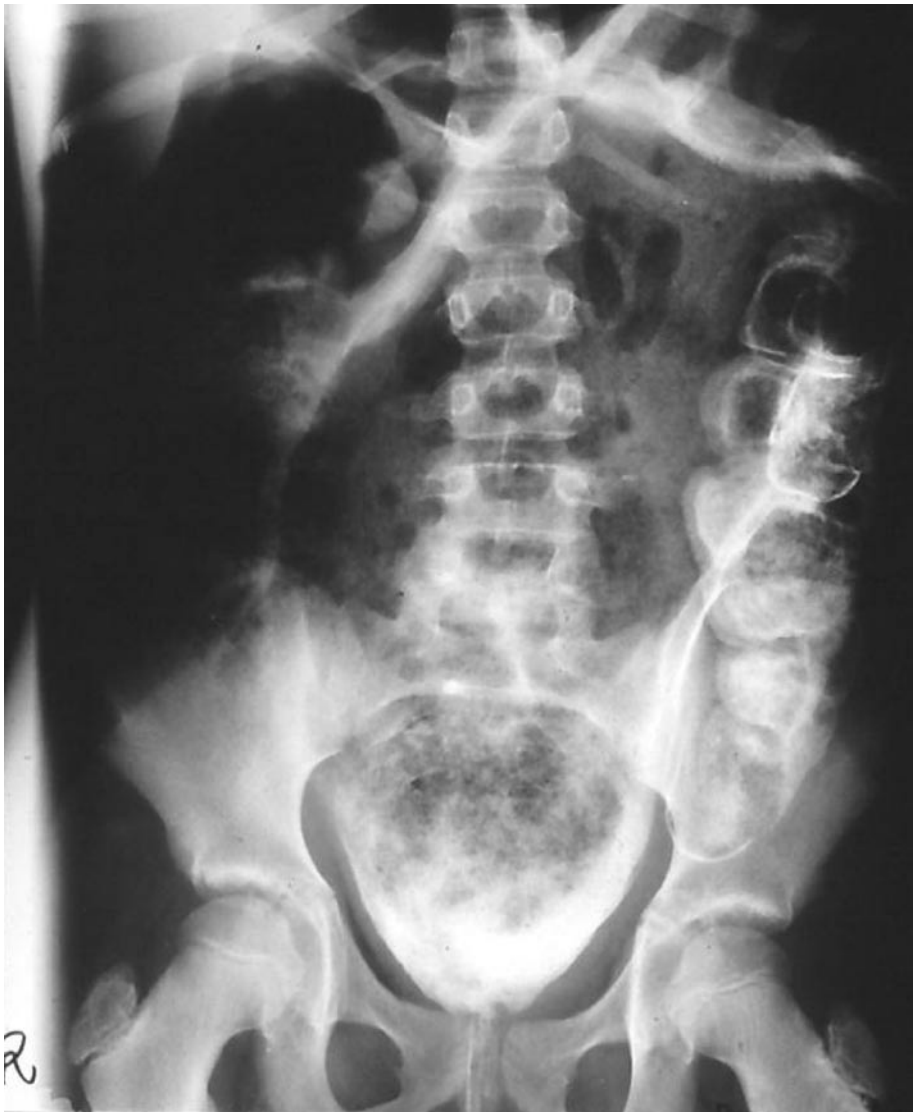

Fig. 22.9 Defecography in myogenic anal sphincter achalasia. Ther was incomplete evacuation of contrast material 36 hours later. Note the huge megarectum and spasticity of the colon descendens. The megarectum needed resection

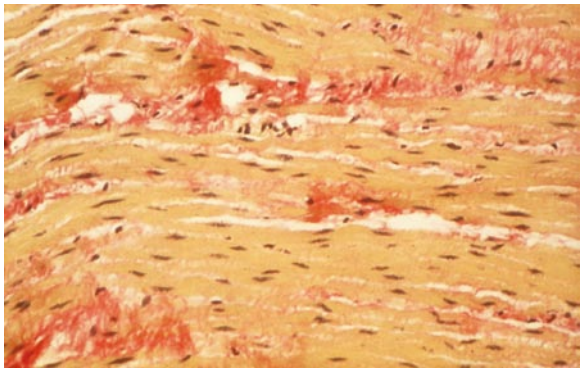

Fig. 22.10 Fibrosis of the internal anal sphincter in myogenic anal sphincter achalasia (van Gieson's staining)

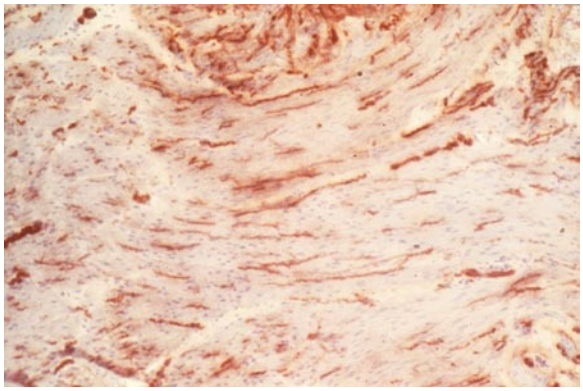

Fig. 22.11 Increased AChE staining in ultrashort Hirschsprung's disease

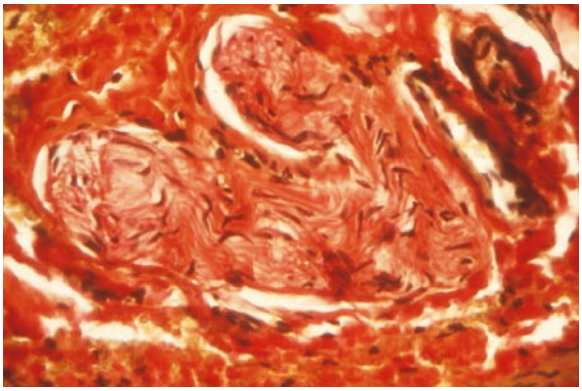

Fig. 22.12 Giant nerve fiber in neurogenic anal sphincter achalasia (H&E staining)

regularly in HD and often in combination with other forms of neuronal intestinal malformations such as hypoganglionosis, hypogenesis, immaturity of ganglion cells, and some true IND. However, it may also occur as a separate entity when the intestinal neuronal malformation is restricted to the internal anal sphincter (Fig. 22.13).

Neurogenic anal sphincter achalasia as a separate entity is very rare. Kubota et al. [38] demonstrated that the colon or anal sphincter from congenitally aganglionic rats is innervated by intrinsic cholinergic excitatory and noncholinergic inhibitory nerves. In addition, many authors have reported that a lack of nonadrenergic–noncholinergic (NANC) nerve fibers is responsible for the inability of the sphincter to relax [39–43]. The same seems to be true for a deficiency of vasoactive intestinal polypeptide (VIP) which has also been suggested to be an inhibitory neurotransmitter for internal sphincter relaxation [44–46]. Rattan et al. [47, 48] demonstrated the possibility of nitric oxide as an inhibitory mediator of neurally mediated relaxation of the internal anal sphincter in the opossum. The authors suggested that it might be possible that a part of the NANC neuron-mediated relaxation in the sphincter occurs via VIP or NO release from the inhibitory neurons. Vanderwinden et al. [49] observed NO synthase was selectively absent from the plexus area and from the musculature of the aganglionic segments, whereas moderate staining was observed in the hypertrophied nerve bundles in the submucosa. In contrast, in the ganglionic segment NO synthase was abundantly present in a pattern similar to that of normal colon. These findings suggest the involvement of NO in the pathophysiology of HD.

Kobayashi et al. consider that a developmental abnormality of innervation of the muscle coat of the gut is most likely responsible for the spasticity of the aganglionic segment [50]. The same authors reported complex neural abnormalities in internal anal sphincter achalasia as well, including prominent AChE-positive nerves fibers and absent or scanty NAPDH-diaphorase activity [12]. Furthermore, Fujimoto et al. observed marked differences in the motor activities of the circular muscle of the rectum and the internal anal sphincter [11]. The various peptide-containing nerves were increased in internal sphincter achalasia compared to normal controls and the circular muscle coat of children with HD. In detail, fibers immunoreactive for neuropeptide Y were abundant in patients with IAS achalasia, and VIP immunoreactivity was also increased. In the sphincter muscles of the controls, however, only scanty VIP-positive nerve fibers were present. Nerves containing substance P were significantly more widely distributed in achalasia patients than VIP nerves, and much more widely distributed than in normal controls. Nerves containing substance P and VIP were reduced in the circular muscle coat of aganglionic segments in HD. Hutson et al. [51] also observed markedly reduced substance P and VIP fibers in patients with severe chronic constipation, and excluded HD.

These findings suggest that the pathophysiology of internal anal sphincter achalasia is different from that of the aganglionic segment in HD. Neurogenic anal sphincter achalasia should therefore be regarded as a distinct clinical and pathophysiological entity occurring in addition to HD [52].

In the internal anal sphincter of patients with IND Müntefering et al. [5] and Fadda et al. [6] found scanty ganglion and Schwann cells and a moderate increase in ACE-positive nerve fibers. Kobayashi et al. [53] observed numbers of lactate dehydrogenase-positive neuron cells as also reported by Meier-Ruge [25].

22.3.4 Cells of Cajal

In addition, Taguchi et al. observed an abnormal distribution of c-kit-positive cells which could be responsible for severe constipation and severe enterocolitis [54]. Interstitial cells of Cajal (ICCs) are pacemaker cells in the smooth muscle of the gut. It has been suggested

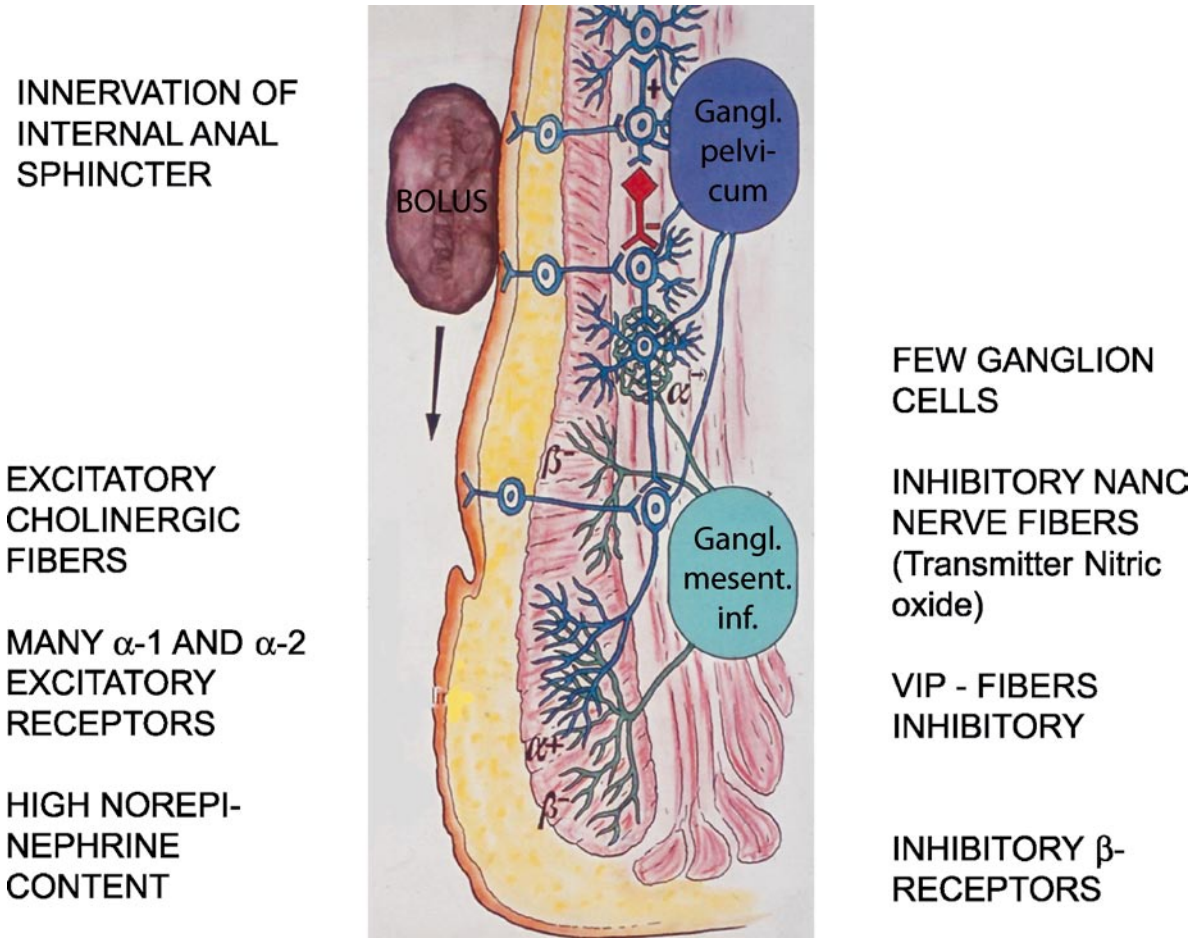

Fig. 22.13 Schematic drawing of innervation of the internal anal sphincter. Note: alpha-stimulating, beta-relaxing receptors and especially NANC-relaxing nerve fibers. Relaxation is also mediated by VIP fibers. In contrast, in the proximal bowel there are beta- and alpha-relaxing influences

that ICCs in the internal anal sphincter mediate the inhibitory innervation of the rectoanal reflexes. Piotrowska et al. investigated the distribution of ICCs in the normal internal anal sphincter and in the internal anal sphincter of children diagnosed with internal anal sphincter achalasia and HD. Altered distribution of ICCs in the internal sphincter in internal anal sphincter achalasia and HD may contribute to the motility dysfunction in these patients [55].

22.3.5 Desmosis

The peristaltic movement of the gut is a function of the alternating contraction and relaxation of the circular and longitudinal muscles. This movement is induced by a tendon-like connective-tissue net (TCTN) in the circular and longitudinal muscles, which are both rooted in a connective-tissue plexus layer (CTPL). In children with a therapy-resistant aperistaltic or hypoperistaltic syndrome who had normally developed ENS, a lack of the TCTN in the muscularis propria was observed. Independent of a well-developed ENS a lack of TCTN in longitudinal and circular muscles and missing CTPL (aplastic desmosis) abolishes the coordinated peristaltic movement of the gut. An isolated lack of the CTPL in the myenteric plexus (hypoplastic desmosis) results in a hypoperistaltic syndrome. This could probably also affect the internal anal sphincter.

22.4 Symptoms

The most prominent symptoms of neurogenic anal sphincter achalasia are recurrent constipation and enterocolitis. Both are also the most common postoperative complications. The constipation can reappear shortly after surgery, or after an interval of weeks to months, even

years. It begins insidiously with increasingly long defecation intervals, and the child is not brought to examination until the parents notice the greatly dilated abdomen, loss of appetite, colicky abdominal pain and occasional vomiting. The parents of two of our patients consulted a physician only after the defecational interval had reached 14 days and more. At that time, we found a maximally dilated rectum with inspissated fecal masses which required manual removal. In myogenic and even more in psychogenic anal sphincter achalasia, overflow incontinence is the leading symptom. The children retain their stool by voluntary contraction and avoid defecation.

Roentgenographic defecography in neurogenic anal sphincter achalasia reveals a dilated rectum with a narrow sphincter, which appears to act obstructively even during defecation. Even definite evagination of the posterior rectal pole during defecation may occur. At rectal examination, a hypertonic sphincter may occasionally be palpated; however, sphincter tone is not always increased. In every patient, one traverses a shortened anal canal into a wide ampulla filled with substantial quantities of stool.

In infants and children up to 2 years of age, enterocolitis is superimposed on the constipation. Dilatation of the sphincter during rectal palpation leads to the typical explosive evacuation of partly firm but primarily liquid stool with marked flatulence. When a rectal tube is inserted, large quantities of air and watery stool flow out as soon as the tube is passed beyond the sphincter or the ultrashort aganglionic segment. The bloated abdomen collapses. In all probability, the enterocolitis is the result of retention with ischemic over-distension of the bowel, chemical damage to the mucosa and bacterial ingrowth, always secondary in nature. Later radiographs, at 6 h, 12 h or 24 h after the contrast enema, give further information on bowel motility and the function of the sphincter (see Figs. 12.29 and 12.30).

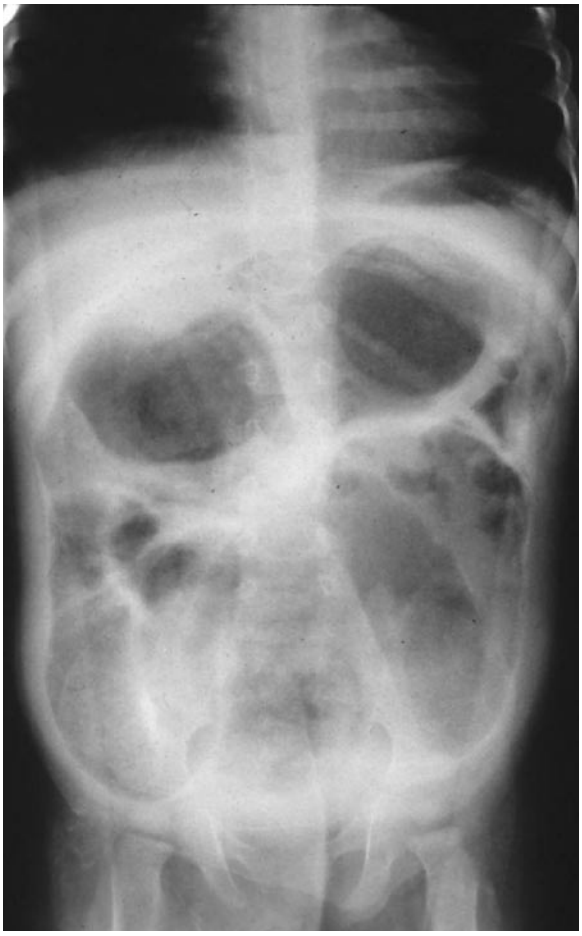

Fig. 22.14 Plain radiograph shows huge extended colon loops in neurogenic anal sphincter achalasia after Rehbein's procedure

22.5 Anal Sphincter Achalasia in Combination with Hirschsprung's Disease

HD is always associated with anal sphincter achalasia and should therefore be treated simultaneously with resection of the aganglionic segment. Sphincteromyotomy in Duhamel's procedure and sphincter dilatation in Rehbein's technique [55] are therefore ongoing parts of these procedures. However, the achalasia can reappear years after the resection of the aganglionic segment (Figs. 22.14–22.17).

The deeper the anastomosis in Rehbein's anterior resection, the weaker becomes the tone of the internal anal sphincter muscle. Holschneider et al. reported in 1980 the results of an international study on 427 patients with HD [8, 56]. Sphincter dilatation had to be performed postoperatively in 31.9% and sphincteromyectomy in 12.9% of the children due to recurrent anal sphincter achalasia (Table 22.1). There was no significant difference between the different operative techniques used. In addition, the obstructive functional disturbance produced by the narrow segment varies in its severity. Patients have been reported in whom long aganglionic segments or extensive neuronal colonic dysplasia are present for years without producing severe symptoms of subileus or ileus, so that conservative treatment is adequate, and conversely, patients are known in whom megacolon with an ultrashort segment has led to ileus soon after delivery. The same is true for the variability of symptoms in anal sphincter achalasia

Numerous theories have been developed to explain these varied manifestations, but no definitive explanation has been found. The reason for this recurrence of internal anal sphincter achalasia is not clear. Probably a varying individual expression of peptidergic nerves containing VIP, substance P, or enkephalin and gastrin-releasing peptide or glial fibrillary acidic protein, or an abnormal expression of neuronal cell adhesion molecule or an absence of

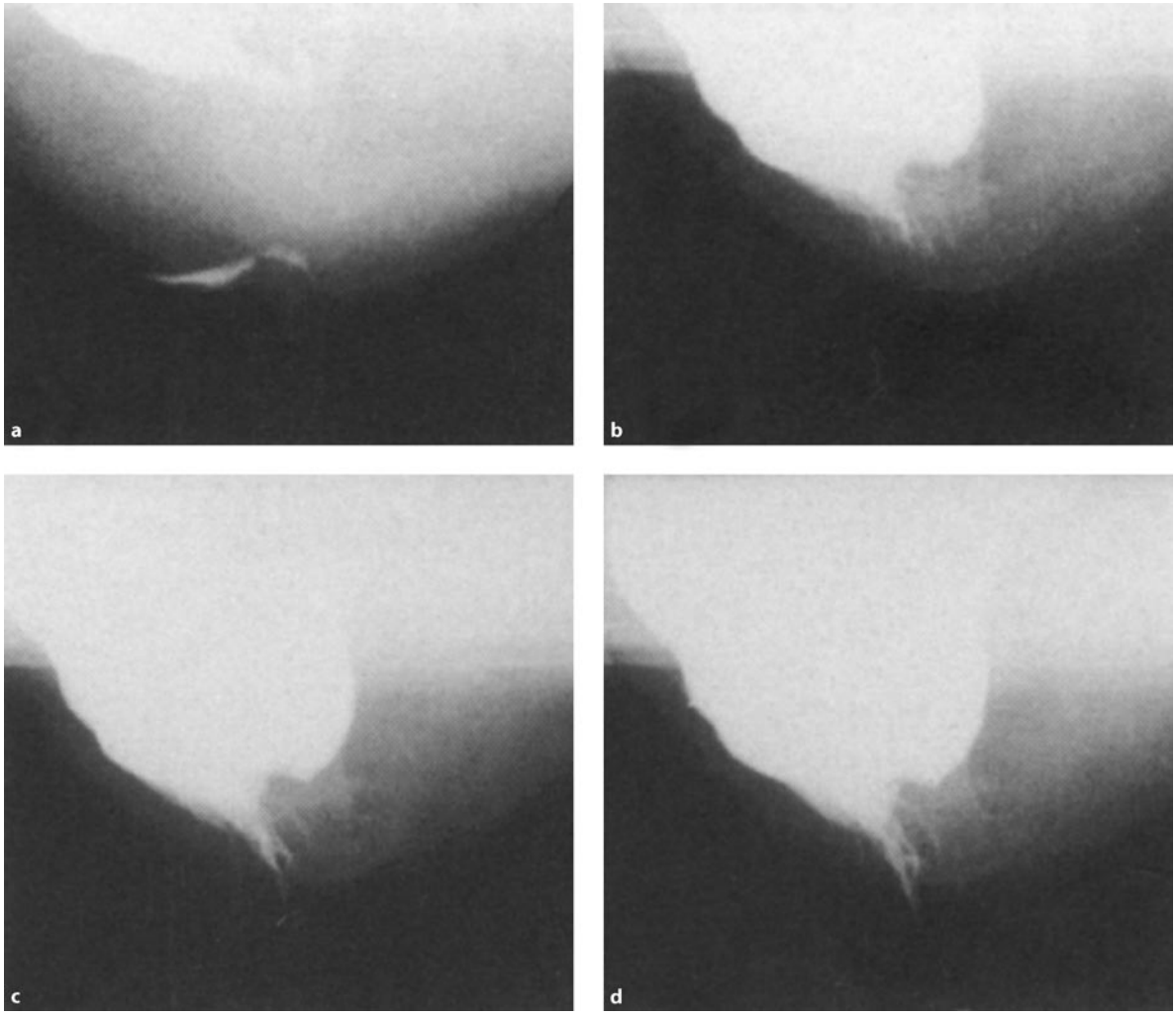

Fig. 22.15a–d Defecography in neurogenic anal sphincter achalasia 2 years after Rehbein's procedure. There is no opening of the internal anal sphincter during the different steps of defecation. Dilatation of the rectum can be seen with evagination of the posterior rectal pole

ICCs might be responsible for the differences in severity of symptoms in HD and allied disorders [55, 57].

Since the report of Richardson [58], we know that the cause of the "spasticity" of the narrow segment lies in the loss of the NANC inhibitory regulating system. Since according to Richardson [58], the presence of a few ganglion cells in the hypoganglionic bowel segment is sufficient to produce a certain degree of relaxation, the presence of a few ganglion cells or a few fibers of the extrinsic innervation of the lower gastrointestinal tract alone could explain the variability in the clinical picture of HD and allied intestinal disorders.

Concerning IND type B, Munakata et al. [59] observed recently that in the IND colon the number of myenteric ganglia is decreased. There were fewer and morphologically abnormal synaptic vesicles, identical with peptidergic nerves, in the circular muscle layer, while there were many synaptic vesicles in the longitudinal muscle coat. In healthy subjects, however, synaptic vesicles were much more numerous in the circular muscle than in the longitudinal muscle layers. The innervation of circular muscle layers of the IND colon by met-enk-, GRP-, and SP-immunoreactive fibers was reduced, but longitudinal muscles were more strongly innervated than in the normal colon. The authors concluded that not only a varying alteration of NANC neurons but also an imbalance of peptidergic innervation in both muscle layers of the colon may be the underlying cause for abnormal peristalsis of IND colon. This might also be the reason why Ure et al. were unable to find any relationship between clinical outcome, transit-time studies, and the histological morphology of the affected bowel segment [60].

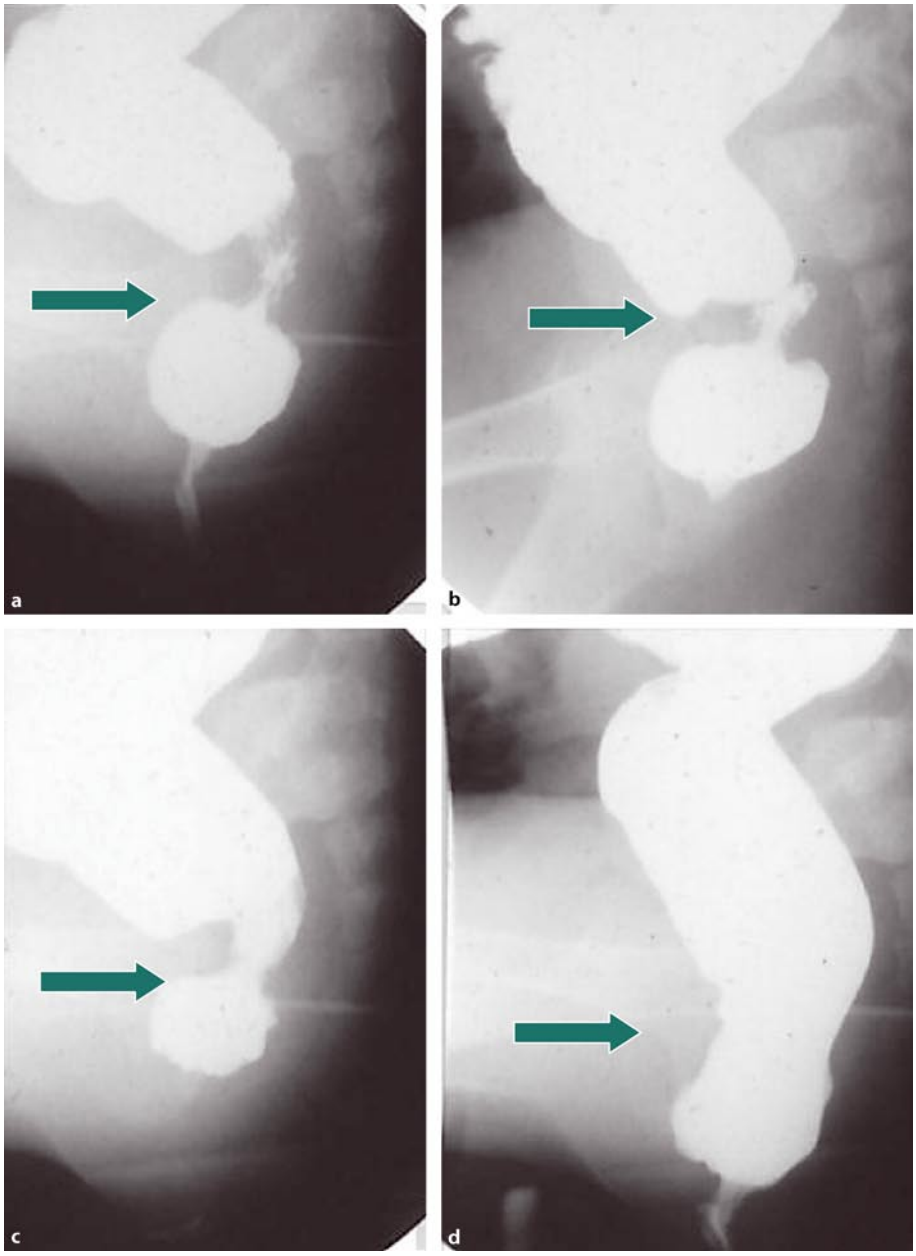

Fig. 22.16a–d Neurogenic anal sphincter achalasia and narrowing of the endorectal pulled-through colon by the rectal muscle coat 3 years after endorectal pull-through by the Soave procedure. The stenosis widens during defecation (*arrows*), but the proximal colon remains dilated and the anal sphincter achalasia persists

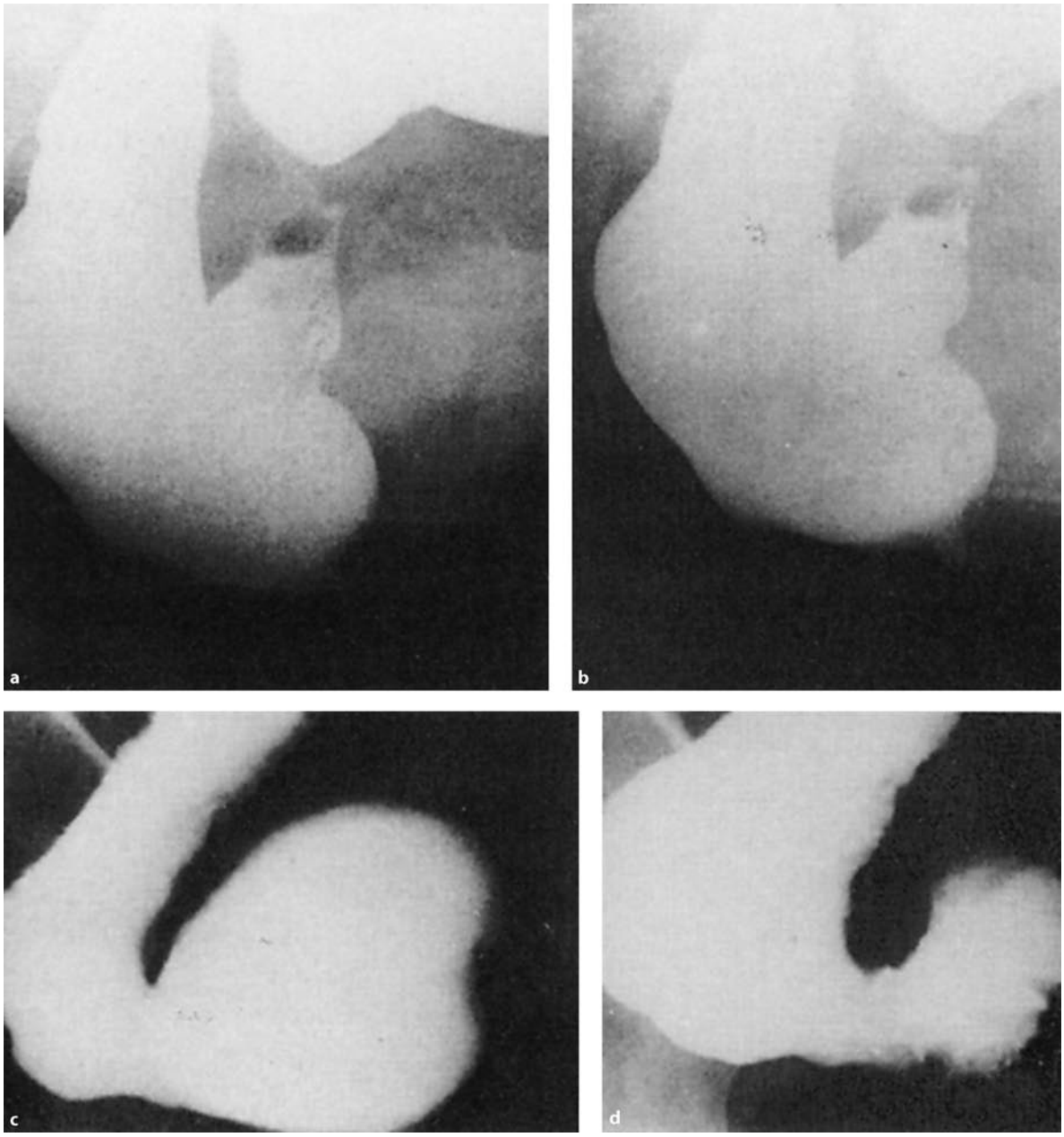

Fig. 22.17a–d Neurogenic anal sphincter achalasia 3-years after Duhamel's operation for Hirschsprung's disease. **a**, **b** no opening of the anal channel during defecation, but the puborectalis loop relaxes. **c**, **d** Undulation during defecation: inadequate evacuational function due to shifting of the contrast material from the dilated rectal pouch into the dilated colon

Table 22.1 Frequency of postoperative sphincter dilatations and sphincteromyectomies in 427 patients with Hirschsprung's disease from reference [8]

Surgical treatment	Sphincter dilatation	Sphincteromyectomy
Rehbein	87 (49.4%)	24 (13.7%)
Swenson	18 (22.5%)	12 (15.0%)
Soave	13 (12.3%)	12 (11.4%)
Duhamel	11 (24.5%)	6 (13.0%)
Others	2	0
Total	131 (31.3%)	54 (12.9%)

Wedel et al. [61] recently performed electron-microscopic investigations of specimens from patients with HD. Varying degrees of extramural, polyaxonal, myelinated nerve fibers invading the aganglionic segment were observed. This could be a primary preoperative or secondary postoperative phenomenon. In any case, these nerve fibers could also be responsible for the varying degrees of severity of the disease and in all probability of postoperative reinnervation of the internal anal sphincter.

22.6 Reinnervation of the Internal Anal Sphincter

In some cases reinnervation of the internal anal sphincter after resection of the aganglionic segment postoperatively may also lead to an improvement in chronic constipation and enterocolitis (Figs. 22.18–22.20).

In studies of patients who underwent Rehbein's anterior resection we observed no differences in the frequency or morphology of the internal sphincter relaxations whether the balloon was insufflated above or below the anastomosis [62]. Ikeda et al. [63] were able to confirm the development of internal sphincter relaxation after a modified Duhamel operation. Mishalanay and Woolley [64] found a normal anorectal reflex in 10% of patients postoperatively which seemed not to be related to clinical fecal continence. Suzuki et al. [65] found a normal rectosphincteric reflex response in patients with postrectal myotomy or myectomy. They found no reflex response in patients who had undergone Swenson's procedure. Meunier and Mollard [66] were also unable to demonstrate internal sphincter relaxations in three patients up to 8 years after Swenson's procedure and in three patients up to 9 years after Soave's operation. In an international study, we observed the reappearance of internal sphincter relaxation after definitive operation for HD in 26.2% to 48.6% of patients depending on the technique used [8, 56]. There was no relationship between internal sphincter relaxations appearing postoperatively and the occurrence of postoperative enterocolitis or encopresis. Yamamoto et al. [67], Schweizer et al. [68] and Nagasaki et al. [69] observed normal internal sphincter relaxations after Duhamel's, Rehbein's, Ikeda's and Swenson's procedures. Varma and Stephens [70] found reflex contraction of the external anal sphincter to be absent in patients who had undergone Swenson's rectosigmoidectomy, while this rectoanal reflex remained intact in patients after Duhamel's colorectal anastomosis.

Persistent anal sphincter achalasia in HD thus depends on three factors:

1. Individual variations in residual innervation of the aganglionic or dysganglionic segment as a result of variability in the extension of the extrinsic nervous system, or variations in the extent of the hypo-, dysor aganglionosis, or absence of ICCs.
2. Traumatization of the vascular and nerve supply of the residual aganglionic and the proximal normal bowel segments, and the hypoganglionic or dysganglionic bowel segment after its shift into the small pelvis.
3. Regeneration of the rectoanal reflex mechanisms.

22.7 Diagnosis

In order to meaningfully diagnose postoperative anal sphincter achalasia, the bowel must first be emptied completely. For this reason, we introduce a small rectal tube at 4-hour intervals and irrigate the anal canal and rectum with physiological saline solution. At the same time, the child is fed only fluids and given a mild laxative. It is occasionally necessary to manually evacuate the inspissated fecal masses under anesthesia.

When the rectum is completely empty, anorectal manometry is performed as a screening examination. Typical findings in the anal sphincter achalasia of HD are the absence of internal sphincter relaxations with no signs of any maturational process, an elevated anorectal resting pressure profile, though not in all patients, and a high compliance in the greatly dilated rectum with essentially no adaptation reaction, and the persistence of mass contractions. In psychogenic anal sphincter achalasia, internal anal sphincter relaxation is not impaired but is interrupted by voluntary contractions of the striated pelvic floor muscles and sphincters. Myogenic achalasia shows rudimentary sphincter relaxations with reduced amplitude and shorter duration.

Since the degree of rectal dilatation cannot be determined using anorectal manometry, and thus the question of further resection cannot be resolved by this means, we also perform roentgenographic defecography. In typical anal sphincter achalasia, the internal anal sphincter does not open on the defecogram. The anal canal remains closed, and the posterior pole of the rectum arches up, pouch-like, over the anal canal. A radiographic contrast

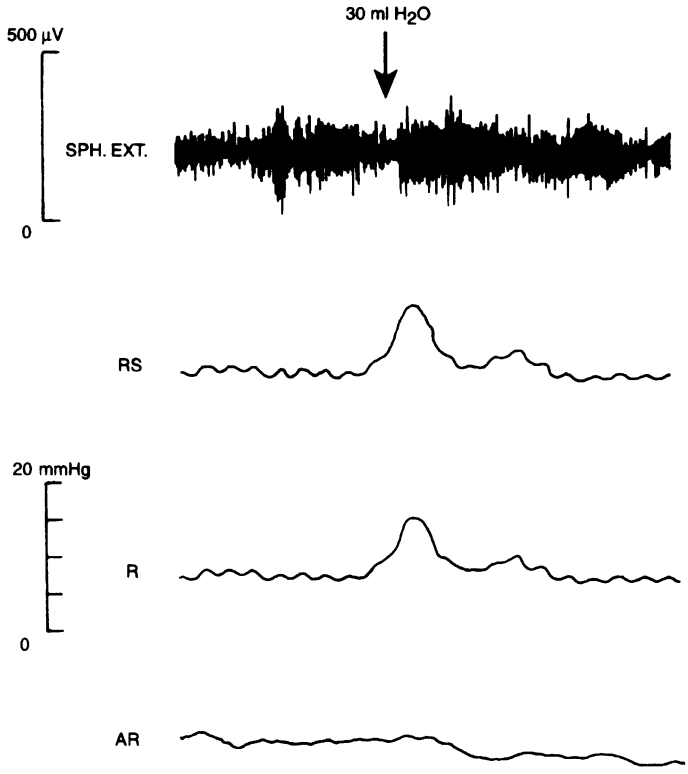

Fig. 22.18 Electromanometric study indicating disturbed rectal motility (multisegmental mass contractions) and missing internal sphincter relaxation (neurogenic achalasia) in Hirschsprung's disease before resection, (*SPH EXT* = external anal sphincter, *RS* = rectosigmoid, *R* = rectum, *AR* = anorectum)

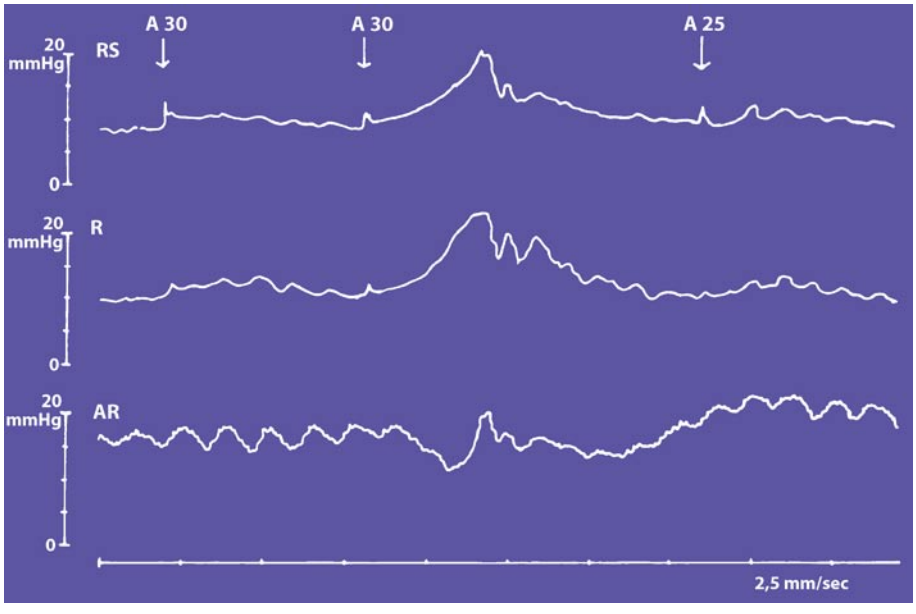

Fig. 22.19 Electromanometric study indicating reinnervation of the internal anal sphincter after anterior resection for Hirschsprung's disease. Note the simultaneous occurrence of multisegmental mass contractions and internal sphincter relaxation 3 years after anterior resection by the Rehbein procedure (*A30* = 30 ml air injected into the rectosigmoid; *RS* = rectosigmoid, *R* = rectum, *AR* = anorectum)

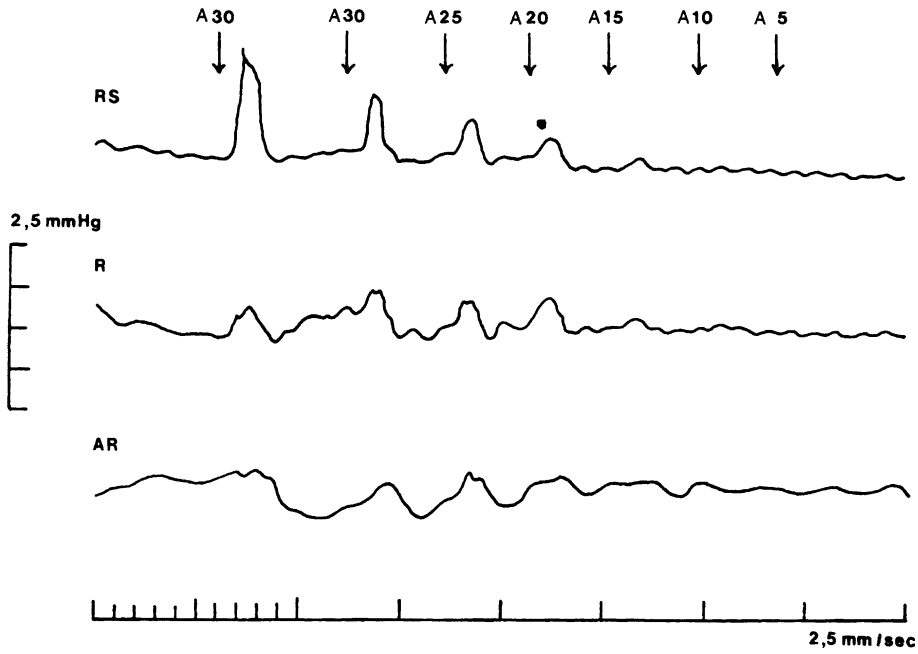

Fig. 22.20 Electromanometric study indicating complete postoperative reinnervation of the internal anal sphincter 7 years after anterior resection with normal internal sphincter relaxations. The amplitude of the relaxations is directly proportional to the distending volume (*A* insufflated air indicated in milliliters)

study also shows the degree of rectal dilatation, which can extend as far as the pouch-like enlargement of the dorsal rectal pole, so that the expulsive pressure vector no longer lies in the direction of the anal canal, but rather is directed towards the posterior rectal wall.

A further possible cause of postoperative constipation, siphon formation due to kinking of the descending colon proximal to the anastomosis, can also be detected roentgenologically.

22.8 Therapy of Anal Sphincter Achalasia

22.8.1 Conservative treatment

Conservative treatment of anal sphincter achalasia (and chronic constipation) consists of a laxative diet, administration of laxatives and an alpha-excitatory blocking agent such as phenoxybenzamine or dihydroergotamine in low doses [71]. Out of 47 patients with anal sphincter achalasia treated by us in 1980 with phenoxybenzamine, 29 became completely cured. There was a significant improvement in a further 13 children, and only 5 patients showed no change in their symptoms (Fig. 22.21).

Biofeedback training is very effective, especially in patients with functional anal sphincter achalasia [72] (Fig. 22.22). Berquist [73] reported a success rate of over 70% in the manometry-guided biofeedback management of children with functional or even organic anorectal disease refractory to conventional medical and behavioral therapy. Cox et al. [74] also reported good results using electromyographic biofeedback training. One should introduced a complete program designed to clear stools, to prevent further impacting, and to promote regular bowel habits. The majority of patients (65–70%) will be cured after 2 years. In cases of fecal soiling, the use of long-term daily enemas will be of great benefit [75]. Cisapride, a prokinetic substance, had a significant effect on the sensation threshold of the inhibitory relaxation reflex of the internal anal sphincter, allowing chronic idiopathic constipation to be normalized or improved in 15 out of 16 patients studied by Reboa et al. [76]. Krevsky et al. [77] also reported good results with cisapride. Unfortunately, the drug is not available in all countries. Loperamide seems to have a specific continence-improving action on the anal sphincter in incontinent patients. Loperamide significantly increases the threshold volumes for minimal perception and urgency

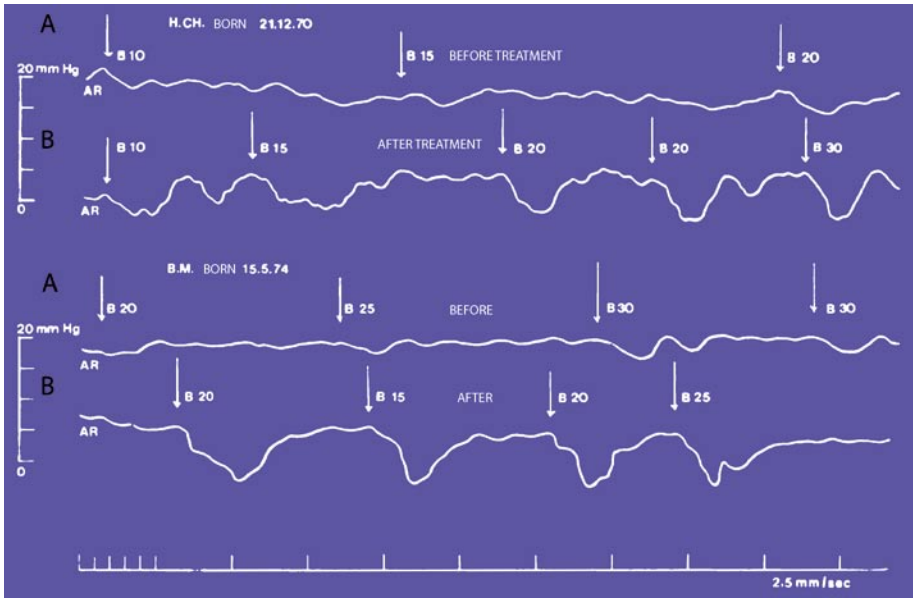

Fig. 22.21 Electromanometric study in two children before *A* and *B* after 3 months of treatment with phenoxybenzamine (*B* rectal balloon with volume of insufflated air indicated in milliliters; *AR* anorectum). Note the increasing duration and amplitude of internal sphincter relaxations after treatment, (*RS* rectosigmoid, *B* ballon, *AR* anorectum)

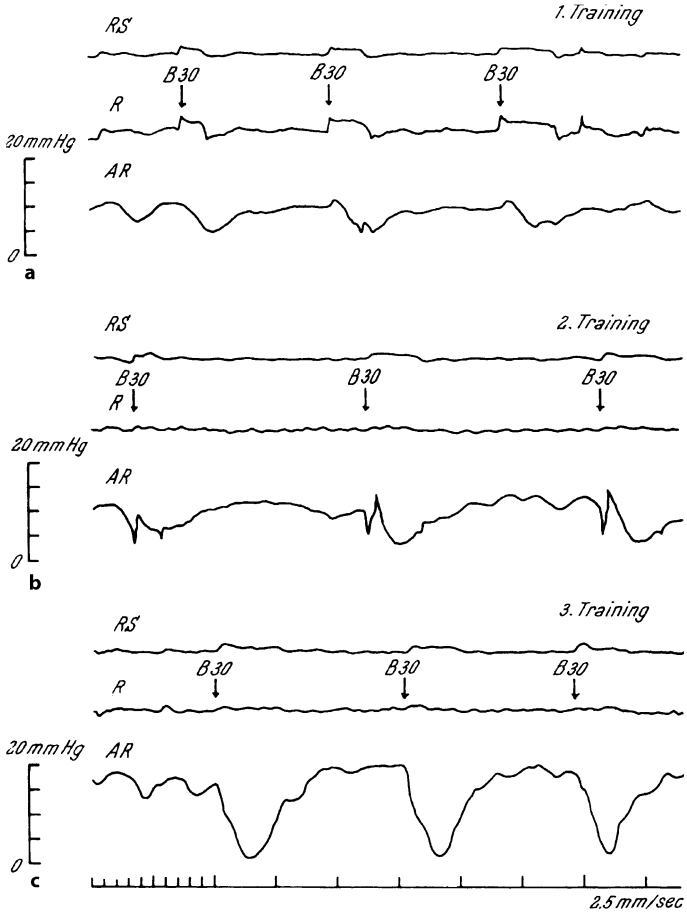

Fig. 22.22 Effect of biofeedback-training in a 12-year-old girl with neurovegetative anal sphincter achalasia. Three training episodes with 1 month duration each led to continuous improvement. *Top* First training: rudimentary internal sphincter relaxations are still visible. *Center* Second training: internal sphincter relaxations interrupted by voluntary contractions. *Bottom* Third training: normal inhibitory reflex response of the internal anal sphincter (*B30* rectal balloon insufflated with 30 ml of air) (*AR* anorectum, *R* rectum, *RS* rectosigmoid)

to defecation and raises the volume required to abolish recovery of the rectoanal inhibitory reflex [78]. Reasons for stool incontinence in children treated for HD may be damage to the internal anal sphincter by too-extensive myectomy or neurogenic damage to the external anal sphincter after a pull-through procedure [79]. Whether nitroglycerin which reduces anal sphincter pressure significantly in all patients with terminal constipation [80] is of great clinical value with special regard to side effects is still under discussion.

Millar et al. investigated the effect of topical isosorbide dinitrate (DTN) on anal achalasia after a pull-through operation for HD [81]. After application of the DTN paste the maximum pressure dropped, and the total length and the high pressure zone shortened. Messino et al. showed that local injection of botulinum toxin in children with IAS achalasia decreased the resting pressure and allowed the sphincter to relax, thereby making spontaneous defecation possible. Both methods can be used as a temporary form of treatment for internal anal sphincter achalasia [82]. Ciamarra et al. [83], however, described a very varied response in 20 patients with anal sphincter achalasia treated with botulinum toxin. In 60% of patients the result was excellent according to the parents, but in only 35% according to the physicians. The duration of response varied between 1 week and 18 months. One has to keep in mind that botulinum toxin blocks acetylcholine release and has therefore no direct influence on the internal anal sphincter. However, it could help to decrease the influence of the striated external anal sphincter on the anorectal pressure barrier.

In every patient, however, the first priority is to obtain relief of impacting before any further conservative or surgical treatment can be introduced. Very good results can be obtained with pineapple-flavored isotonic intestinal lavage solution containing polyethylene glycol 3350 (20–40 ml/kg per hour) until the stool is clear, which will be achieved in 2.5–4 hours [84, 85]. However, the serum glucose concentration and electrolytes (potassium values) have to be controlled.

22.8.2 Sphincter Dilatation

Sphincter dilatation is a part of every operative procedure for congenital Hirschsprung's megacolon and the treatment of choice in functional or psychogenic sphincter achalasia. It is not only performed preoperatively in order to eliminate sphincter achalasia and to prevent sphincter spasm associated with postoperative ileus, but is also a component of the surgical technique. Thus, in Duhamel's operation the sphincter must be dilated in order to introduce Kocher clamps or the stapler for crushing the colorectal septum. In Soave's procedure, the sphincter is stretched to make possible the evagination of the rectal mucosa and the pull-through of the colon. The same is true in Swenson's procedure. In the Rehbein procedure the anterior resection also begins with dilatation of the sphincter originally using an instrument similar to a nasal speculum specifically designed for this purpose [86].

In most patients, no further dilatation of the sphincter is necessary postoperatively, regardless of whether or not sphincteromyectomy was included in the procedure. According to Rehbein, myectomy is only indicated when two or three gentle sphincter dilatations using Hegar bougies have been unsuccessful.

22.8.3 Sphincteromyectomy

Sphincteromyectomy was first employed by Copeland [87] for the treatment of so-called sphincter spasm. Dupuytren [88], Demarquay [89] and Bodenhammer [90] modified the method. In the English literature, Brodie [91] and later Allingham and Allingham [92] were the first to describe radical sphincteromyotomy for the treatment of sphincter spasm associated with hemorrhoids and fistulas. Martin and Burden [93] subsequently introduced sphincteromyotomy, analogous to Ramstedt's pyloromyotomy and Heller's cardiomyotomy and based on Hurst's [94] concept, as a therapeutic procedure for megacolon with ultrashort segment and chronic constipation. Sphincteromyectomy was recommended for the treatment of anal sphincter achalasia in 1960 by Swenson et al. [95]. Its use has spread progressively since then [96–103].

22.8.3.1 Classical Sphincteromyectomy Technique

Younger children are placed in the lithotomy position on a sandbag with the legs flexed sharply to the right and left of the trunk at the hips and fixed to the operating table with wide adhesive tape. Standard leg supports are suitable only for older children.

Preoperatively the bowel of the child should be treated with Golitely solution and enemas for at least 2 days before the operation. The anus and rectum are then held open by two specula. Any stool still present in the anorectum despite vigorous laxative preparation is suctioned. The anorectum is disinfected and packed proximally with two thick paraffin sponges attached to long threads. The instruments are changed, a self-holding speculum with an endoscopic light is inserted and the anal canal carefully spread open. A transverse incision is made 1 to 2 cm above the dentate line (Fig. 22.23a). The mucosa is dissected free from the underlying circular muscle to an extent 2 to 3 cm cranially, with the mucosa retracted with a curved Babcock clamp (Fig. 22.23b). The border between the internal and external sphincters is then pal-

pated. This can easily be determined by the difference in tissue tone of the two sphincters. The internal anal sphincter is usually harder, while the external sphincter has a softer consistency. In addition, there is a sulcus between the two muscles which can be readily felt in the open anal canal. After exposure of the lower two thirds of the internal anal sphincter, the internal sphincter is bluntly undermined—proceeding from the intermuscular sulcus—so that the glistening, whitish intermuscular septum becomes visible.

A segment of the internal anal sphincter about 2–3 cm long and 3–5 mm wide is then excised. The edges of the circular muscle recede laterally (Fig. 22.23c). After careful electrical hemostasis, the mucosal edges are re-adapted using Vicryl or Dexon vertical mattress sutures (Fig. 22.23d). Finally, the anal canal is tamponaded for 12 hours with a rectal tube surrounded by a paraffinized pack. The long threads of the paraffinized sponges in the anorectum are tied around the tube and removed along with the whole pack the day after surgery.

22.8.3.2 Technical Variations

This technique described by Bentley in 1964 [97] and Lynn in 1966 [98] was adopted by many others [33, 100, 104, 105]. Variations concern only the incision at the dentate line, which is performed by some 1 cm above it, by others exactly at the line, and by still others below it. In the procedure of Bentley, under local anesthesia a triangular postanal skin flap is excised about one finger-width posterior to the anus, the internal anal sphincter and the border of the external sphincter are exposed, and then the myectomy is carried out. The skin is closed with a continuous suture, leaving the most distal portion of the wound open. Notaras [106] suggests lateral subcutaneous

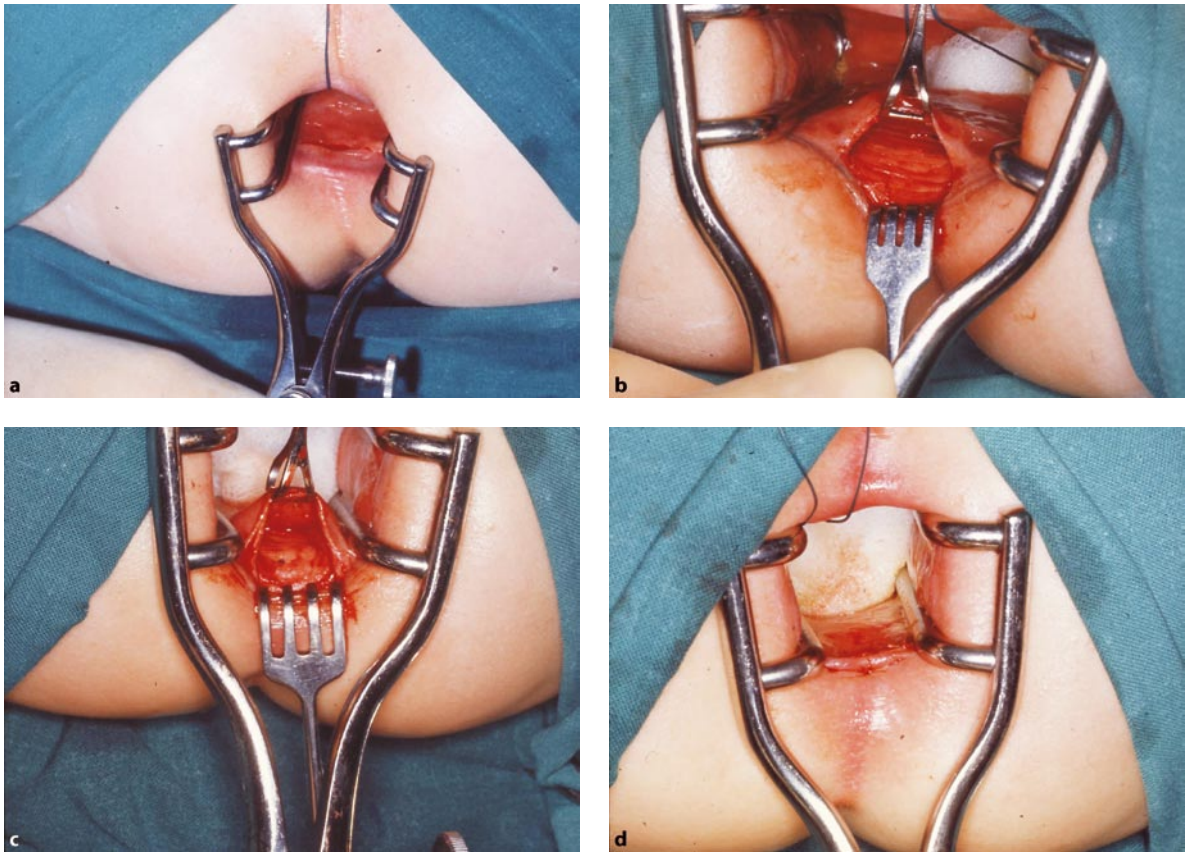

Fig. 22.23a–d Sphincteromyectomy technique. **a** Transverse incision at the dentate line. **b** Dissection of the mucosa from the underlying internal anal sphincter. **c** Excision of a muscle segment 2–3 cm long and 0.5 cm wide. Retraction of the muscle edges. **d** Re-adaptation of the mucosa to the skin using vertical mattress sutures

sphincteromyectomy with the incision at the mucocutaneous junction, preferably in the form of a small puncture incision. Especially for the correction of chronic anal fissures open or closed, midline or lateral sphincterotomy is very common. However, the midline fissurectomy shows the worst results: 25% of patients experience problems with anal competence [107], 27.6% with sporadic loss of continence, and 9.2% with soiling [108]. In lateral subcutaneous sphincteromyotomy, which is similar to the authors' technique, the results vary in the literature: 17% with incontinence [109], 15% long-term morbidity [110], 8% with disorders of fecal continence [107], and 1.3% to 1.5% with soiling [111, 112]. However, anal dilatation can be followed by infection and postoperative incontinence: 24.3% of the patients of Saad and Omer [113].

Thomas [99] recommends operating further away from the anal opening. In this procedure, sphincterotomy is performed from an incision in the middle anococcygeal raphe. The external sphincter is pulled caudally and the puborectalis fibers cranially so that the internal sphincter is exposed and a segment 3–6 cm long can be removed. This procedure has been modified by Alexander and Aston [114], who introduced a Foley catheter with a 30-cm3 balloon into the rectum. Rehbein's experience [86] has shown that myectomies of 2.5 to 3 cm are sufficient to ensure normal postoperative continence and to relieve related symptoms. Our experience is fully in accordance with this. Other authors excise substantially longer muscle segments: Lynn and van Heerden [98] from 4 to 14 cm, and Bentley et al. [115] from 4 to 10 cm. Myectomy of the distal two-thirds of the internal anal sphincter is undoubtedly decisive, since a significant reduction of pressure occurs after sphincterotomy [115, 116].

22.9 Results

Of 189 myectomies reviewed by us up to 1982, performed mainly for HD, 144 (76.1%) had excellent results. In 38 patients (17.9%), the constipation, enterocolitic symptoms and soiling had definitely improved, and the result was unsatisfactory in 11 children (6%). In 13 patients, repeated sphincteromyectomies or resections were necessary before a good or satisfactory result could be achieved [7, 8, 34]. Since 1982 we have preferred repeated sphincter dilatations instead of myectomies in patients suffering from neurogenic anal sphincter achalasia after Rehbein's anterior resection or Soave's endorectal pull-through. Sphincteromyotomy has been performed rarely since that time. Rehbein reported that he had to perform myectomies in only 40 of 370 operations in patients with Hirschsprung disease up to 1976, that is in 10% of patients. As mentioned above, in our international study of 439 patients with HD, sphincteromyectomy had to be performed in 13.6 % of the patients after Rehbein's procedure, in 15% after Swenson's procedure, in 11.3% after Soave's procedure, and in 10.8% after Duhamel's procedure [8, 56]. Sphincter dilatation had to be performed in 49.4% of the children after Rehbein's procedure, in 22.5% after Swenson's procedure, in 12.3% after Soave's procedure, and in 28.1% after Duhamel's procedure (Table 22.1). In a recent study we reported on 203 patients with neuronal intestinal malformations. Sphincteromyotomy was necessary in 6% of the patients. However, 13% needed a second resection instead of sphincteromyotomy due to persisting severe constipation caused by additional aganglionic, hypoganglionic, or neuronal dysplastic segments not diagnosed before the initial treatment [60].

Abbas Banani and Forootan [117] reported on 37 patients with HD and endorectal pull-through. In six patients partial rectal myectomy and sphincterotomy was performed. Five patients showed marked improvement and one had a partial response. Hata and Sasaki [118] treated 11 patients with idiopathic chronic constipation with posterior rectotomy and sphincteromyotomy. Complete cure was obtained in eight patients, and three showed improvement. Freeman [119] reported that anorectal myotomy was beneficial in 85.7% of 61 children with intractable constipation. Bourdelat et al. [103] noted good results after sphincterotomy in 21 out of 22 children with anal sphincter achalasia. In all patients, the sphincteromyectomy was made in the lower two-thirds of the sphincter and in a few—particularly those undergoing repeat sphincteromyectomy—in the upper sphincter and beyond as well. All the reports concur in the view that permanent success cannot be achieved with simple sphincter dilatation in neurogenic anal sphincter achalasia. Incontinence was observed in only 6.0 % of the children. However, Joosten et al. [120], in 25 out of 51 children operated on for classic HD, observed recurrent complaints such as constipation and diarrhea. Recently, Heikkinen et al. [121] observed no soiling in six out of ten patients treated with sphincteromyectomy. However, three patients showed occasional, and one child daily, soiling. Three out of ten children needed further treatment, and in seven the constipation disappeared. After redilatation of the internal anal sphincter, problems persisted in 40% of patients. The authors suggest that innervation abnormalities, demonstrated with the aid of polyclonal antibody staining, were responsible for these failures.

The success or failure of myectomy depends on the length of the incision in the sphincter. Bennett and Duthie [122] observed a reduction in anorectal resting pressure of 50% in their patients following total division of the fibers of the internal anal sphincter. Subtotal sphincteromyotomy lowered the resting pressure only when performed in the caudal 2 cm. The resting pressure decreased by an average of 7±4 mmHg. This corresponds to Stelzner's view [123] that division of the entire sphincter system causes total incontinence while indentation of a maximum of one-third to two-thirds of the sphincteric ring up to the crypts does not endanger continence.

Our own studies [8] showed that the decrease in resting pressure profile is a good measure of myectomy success [56, 62]. Thus, a resting pressure which remains above 30 mmHg indicates an inadequate myectomy result. Conversely, however, even if the anorectal resting pressure profile falls below 10 mmHg postoperatively, this does not necessarily mean that incontinence will occur, although this value indicates that too much sphincter tissue was removed at myectomy. The anorectal pressure profile should, of course, be performed under constant irrigation and using a catheter-withdrawing machine as recommended by Kaiser and Reuter [124], Maie et al. [125] and Holschneider [72]. Since the resting pressure profile of the internal anal sphincter can be decreased by oral application of dihydroergotamine, drug therapy in combination with a laxative diet and stool training as mentioned above should always precede sphincteromyectomy [71]. In our own experience the frequency of sphincteromyectomy decreased from 103 myectomized patients in 1976 to 18 patients operated on during the last 29 years [34].

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Laparoscopically Assisted Anorectal Pull-Through

K. E. Georgeson and O. J. Muensterer

23

23.1 Introduction

Posterior sagittal anorectoplasty (PSARP [3]) is the current standard surgical management for patients with imperforate anus. Despite the excellent exposure of the anatomy and the exact placement of the distal rectum within the muscle complex with this operation, postoperative fecal continence is less than ideal [4, 7]. Tsuji et al. reported a careful analysis of postoperative anorectal function, comparing posterior sagittal anorectoplasty with older, conventional operations [10]. He found that patients in both groups had a similar manometry and long-term function. Most of the patients needed bowel management. Other authors have reported similar findings [1, 9]. Increased constipation after posterior sagittal anorectoplasty compared to a more limited surgical approach has also been reported [2].

The goals of laparoscopically assisted pull-through for anorectal malformations include avoiding the dividing and weakening of the external sphincters and diminishing perirectal scarring, while allowing precise placement of the rectum through the external sphincters and the potential development of a primary procedure in the newborn, which would avoid the morbidity of a colostomy.

23.2 Operative Technique

A standard proximal sigmoid colostomy is performed in the newborn. Two to four months later, the patient is positioned transversely at the end of the operating table (Fig. 23.1). A circumferential prep is performed from the nipples down to the toes. A catheter is passed into the bladder in all cases, even if cystoscopy is required to do so. A pneumoperitoneum with pressures of 12 cm of water is established. A 4-mm trocar is placed in the anterior-axillary line just below the liver. A 5-mm port is placed through the umbilicus using an open technique. A 3 or 4-mm port is placed in the anterior-axillary line just above the anterior superior iliac spine (Fig. 23.1).

Laparoscopic rectal dissection is initiated at the peritoneal reflection. Using a hook cautery, the distal mesorectum is divided. The dissection is continued circumferentially around the rectum down to the rectourethral or rectovesical fistula. It is important to keep this dissection in the definitive plane between the longitudinal rectal muscles and surrounding tissues. Just proximal to the entrance of the fistula into the urinary tract, a loop ligature is preloaded through the 5-mm trocar in the umbilicus over a Maryland clamp placed through the right lower quadrant trocar. This clamp is placed on the fistula several millimeters proximal to the entrance of the fistula

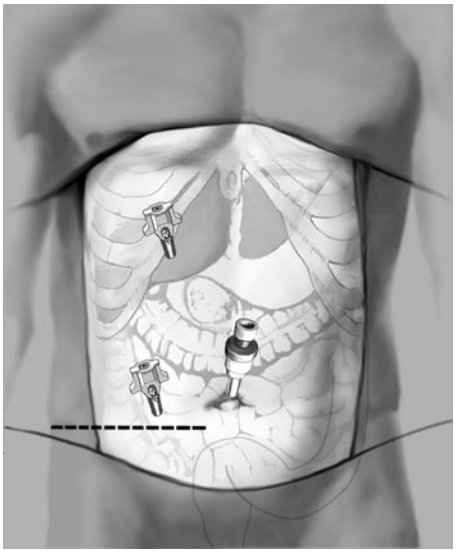

Fig. 23.1 The patient is positioned transversely on the operating table, a bladder catheter is placed, and the trocars are positioned in the illustrated sites (umbilical, 5 mm; right upper and lower quadrant, 4 mm)

into the urinary tract (Fig. 23.2). The fistula is divided proximal to the placement of the Maryland clamp. The loop ligature is then passed around the Maryland clamp and the fistula and snugged in place, adjacent to the urethra. A second loop can be placed on the rectal fistula proximally in a similar fashion (Fig. 23.3). The rectum is then retracted out of the pelvis. The pubococcygeus muscle can often be visualized when it is present (Fig. 23.4). In some patients with anorectal malformations, particularly the higher lesions, the levator ani muscle is poorly developed. However, in many patients with a rectoprostatic fistula, the muscle is quite well developed and can be seen from above with the endoscope.

A transperineal dissection follows division of the rectourethral fistula. The external anal sphincters are mapped using a transcutaneous electrostimulator. The area of maximal contraction is identified and marked appropriately with sutures. A 1-cm vertical midline incision is made at the site of the maximal muscle contraction. The intersphincteric plane is gently dissected from below the

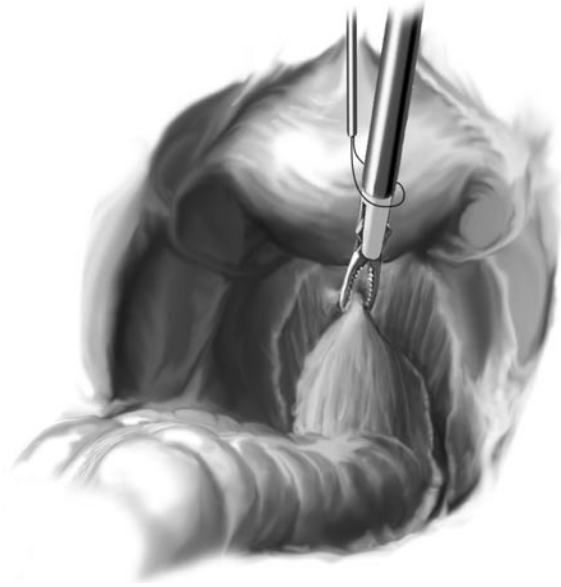

Fig. 23.2 After circumferential dissection of the rectum, the fistula is grasped with a Maryland clamp preloaded with a loop ligature. The fistula is then divided on the rectal side of the clamp, and the ligature is tightened around the urethral side of the clamp

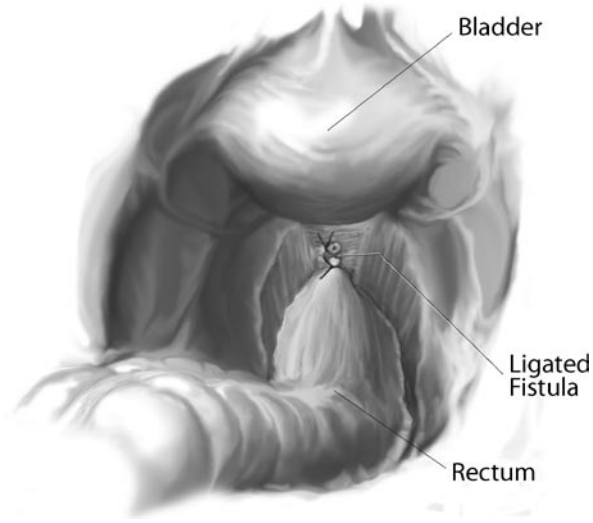

Fig. 23.3 A second loop ligature is used to close the fistula on the rectum

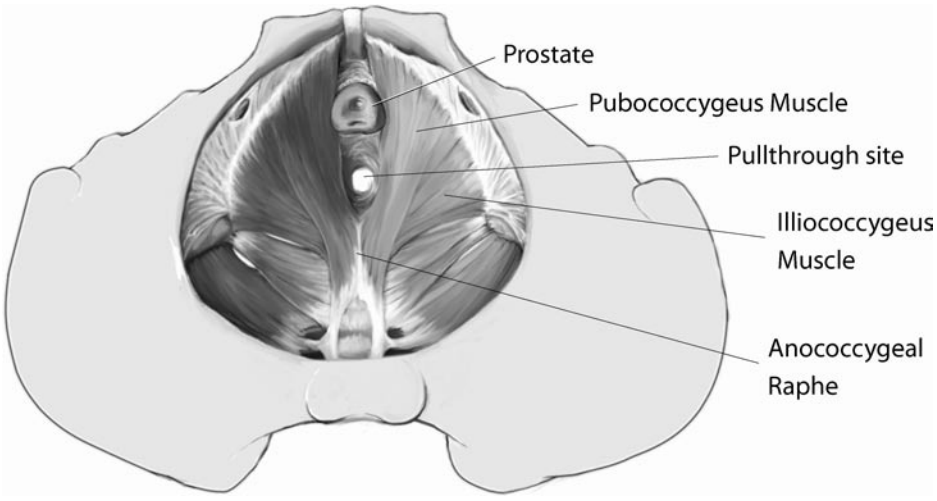

Fig. 23.4 Anatomic diagram of the pull-through site in relationship to the pubococcygeus muscle

level of the levator sling up through the muscle complex bluntly (Fig. 23.5). A radially expanding trocar is then passed over a Veress needle through this intersphincteric plane and advanced between the two bellies of the pubococcygeus muscle in the midline just posterior to the urethra using laparoscopic guidance. If the needle is inaccurately passed to either side of the midline it is readily apparent due to the laparoscopic surveillance. The Veress needle is redirected to a correct position prior to the dilatation of the tract through the expandable trocar sleeve. The tract is dilated radially up to 10–12 mm. The rectal fistula is then grasped through the transperineal trocar and is pulled down onto the perineum trailing the trocar (Fig. 23.6). The anastomosis between rectum and anus is completed with a polyglycolic acid suture. The rectum is retracted cephalad laparoscopically and secured in this retracted position with 2–0 silk sutures (Fig. 23.7). It is important to place these hitch stitches to avoid prolapse of the rectal mucosal wall through the anus and also to lengthen the skin-lined anal canal.

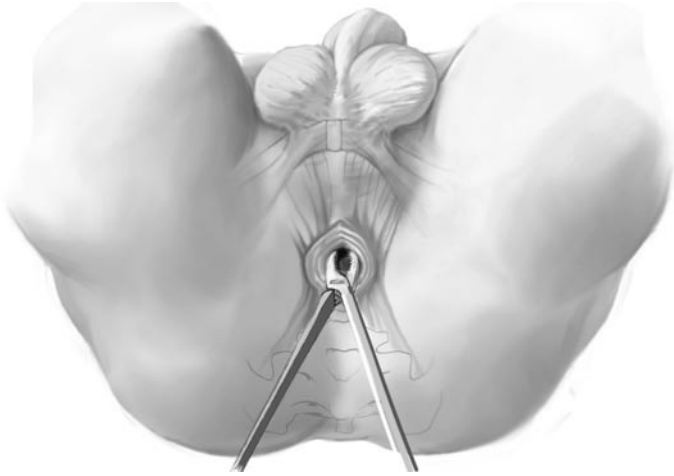

Fig. 23.5 Transperitoneal blunt dissection of the intersphincteric plane is performed through a 1-cm vertical midline incision using a clamp (the underlying external sphincter muscle complex and the pubococcygeus muscle are dotted)

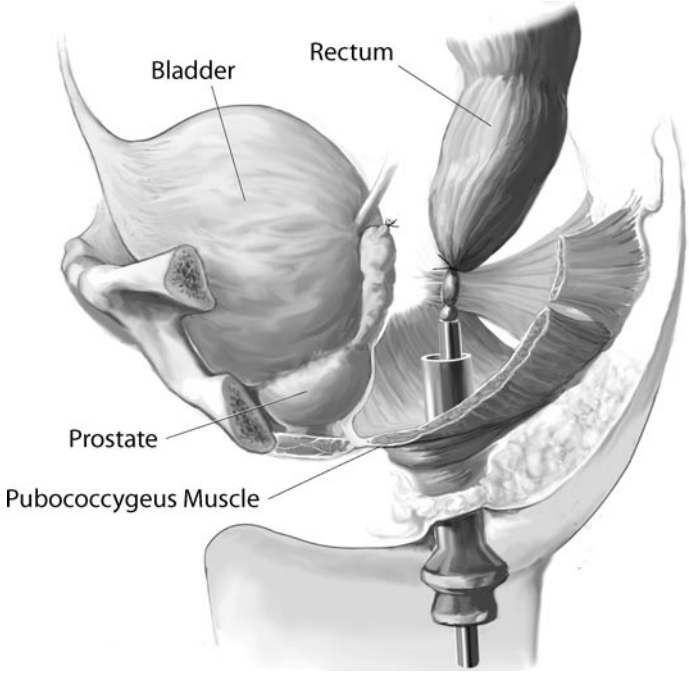

Fig. 23.6 Schematic diagram of the trocar passing from the peritoneum between the two bellies of the pubococcygeus muscle into the abdomen. A grasper is advanced through the port to grasp the distal end of the dissected rectum. The rectum is then pulled down through the perineum trailing the trocar

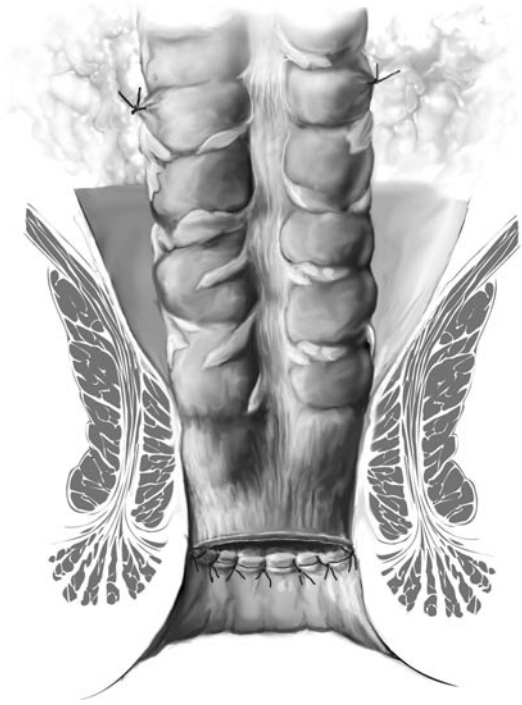

Fig. 23.7 After performing the anastomosis between rectum and anus, the rectum is retracted cephalad and secured to the presacral fascia using lateral hitch stitches to avoid prolapse of the rectal mucosa through the anus

Patients are fed on the first or second postoperative day. Graduated anorectal dilatation is started 2–3 weeks after surgery. The colostomy is closed 2–3 months after completing the pull-through procedure.

23.3 Results

The best comparative study between the laparoscopically assisted anorectal pull-through and the posterior sagittal anorectoplasty has been reported by Lin et al. [6]. Nine patients had a laparoscopically assisted pull-through and 13 had a posterior sagittal anorectoplasty. Lin et al. reported equal centrality of the pull-through segment when comparing the posterior sagittal approach and the laparoscopic approach. However, sphincter asymmetry was much greater in the posterior sagittal approach as was sphincter irregularity. Megarectum and constipation were also greater in the posterior sagittal group. Eight of the nine patients developed an anorectal reflex after laparoscopic pull-through while only 4 of 13 developed an anorectal reflex after posterior sagittal anorectoplasty. As many other authors have noted, eventual continence is related to a positive anorectal reflex [5, 8]. Lin et al. also reported similar anal resting pressures after the two operations. However, rectal compliance was much better following the laparoscopically assisted approach when compared to the posterior sagittal approach [6].

23.4 Discussion

Laparoscopically assisted anorectal pull-through seems to achieve some of its stated goals. There does not seem to be any question that the centrality of the pull-through inside the sphincter complex is achieved successfully. Additionally, there appears to be less scarring with the laparoscopic approach when compared to the posterior sagittal approach, as demonstrated by magnetic resonance imaging [11]. Long-term follow-up for fecal continence has yet to be determined. Because the internal sphincter is not well-developed in these patients, long-term continence may not be significantly improved. A normally functioning internal sphincter is certainly a great aid to fecal continence.

In summary, laparoscopic anorectal pull-through is anatomically sound and leaves the external sphincter muscles intact. This technique allows for the centrality of the pull-through inside the sphincter complex. There is a higher incidence of the anorectal reflex in patients after laparoscopically assisted pull-through than after posterior sagittal anorectoplasty. There is less scarring in the pelvic floor resulting in better rectal compliance. Long-term follow-up for continence is needed for further evaluation of this technique.

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Swenson's Procedure

P. Puri

24

24.1 Swenson's Procedure

Once the diagnosis of Hirschsprung's disease (HD) has been confirmed by rectal biopsy examination, the infant should be prepared for laparotomy. Biopsies for frozen sections are taken to determine the level of transition and a colostomy is placed just proximal to the transition zone.

If the newborn has enterocolitis complicating HD, he will require correction of dehydration and electrolyte imbalance by infusion of appropriate fluids. Thomas et al. [1] have demonstrated a relationship to *Clostridium difficile* and its toxin in about 30% of patients with enterocolitis in HD and suggested treating these patients with vancomycin during acute episodes. It is essential to decompress the bowel as early as possible in these babies. Deflation of the intestine may be carried out initially by rectal irrigations and when the baby is clinically stable a colostomy could be performed.

Traditionally, a definitive pull-through operation for HD has been performed when the infant is 6 to 12 months old. This approach evolved during the 1950s when major operations on neonates were considered unsafe and neonatal HD was associated with a high mortality [2, 3]. Advances in neonatal anesthesia, monitoring and surgical care together with parenteral nutrition and effective antibiotics have allowed primary prolonged reparative procedures to be undertaken safely in the neonate. In recent years, the vast majority of cases of HD are diagnosed in the neonatal period. Many centers are now performing a one-stage pull-through operation in the newborn with minimal morbidity and encouraging results [4–7].

A number of different operations have been described for the treatment of HD. The three most commonly used are the rectosigmoidectomy developed by Swenson, the retrorectal transanal approach developed by Duhamel and the endorectal procedure developed by Soave. The basic principle in all these procedures is to bring the ganglionic bowel down to the anus. Long-term results of any of these operations are very satisfactory if they are performed correctly.

24.1.1 Primary Swenson's Pull-Through Operation

Many surgeons have reported good results with primary neonatal pull-through operation for HD. The author like many others prefers Swenson's pull-through operation in the neonatal period because of its simplicity and lack of complications. We have not used diversionary colostomy for usual cases.

Once the diagnosis of HD is confirmed, the neonate is started on total parenteral nutrition 2 to 3 days prior to operation. Rectal irrigations are carried out twice a day for 3 days before surgery. Intravenous gentamicin and metronidazole are started on the morning of operation.

24.1.2 Operative Technique

The patient is positioned on the operating table to provide simultaneous exposure of the perineum and abdo-

men. The pelvis is allowed to drop back over the lower end of the table and legs are strapped over sandbags. A Foley catheter is inserted in the bladder.

We prefer a Pfannenstiel incision when performing a Swenson's pull-through operation in the neonate (Fig. 24.1a). Some surgeons use a left paramedian incision. A Denis Browne retractor is applied and the urinary bladder is lifted forward out of the abdomen by stay sutures. Extramucosal biopsies are taken at intervals along the antimesenteric border and assessed by frozen section to determine the level of ganglionated bowel. The sigmoid colon is mobilized by dividing the sigmoid vessels and retaining the marginal vessels. It may be necessary to mobilize the splenic flexure to obtain adequate length. The proximal level of resection above the ganglionated level previously determined by frozen section is selected and the bowel is divided between intestinal clamps or staples (Fig. 24.1b).

The peritoneum is divided around its lateral and anterior reflection from the rectum exposing the muscle coat of the rectum. At this point, the bowel is divided at the rectosigmoid junction and removed (Fig. 24.1c). Dissection extends around the rectum keeping very close to the bowel wall. It is essential to maintain the dissection close to the muscular wall in order to prevent damage to the pelvic splanchnic innervation. All vessels are electrocoagulated under direct vision. A sufficient tension-free length is obtained by dividing the inferior mesenteric pedicle, carefully preserving the marginal vessels. Dissection is carried down to the level of external sphincter posteriorly and laterally, but does not extend as deeply anteriorly leaving around 1.5 cm of intact rectal wall abutting against the vagina or urethra. The extent of dissection can be confirmed by putting a second glove over that on the left hand and by manual palpation with a finger in the anus.

The mobilized rectum is intussuscepted through the anus by passing a curved clamp or a Babcock forceps through the anal canal, and an assistant places the closed rectal stump within the jaws of the clamp (Fig. 24.1d). The mucosal surface is cleaned with Betadine. When the dissection has been completed it should be possible to evert the anal canal completely when traction is applied on the rectum. An incision is made anteriorly through the rectal wall about 1 to 2 cm from the dentate line, extending halfway through the rectal circumference. A clamp is inserted through this incision to grasp multiple sutures placed through the cut end of the proximal colon (Fig. 24.1e). An outer layer of interrupted 4-0 Vicryl sutures are placed through this incision to grasp multiple sutures placed through the cut muscular edge of the rectum and the muscular wall of the pull-through colon (Fig. 24.1f, g). When the outer layer has been completed, the proximal bowel is opened and an inner layer of interrupted 4-0 Vicryl sutures is placed (Fig. 24.1h). When the anastomosis is completed, the sutures are cut, allowing the anastomosis to retract within the anus (Fig. 24.1i).

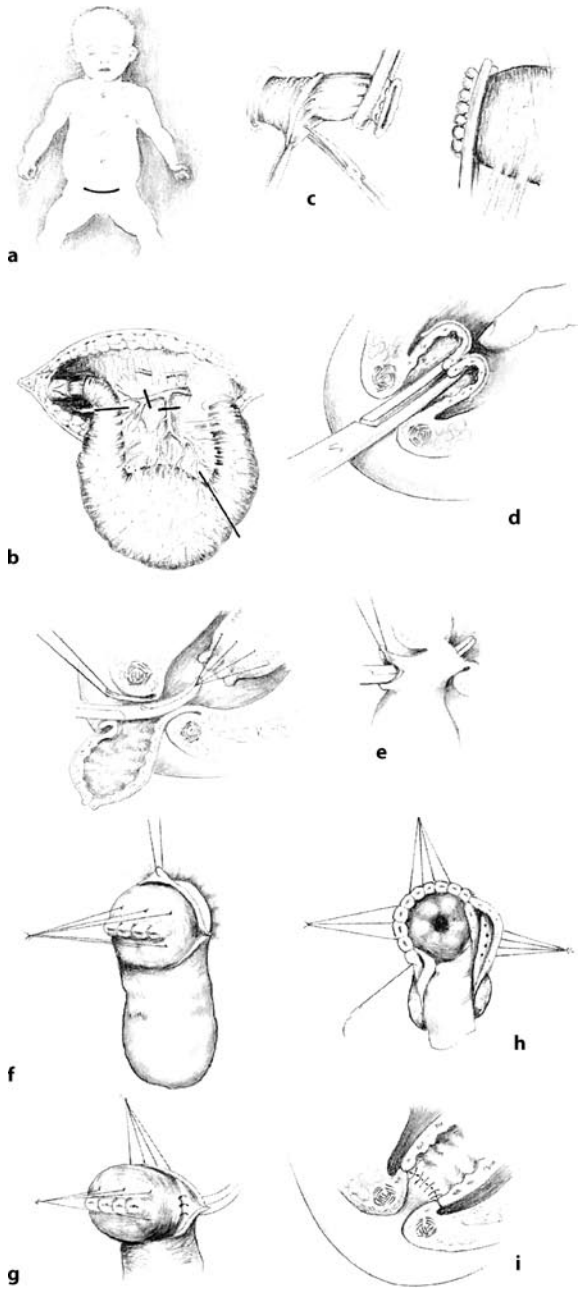

Fig. 24.1 **a** Incision. **b** Proximal and distal level of resection of colon to provide more room for dissection in the pelvis. **c** It is essential to maintain dissection close to the rectal wall in order to prevent damage to the splanchnic nerves. **d** The mobilized rectum is intussuscepted through the anus. **e** A clamp is inserted through the incision in the anterior rectal wall to grasp the proximal colon. **f** Pulled-through colon. **g** Outer layer of sutures. **h** Inner layer of sutures. **i** Anastomosis retracted within the anus

We keep the infant on total parenteral nutrition for 7 days postoperatively and then gradually start oral feeds. The urethral catheter is removed after 3 days. Antibiotics are discontinued after 5 days. Rectal examination is performed 2 weeks later during an outpatient visit.

24.2 Experience with Swenson's Operation

24.2.1 Mortality

The mortality after Swenson's operation is reported to be 0–5.8% [6, 8, 9, 10–15] (Table 24.1). Reviewing the literature, Joppich [16] found a total mortality of 5.2% in 5646 patients operated on with various procedures. In the 3506 patients operated on with Swenson's procedure, the mortality was 6.4%; however, the review included several early publications reporting high mortality [16].

Mortality in children with HD has decreased over the years. After Swenson's operation, Sherman et al. reported 2.6% mortality during the early years 1947–1956, 4.8% postoperative mortality for the period 1957–1966, 1.2% for 1967–1976, and 1.3% for 1977–1986 [11]. In recent years, some authors have reported series without mortality after Swenson's operation for HD [6, 9].

Postoperative mortality is considerably increased in patients in whom anastomotic leaks occur [11]. The mortality rate has also been reported to be significantly higher in children with Down's syndrome, which was related to a higher incidence of anastomotic leaks in these patients [11]. Sherman et al. reported a higher mortality in those operated on before the age of 4 months and recommended that resection in these young children should be avoided. However, this suggestion could be questioned considering the excellent results reported by Carcassonne et al. and Shanbhogue and Bianchi, in which primary Swenson's procedure was performed in neonates or before the age of 3 months without any mortality [6, 9].

Late deaths have been reported to occur in 0 to 3.1% of patients [6, 8–10, 12–15] (Table 24.1). Joppich found 2.7% late deaths after Swenson's pull-through in 3506 patients in the literature [16].

Table 24.1 Mortality

Reference	Mortality (%)		No. of patients
	Total	Late	
12	5.4	2.1	483
10	5	0	84
8	2.5	1.0	390
14	2.2	2.2	89
13	3.1	3.1	65
11[a]	2.4	–	880
9	0	0	25
6	0	0	25
15	5.8	2.9	69

[a]An additional 2.2% died of causes unrelated to Hirschsprung's disease or their operation.

24.2.2 Bowel Control

For the evaluation of the patient's final status, long-term follow-up is necessary, and Sieber stated that the follow-up period should be at least 5 years to provide meaningful final evaluation after the various procedures [17]. Evaluation of bowel habits is highly subjective, and whether they are normal or abnormal can be difficult to assess. It is generally accepted that bowel control improves with age after surgery for HD [13]. Heikkinen et al. [18] reported 100 patients, with a follow-up period of 20–45 years after various operations for HD in childhood, and found that in most patients, fecal continence and quality of life did no differ from that of healthy adults. However, Heij et al. found considerable problems with constipation, soiling or fecal incontinence in their series of 49 children over 4 years of age operated on with Duhamel's procedure. They suggested that these children adapt to their bowel dysfunction and subsequently have a tendency to underreport their symptoms [19].

Sherman et al. found that the best predictors of abnormal bowel habits in patients followed up for more than 5 years after definitive surgery were temporary soiling after discharge and rectal stricture. The length of aganglionic bowel, enterocolitis or previous sphincterectomy did not influence the patients' final status concerning bowel habits or soiling [11].

Puri and Nixon reported that 57% of patients were relieved of all symptoms within a year after Swenson's operation [10]. Wilcox et al. [7] reported their results in 21 patients operated on with neonatal one-stage pull-through, with a follow-up period of more than 4 years. Of these 21 patients, 17 patients had normal bowel control in this series. Liem et al. reported that 51 (94%) of 54 patients had normal bowel evacuation after a follow-up period of 2 to 5 years [15]. The number of patients with normal bowel habits, with a follow-up period of more than 5 years after Swenson's operation, has been reported by Sherman et al. [11] to be 89.9%. However, in patients followed up for more than 20 years, 93.7% reported normal bowel habits. Sherman et al. reported one to three bowel movements per day in 97.9% of the patients with more than 5 years follow-up. This number remained virtually unchanged in those with a follow-up period of more than 10 and 15 years, respectively. However, in those who had a follow-up period of more than 20 years 96.7% were reported to pass stools one to three times per day [11]. In the latest

series of Sherman et al. [11], 0.8% and 0.5% of patients had permanent ileostomy and permanent colostomy, respectively.

24.2.3 Constipation

Constipation constitutes one of the most common late complications after surgery for HD [16], and consequently one of the most fundamental criteria of successful management. The literature reveals wide differences in the incidence of constipation, not only between the various surgical procedures but also between different authors using the same procedure. In the publications reviewed by Joppich, constipation occurred in 452 (9.1%) of 4783 patients who underwent surgery for HD with various techniques [16]. Puri and Nixon reported that constipation was the most common problem in their series and required treatment in 29 (34.5%) of their 84 patients [10]. Holschneider reported similar figures in 80 patients, among whom 32.5% suffered from constipation after operation [20]. However, the number of patients with constipation decreased with time. Puri and Nixon in a long-term follow-up study reported that 6.0% of patients required treatment for constipation regularly, while another 14.3% used laxatives occasionally [10]. This is similar to the figures reported by Liem et al., who also found constipation in 6% of their patients at follow-up [15]. In Holschneider's series 10.0% of patients suffered from constipation at follow-up [20].

Swenson et al. reported a 5.7% incidence of constipation in 282 patients followed up for more than 5 years [12]. Quinn et al. investigated the long-term incidence of constipation after Duhamel, Soave and Swenson's procedures, and found that the Duhamel procedure was associated with the highest rate of constipation (54%), followed by the Soave procedure (43%) and Swenson's operation (4%) [21].

24.2.4 Soiling

Swenson et al. found temporary soiling in 13.3% of patients in 1975 [12], which is similar to the incidence of 12.9% reported by Sherman et al. in the same extended series in 1989 [11]. Temporary soiling after discharge was the only factor which influenced the occurrence of soiling at follow-up [11]. Of the patients followed up for more than 5 years, 8% were reported to have temporary soiling at follow-up. A continuous improvement was found, and in those followed up for more than 20 years, only 1.6% suffered from soiling [11]. Puri and Nixon reported that 45 (54%) of 84 patients achieved normal continence soon after operation, while 39 (46%) of the 84 suffered from lack of control. However, in only 9 (10.7%) of these patients was soiling troublesome. In the remaining 30 it was only occasional soiling or staining. A considerable number of patients continued to have some degree of soiling for several years before acquiring complete control, which all had done by follow-up [10]. In the series of Holschneider, encopresis was reported in 45.5% of patients after operation and in 15.1% at follow-up [11]. Furthermore, temporary soiling was reported in 26% of patients at follow-up. Liem et al. reported fecal soiling in 8% of patients after a follow-up period of 2 to 5 years [15]. In 21 patients followed up for more than 4 years after a neonatal primary procedure. Wilcox et al. reported soiling at least once a week in four patients (19%). However, in this series, 13 patients were operated on with Soave's procedure, while Swenson's operation was performed in 38 [7].

24.2.5 Influence of Trisomy 21 on Bowel Control

The association of HD and trisomy 21 has been well documented. Although in early reports it was suggested that less than 5% of children with HD had trisomy 21, more recent studies have shown a 10% to 15% incidence of this association [22, 23]. Although many children with HD have bowel problems, the majority eventually have a satisfactory outcome. The attainment of normal postoperative defecation is clearly dependent on the intensity of bowel training, the social background and intelligence of the patient, and the motivation to be socially clean. However, this motivation is probably low or absent in children with trisomy 21. In the series reported by Quinn et al., with a mean follow-up period of 8 years (4 to 15 years) after Swenson's operation, more than two-thirds of the patients with trisomy 21 were soiling constantly up to 14 years after surgery, and two others had reverted back to a permanent stoma after failure of surgical management of the HD. Thus more than three-quarters of the patients with Down's syndrome had a completely unsatisfactory outcome following treatment of HD [22].

Sherman et al. reported temporary soiling in 24% and 37.5% of patients with Down's syndrome and mental retardation, respectively [11].

24.2.6 Persistent Bowel Symptoms due to Associated Intestinal Neuronal Dysplasia

Constipation may occur postoperatively, despite complete resection of the aganglionic segment and without anastomotic stricture. These patients should be re-evaluated with rectal biopsies, since HD is associated with intestinal neuronal dysplasia (IND) in about 25% of patients [24, 25]. In 31 consecutively treated patients who underwent Swenson's pull-through for HD, Kobayashi et al. found

histological features of IND in ten patients. All ten patients had persistent bowel dysfunction (constipation, enterocolitis or soiling) after their pull-through operation [26]. Moore et al. assessed the postoperative outcome in 178 patients operated on with various procedures for HD, 16 of whom had clinical evidence of a degree of persisting obstruction. Rectal biopsies were performed in these 16, 14 of which were abnormal. There was aganglionosis in four, features of IND in nine and ganglioneuromatosis of the colon in one [27].

24.2.7 Enterocolitis

Enterocolitis remains the most serious complication of HD resulting in considerable morbidity and mortality [8, 11, 14]. The pathogenesis is still unclear, although, traditionally, mechanical dilation and fecal stasis have been considered to be critical factors [28]. However, in recent years, various theories have been put forward, including alterations in mucin components [29–31]. infection with *Clostridium difficile* [1, 32] and rotavirus [33], and defects in mucosal defense mechanisms [34–36].

After Swenson's operation, postoperative enterocolitis has been reported to occur in 11.5–33.7% of patients [8, 11, 14, 20]. In the series reported by Sherman et al., late enterocolitis occurred in 22.5% of patients. However, only five patients required hospitalization for enterocolitis more than 3.5 years after their definitive operation [11]. Holschneider found that 3.7% of 80 patients had recurring enterocolitis at the time of follow-up 1 to 18 years after Swenson's procedure [20]. Late enterocolitis also results in late morality, reported to be the cause of late death in 62% of patients [16]. Sherman et al. reported that 9 (1.0%) of 859 patients died from late enterocolitis [11]. In the series reported by Liem et al., 2 (2.9%) of 69 children died from late enterocolitis [15].

24.2.8 Rectal Stricture

Kleinhaus et al. reported that rectal stricture occurred in 9.5% of patients after Swenson's pull-through operation; 5.2% were mild, while 4.3% required further surgical procedures [8]. This is similar to the occurrence reported by Puri and Nixon. In their series, 9.5% of patients developed rectal stricture, seven of these during the early postoperative period. In one patient the stricture was delayed for months. Six of the patients recovered after dilatation, but two required a secondary resection, one of whom ended up having a permanent ileostomy [10]. Sherman et al. found development of stricture in 65 patients (7.6%), 49 of whom responded to dilatation, while 12 required surgical division. These authors also reported a higher incidence of stricture in patients operated on before 4 months of age (22.2%) compared to those operated on later (7.5%) [11]. In Weitzman's series of 65 patients, no rectal strictures occurred [13].

24.2.9 Fistulae

Fistulae are rare complications after Swenson's operation. Kleinhaus et al. found a relatively high incidence of fistulae (6.2%) in 390 patients. In this series the occurrence was considerably higher than in the cases operated on with Duhamel's procedure (2.9%) and various modifications of Soave's procedure (1.1%) [8]. Puri and Nixon reported fistulae in 2 (2.4%) of 84 patients. A rectovaginal fistula was found in one patient after secondary resection, which was successfully repaired. Another patient had a rectourethral fistula detected 5 years after rectosigmoidectomy [10]. Shanbhogue and Bianchi reported that one rectourethral fistula occurred after dilatation of an anastomotic stricture in their series of 25 neonates [6].

24.2.10 Intestinal Obstruction

Ikeda and Goto reported intestinal obstruction in 18% of patients after Swenson's operation [14]. which is considerably higher than the incidence reported by other authors. Puri and Nixon reported intestinal obstruction in 7 (8.3%) of 84 patients, 5 of whom had peritoneal adhesions and all of whom recovered after surgery. The remaining two had strictures at the colostomy site and small-bowel volvulus, respectively [10]. In the series reported by Holschneider, ileus occurred in 11.8% of the patients [20], which is similar to the incidence of 9.0% reported by Kleinhaus et al. in the survey of the Surgical Section of American Academy of Pediatrics [8]. Postoperative small-bowel obstruction occurred in 53 (6.0%) of the 880 patients in the series reported by Sherman et al. [11]. The earliest obstruction occurred on the second postoperative day and the latest 17.3 years after the operation. Obstruction was more common in those resected before the age of 2 years [11].

24.2.11 Urinary Incontinence

Puri and Nixon reported that 9 of 84 patients over the age of 3 years at the time of Swenson's operation had incontinence of urine from 3 to 11 years after the operation. Seven had only nocturnal enuresis, while two had incontinence day and night. However, all had recovered by the time of follow-up [10]. Liem et al. reported stress incontinence in 1 of 54 patients followed up for 2 to 5 years [15]. Urinary incontinence did not occur in the 282 patients with a follow-up of more than 5 years reported by Swenson et al. [12] or in the same extended series reported later by Sherman et al. [11]. Similarly, Weitzman found

no patients with bladder dysfunction in his series of 65 patients [13].

24.2.11 Sexual Dysfunction

Erectile and ejaculatory dysfunctions are relatively rare complications after Swenson's operation. Puri and Nixon found ejaculatory difficulties in 2 of 84 patients, despite normal erection [10]. Weitzman found no sexual dysfunction in 65 patients [13], and Sherman et al. reported no sexual difficulties in 194 older males [11].

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Soave's Extramucosal Endorectal Pull-Through Procedure

V. Jasonni, A. Pini Prato and G. Martucciello

25

25.1 History of the Endorectal Pull-Through Procedure

The use of the abdominal extramucosal dissection of the rectal pouch was first proposed by Romualdi at the Roman Society of Surgery on 15 May 1955. The technique was first published in 1960 [1]. During the next few years, Rehbein [2] and Kiesewetter and Turner [3] also popularized this operation. In 1957, Soave started using Romualdi's procedure for the treatment of anorectal malformations with urethral fistula. Since his initial experience with Romualdi's technique, he thought that this principle could be applied for the treatment of Hirschsprung's disease (HSCR). So, in 1961, he performed his first operation on a 2-year-old boy with the classic form of HSCR. After treating six children with endorectal pull-through, Soave reported his first results [4, 5]. The technique of separating the mucous coat from the muscular layer of the rectum for the treatment of HSCR was experimented with during about the same years by Soave and Boley. However, Soave's first report [4] on endorectal pull-through without anastomosis dates back to 1963, and other internationally more well-known reports [5, 6] to 1963 and 1964. In the same 1964 issue of *Surgery*, Boley [7] first presented a technique using the same endorectal approach but with a primary anastomosis of the pulled-through colon. In a short time, a large number of pediatric surgeons from all over the world were experimenting with the endorectal pull-through, and the indication for Soave's procedure was extended to other conditions including multiple juvenile polyposis, familial polyposis, and ulcerative colitis.

25.2 Preparation for Soave's Procedure

Up to the 1980s, the endorectal pull-through was generally performed in patients older than 5 months and weighing more than 8 kg. In those days, it was necessary to have a clear clinical and radiological picture to confirm the diagnosis of HSCR. Today, the histochemical preoperative diagnosis based on acetylcholinesterase activity [9, 10] can be made in the neonatal period without anesthesia using a suction rectal biopsy tool, the Solo-RBT, which was developed in 2000 at the Gaslini Institute [11]. The availability of an early and reliable diagnosis has led pediatric surgeons to perform, when possible, radical surgery within the first 3 months of life. In this way, the conservative period of nursing can be reduced to a minimum, decreasing the risk of complications and avoiding chronic mucositis of the rectum and colon due to the prolonged daily evacuating enemas. These alterations are well known to surgeons who have performed Soave's procedure in older children (more than 6–10 years of age) undergoing prolonged daily nursing maneuvers. In these subjects, endorectal dissection is very difficult due to

the tenacious adhesions on the submucous layer caused by chronic proctitis. If Soave's procedure is performed within the first 3 months of life—a very common approach today among pediatric surgeons—chronic inflammatory processes of the rectum are generally avoided and endorectal dissection between the internal submucous layer and the external muscular cylinder is straightforward and rapid.

Soave's procedure does not require any protective colostomy. The aim is to achieve radical treatment without contaminating the operative field at all. Therefore, colostomy is only indicated for the subgroup of HSCR patients presenting with acute enterocolitis or intestinal obstruction in the first weeks of life. When necessary, colostomy should be performed in the most distal portion of the ganglionic colon (level colostomy). In order to achieve this, it is necessary to perform multiple intraoperative seromuscular biopsies of the colon, using, if possible, histochemical techniques to evaluate the length of the aganglionic and hypoganglionic segments. At least four good intraoperative histochemical techniques are available today: succinic dehydrogenase [12] (SDH), lactic dehydrogenase [13] (LDH), alpha-naphthylesterase [14–18] (ANE), and rapid acetylcholinesterase [19, 20] (rapid-AChE). The level colostomy has to be terminal and part of the aganglionic distal colon has to be resected up to the rectosigmoid junction, where it is sutured and plunged. We choose a level terminal colostomy for the following reasons: none of the normoganglionic colon is resected and lost; the colostomy is not formed in an aganglionic or hypoganglionic segment, thus the risk of a second pull-through operation using these segments with abnormal innervation is avoided; and the number of operations is reduced from three to two, since the radical treatment is performed by mobilizing the level colostomy avoiding a subsequent operation for colostomy closure.

The preparation of the bowel the week before Soave's radical treatment is essential to reduce complications. The colon has to be cleaned with special enema preparations (we use a mixture of sorbitol enema or phosphate saline enema, saline solution and Vaseline oil). During the days preceding surgery, rectal probing should be repeated and alternated with evacuating enemas. Perioperative intravenous antibiotic prophylaxis is started 1 hour before surgery.

25.3 Operative Technique

25.3.1 Positioning of the Patient

When the patient is under general anesthesia and a catheter has been inserted into the bladder, before the definitive positioning of the patient for the operation, the surgeon has to perform a wide dilatation of the anal canal. This procedure is performed using the two forefingers: the anorectal canal is dilated by traction in opposite directions. This maneuver is always essential for a successful pull-through procedure, especially in patients under 1 year of age. The patient should be positioned supine with the buttocks lying at the extreme edge of the operating table and the legs hanging freely, wrapped in drapes, and fixed to the table to prevent slipping of the patient's pelvis (Fig. 25.1a).

25.3.2 Laparotomy

Different incisions may be performed for Soave's procedure. A good alternative to the classic paramedian left incision is the Pfannenstiel incision, which leads to better cosmetic results. It is performed above the pubis and is sufficient for the treatment of rectal and rectosigmoid forms of aganglionosis. In patients in whom the disease involves the descending colon, the Pfannenstiel incision can be extended in the left pararectal direction. In patients with level ileostomy for total colonic aganglionosis, a xiphopubic median incision is required in order to perform an endorectal ileal pull-through procedure. The operating field is exposed with malleable retractors, and all mesocolic adhesions to the left parietal peritoneum are dissected up to the splenic flexure (classic form of HSCR). Before starting endorectal dissection, it is essential to perform multiple seromuscular biopsies of the rectum and colon in order to evaluate the length of the aganglionic and associated hypoganglionic segments. A better evaluation of the segment to be resected is possible thanks to specific intraoperative enzymohistochemical techniques [12–20].

25.3.3 Separation of the Seromuscular from the Mucosal Layer of the Rectum

This is the most technically difficult and peculiar step of Soave's procedure. In order to facilitate the initial separation of the seromuscular from the mucosal layer of the rectum, mepivacaina 2% with epinephrine 1:100,000 (10 μg) in 10 ml of normal saline solution is injected between the layers (Fig. 25.1b).

A longitudinal seromuscular incision is made on the previously infiltrated anterior wall of the rectum (Fig. 25.1c). It is important not to start dissection at a more proximal point to avoid a too-large dissection surface. The incision (Fig. 25.1c) is widened with blunt dissection, first on the long axis of the bowel and then progressively laterally and posteriorly (Fig. 25.1d). The edges of the seromuscular layers are held by atraumatic Williams forceps to allow traction (Fig. 25.1d). The mucosal tube is now freed completely (Fig. 25.2) and the blunt dissection of the seromuscular cuff is progressively carried downward, taking great care not to tear the mus-

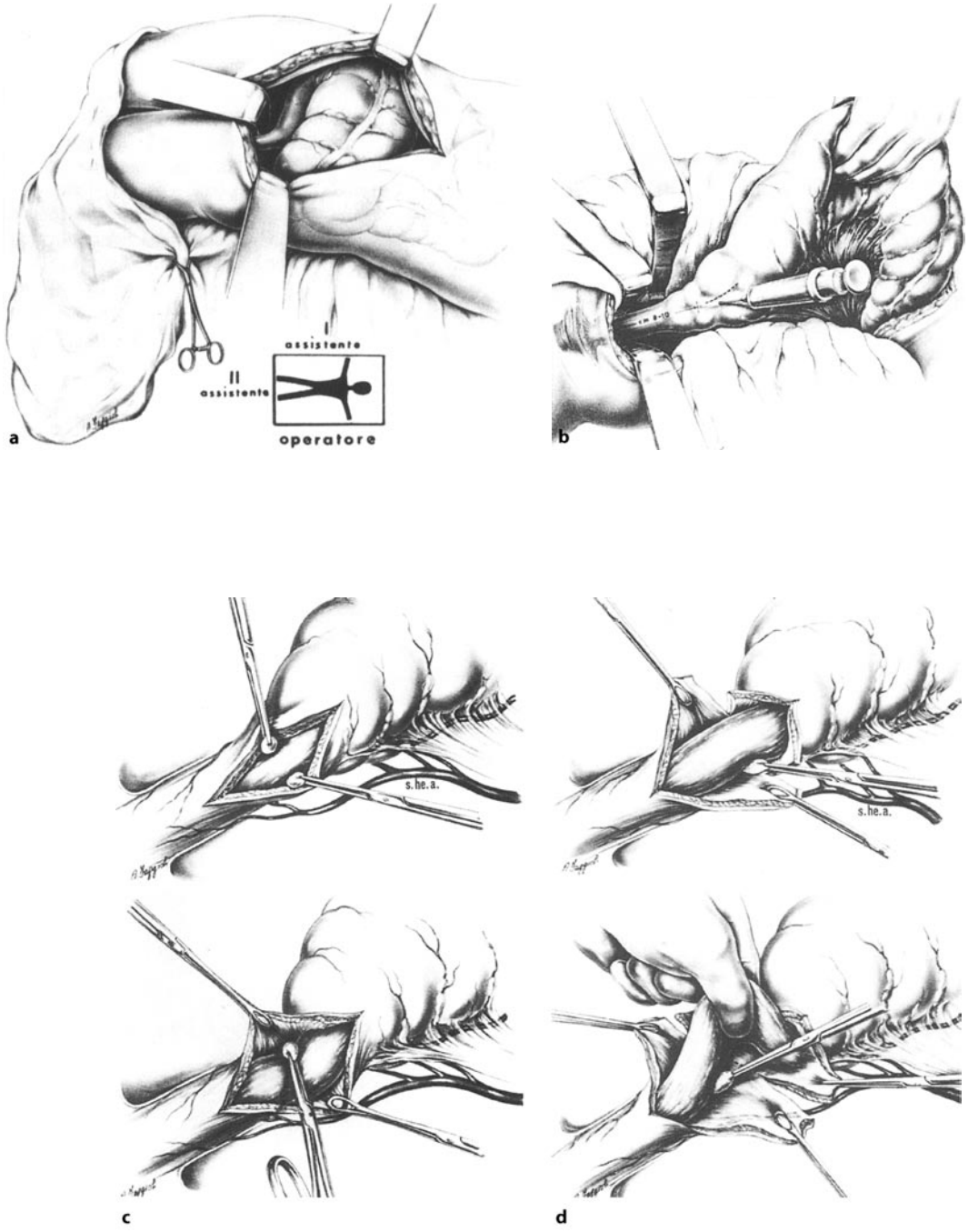

Fig. 25.1 **a** Position of the patient: the surgeon stands on the left, the first assistant on the right, and the second assistant at the feet of the patient. **b** Infiltration. **c**, **d** Incision and dissection of the muscular layer from the mucosa

cular sleeve or perforate the mucosal cylinder. Where the adhesions of the two layers are very tight (in older patients), the experienced surgeon can use scissors in a very delicate manner for dissection. The dissection is easier along the posterior wall of the rectum than along the anterior wall. Overturning of the rectal muscular cuff gives a full view of the adhesions and of the intramural vessels to be coagulated and divided. The use of delicate malleable retractors (Fig. 25.2c) and of the operator's finger (Fig. 25.2d) may be very useful in dividing residual adhesions and in separating the distal 4 cm of the rectal muscular cuff from the internal mucosal tube. The detachment is completed only when a distal level of 1 to 1.5 cm from the pectinate line is reached. A very low endorectal dissection is very important to avoid the transformation of a classic HSCR into a short form thus producing persistent symptoms. To check how far the mucosal dissection has been carried out a finger is inserted into the anus and a finger between the two cylinders (Fig. 25.3a). When the separation is completed, the space between the two tubes is packed with moistened gauzes which are left in place for the time necessary for the extensive mobilization of the colon (Fig. 25.3c).

25.3.4 Pull-Through Procedure

The gauze is removed, the anus is exposed by raising the previously draped lower extremities, a Petzer catheter is introduced into the lumen of the rectal mucosal cylinder, and a strong silk suture is tied around the proximal mucosal tube just below the large head of the catheter (Fig. 25.3d). The catheter is gently pulled down along with the mucosal cylinder which is everted as traction is continued (Fig. 25.4a). The outer layer is cut circumferentially, leaving the Petzer catheter tied to the inner one

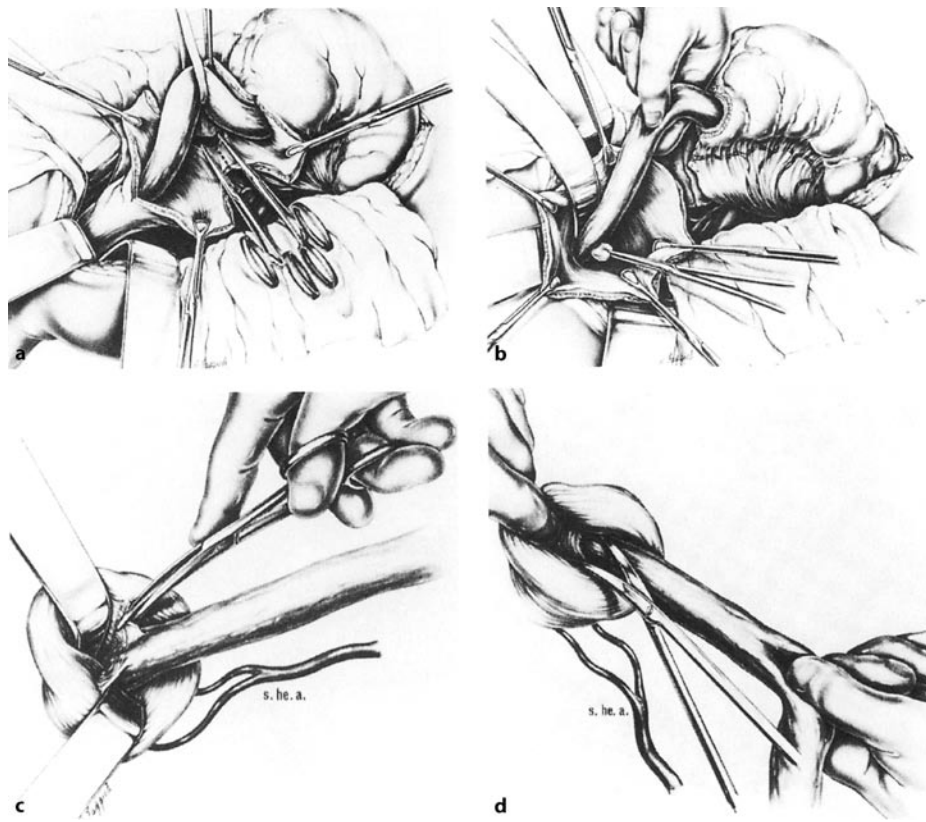

Fig. 25.2 **a** The mucosal tube is freed completely and the muscular cuffs is cut off. **b** Progressive separation of the two layers. **c** Overturning of the muscular coat to dissect strong adhesions. **d** The use of the operator's finger may be useful for cutting residual adhesion

(Fig. 25.4b). Traction on the mucosal tube attached to the proximal bowel pulls the colon (Fig. 25.4c, d) down to the proximal point of resection that corresponds to the seromuscular biopsy that has shown a normoganglionic result. After the pull-through procedure is completed outside the anus there are two concentric cylinders organized as follows: external everted rectal mucosa and internal ganglionic colon.

25.3.5 Completion of the Abdominal Stage

The proximal free edge of the muscular cylinder is sutured to the seromuscular layer of the pulled-through ganglionic colon. No sutures are placed posteriorly between the muscular tube and the seromuscular layer of the colon in order to leave the blood supply intact. In order to better reinforce the fixation of the colon inside the rectal muscular layer, we generally inject fibrin glue between the two cylinders. This injection also ensures adequate hemostasis of the surface of the external cylinder. Any residual opening between the colon and the posterior peritoneum is closed to prevent internal hernias. A Penrose drain is left in place within the peritoneal cavity.

25.3.6 Completion of the Perineal Stage

We prefer to maintain a not too long stump. For this reason we resect the pulled-through colon 5 to 6 cm from the anal verge (length related to the age of the patient). The perineal stage is completed by anchoring the seromuscular coat of the colon to the everted rectal mucosa. A rectal tube is inserted into the lumen of the pulled-through colon only in older patients. When the operation is carried out in newborns or infants it is better not

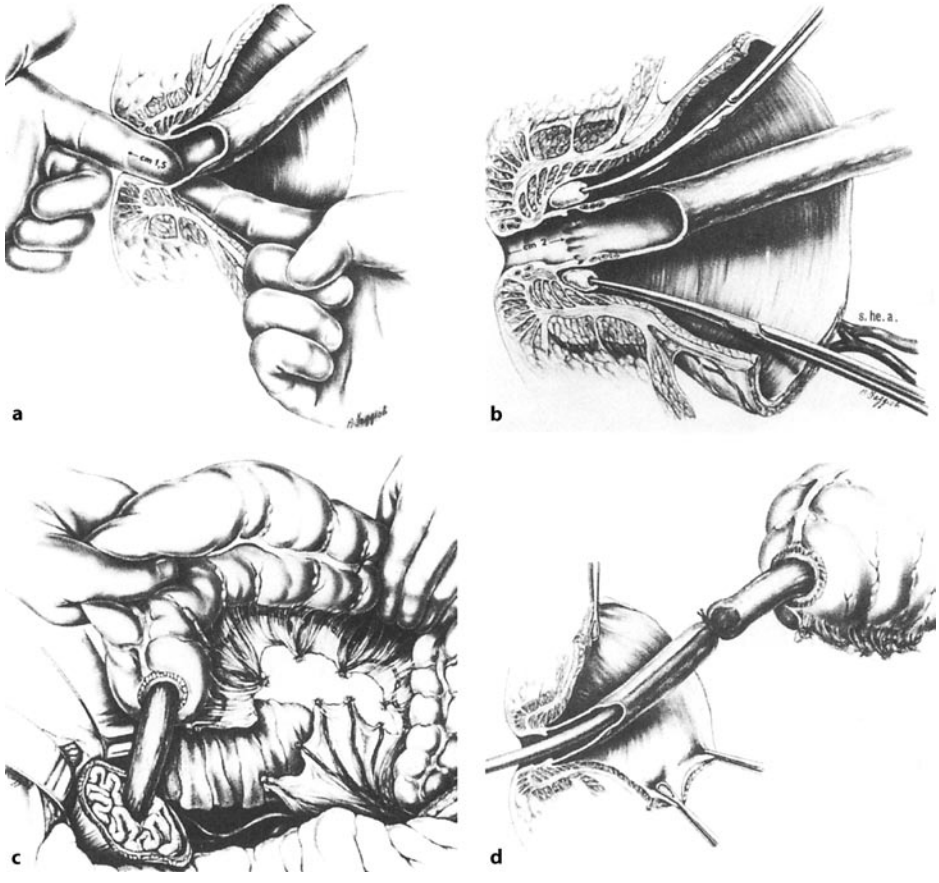

Fig. 25.3 **a** One can check how far the mucosal dissection has been carried out. **b** The mucosal separation is completed. **c** The space between the two cylinders is packed with moist gauze. **d** A Pezzer catheter is introduced into the lumen of the rectal mucosal cylinder and a ligature tied around the mucosal tube

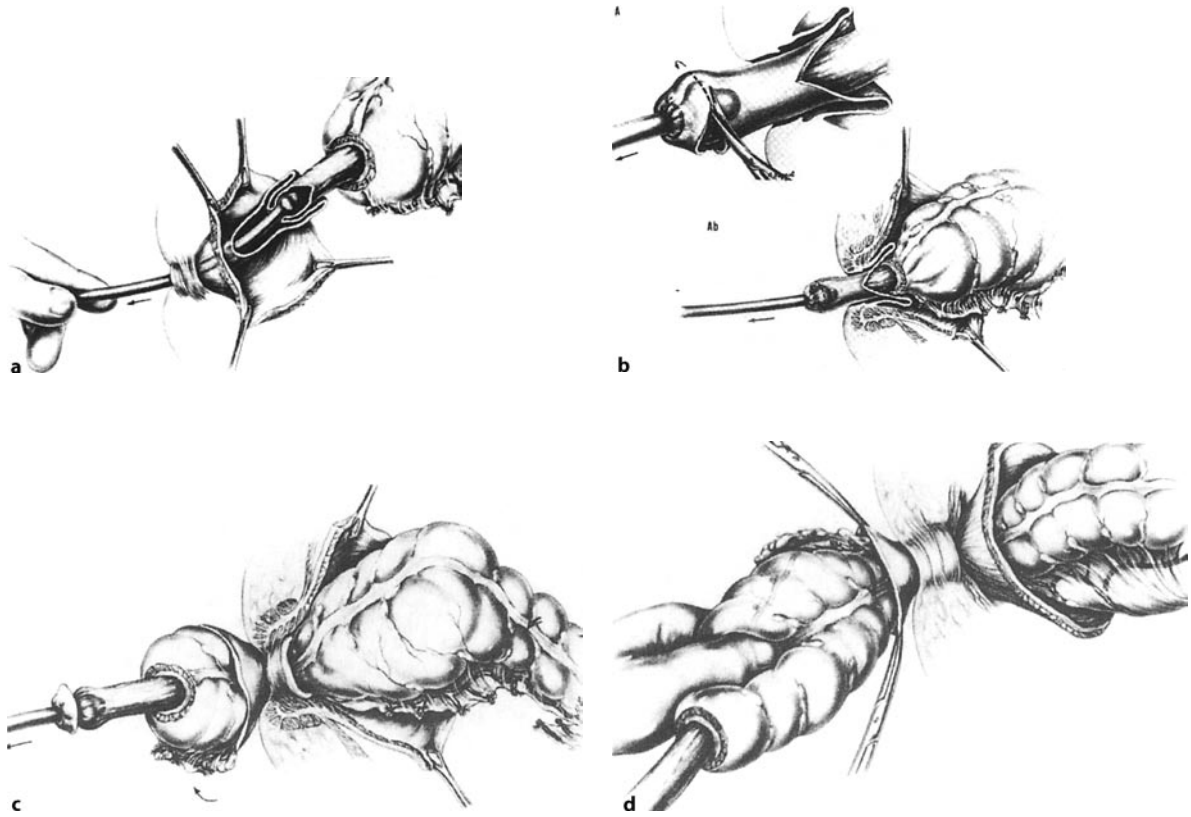

Fig. 25.4 **a** The Pezzer pulls the mucosal cylinder down and everts it. **b** The outer mucous layer is cut. **c**, **d** Pull-through of the colon. Two cylinders are thus formed

to insert any tube to avoid the risk of compression and ischemia of the pulled-through bowel.

25.3.7 Resection of the Protruding Rectal Stump

The second stage of the operation is performed after no more than 7 days. During this period adhesions form over the whole length of the rectal muscular coat and the serosa of the colon, which also adheres to the everted rectal mucosa at the level of the anal canal and outside the anal opening.

Two long curved forceps are applied to the stump and to the everted rectal mucosa for hemostasis and a long longitudinal incision is made with diathermy. A first silk suture (Fig. 25.5a) brings the residual everted mucosa close to the colonic mucosa. In order to re-establish mucosa-to-mucosa continuity, a circular incision of the two cylinders is made step by step as close as possible to the anus (Fig. 25.5b). The protruding stump is resected by cutting the outer mucosal layer as close to the anal verge as possible and the inner colon 1 cm longer. The two mucosal layers are approximated with interrupted sutures, thus avoiding the possibility of a stricture. The stump subsequently spontaneously retracts into the anal canal.

25.4 Anatomic Postoperative Condition

By dissecting endorectally the muscular layer from the mucous coat, the lumbosacral and sacral plexuses are preserved and disorders of the bladder and genital organs avoided. Endorectal dissection also completely avoids any lesion of the ureters and vas deferens in males. The normal residual rectal muscular coat avoids any possible lesions of the sphincteric muscle. This is the reason why fecal incontinence is not a complication of Soave's procedure, when correctly performed. After the procedure, the neorectum presents a double muscle layer. The exter-

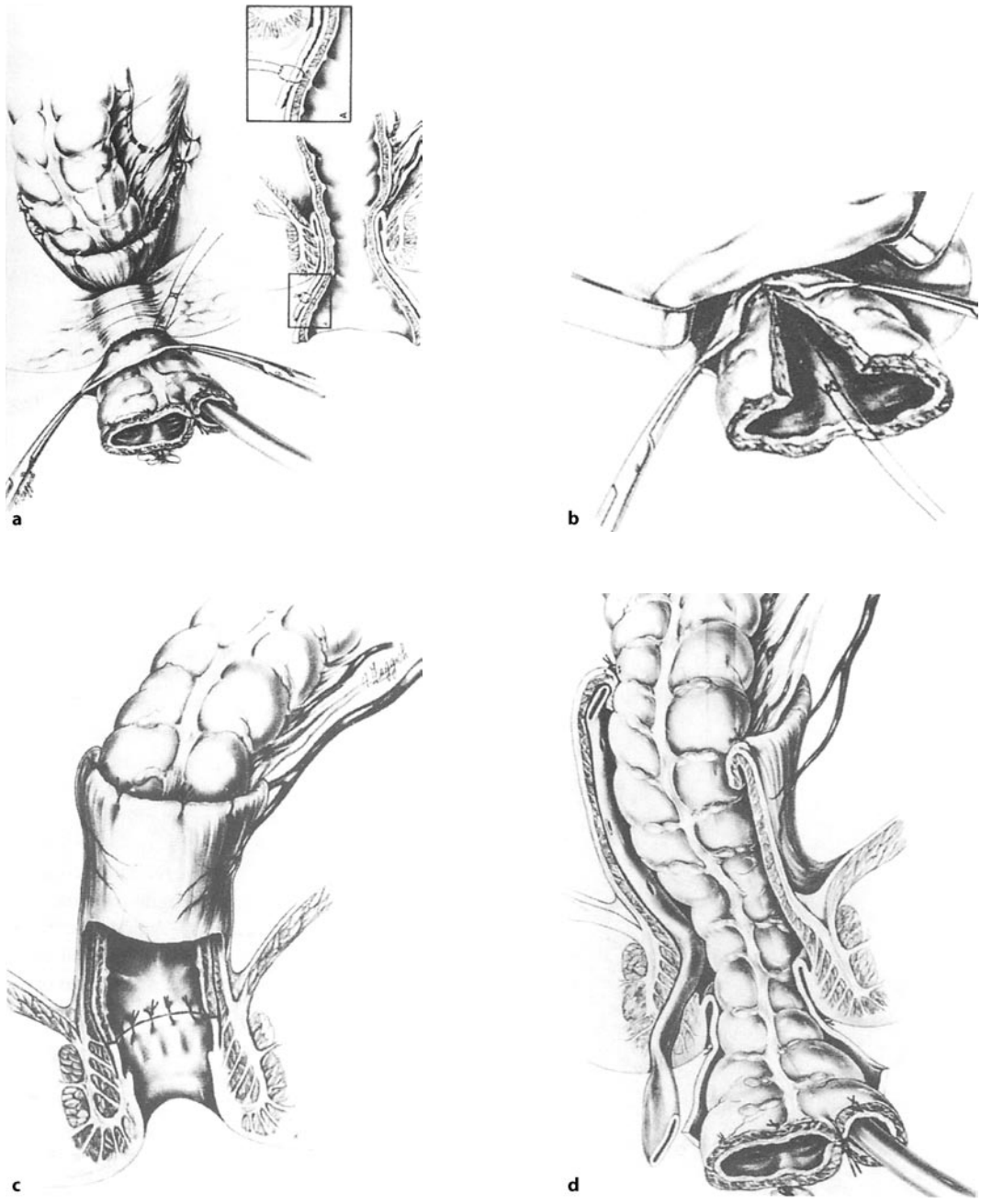

Fig. 25.5 **a** Disposition of the pulled-through colon. **b** Resection of the protruding stump **c** Muco-colonic anastomosis. **d** Drainage

nal coat is represented by the original rectal muscle layer, contiguous with the sphincteric complex, thus ensuring continence. The internal coat is represented by the ganglionic muscle layer of the pulled-through colon whose function is to ensure a normal progression and evacuation of stools.

25.5 Modifications of Soave's Technique

25.5.1 Boley's Primary Anastomosis

To avoid the need for a second procedure for colonic stump resection, Boley proposed a primary coloanal anastomosis. This operation was described by Boley [7] in 1964 and is well known all over the world. During the last 5 years we have adopted the primary coloanal anastomosis (Soave-Boley procedure) in most patients without a significant increase in complications.

25.5.2 Mark's Split Sleeve

In 1972, Kasai et al. [21] proposed a new operation for HSCR defined as "rectal myotomy with colectomy". One year later, Marks [22] proposed some modifications to Soave's endorectal pull-through, which include the following [23]. The mucosa is stripped by blunt dissection as far as possible towards the pectinate line and then divided. Each end is oversewn to preserve the sterile field. The dissected mucosa is grasped transanally and everted externally to ensure denuding of the muscular sleeve that is transected. The peritoneum of the cul-de-sac is incised circumferentially at the reflexion and the sleeve is shortened to lie below the pelvic floor. A posterior median proctomyotomy is performed longitudinally with diathermy in between long clamps from no more than 1 cm above the pectinate line, resulting in a partial internal sphincterectomy. The purpose of these modifications is to avoid postoperative constipation due to internal anal sphincter achalasia and rectal sleeve. However, even though this technique has been adopted by many surgeons, our opinion is that wide preoperative dilatations of the anal canal are sufficient to reach the same goal.

25.6 Treatment of Hirschsprung's Disease

25.6.1 Group 1: From 1955 to 1983

In the pioneering years preceding the 1960s and before Soave's technique was developed, 31 HSCR patients were operated on with the Swenson technique in 14 and the Duhamel procedure in 17. After this period and until 1983, a larger series of 339 patients with HSCR was observed [15]. Of these patients 271 were operated on following the principles of extramucosal endorectal pull-through. From 1961 to 1965, endorectal dissection was completed endoanally as described in the original technique (34 patients treated). From 1965, the whole endorectal dissection was abdominal and extramucosal (237 patients treated). Early complications in the treatment of HSCR with Soave's technique from 1961 to 1983 were seen in 11.2% of the patients. Seven patients died in the early postoperative period: three due to massive bilateral pneumonia and heart-respiratory failure, and four as a consequence of severe peritonitis. Other early complications included rectal cylinder abscess (five patients), rectal stenosis (five), pelvic abscess (five), intestinal obstruction (three), persistent severe constipation (four), persistent enterocolitis (four), evisceration (three), retraction of the stump (three), and necrosis of the endorectal bowel (two). It is definitely important to observe that, in this period no patient under 3 months of age was operated on with radical surgery. In fact, the most suitable age for definitive surgery was considered to be after 5 months. With regard to long-term results of the early period of Soave's procedure (1961 to 1963), the largest series of patients with a proper follow-up was reported by Soave [24, 25] in 1977 and 1978. This series included 147 patients, 71 of whom were aged between 10 and 19 years with a mean follow up of 10 years. Soave reported persistent symptoms in 16% of the patients. The main complaints were chronic enterocolitis (6%), persistent constipation (5.6%) and soiling (5.6%).

25.6.2 Group 2: From 1984 to 2004 (Intraoperative Assessment of Extent of Aganglionosis)

HSCR is a very complex genetic disorder with a wide phenotypic heterogeneity. For a correct interpretation of the results and complications of surgical treatment, it is worth considering not only the possible technical errors during surgery but also the possible misdiagnosis of the underlying disease. The success of surgery is jeopardized by three major types of early and late complications. The first is nonspecific and can occur as a consequence of any abdominal surgery (bridles, adhesions, evisceration, or infections). The second is specifically related to the technique adopted; in the case of Soave's procedure these complications include rectal cylinder abscess (between the muscle sleeve and the pulled-through bowel). These depend on the pediatric surgeon's technical skill and experience with extramucosal endorectal pull-through. Finally, the third type of complication is mainly related to misdiagnosis and therefore to incomplete surgery. These complications can be avoided by the use of intraoperative diagnosis of the extent of the aganglionosis.

In 1983, at Gaslini Children's Hospital, we introduced the alpha-naphthylesterases histochemical technique [16, 17] for intraoperative assessment of normoganglionic, hypoganglionic and aganglionic bowel. This intraoperative diagnosis became routine in 1984 [14–18] and is now used in the Pathology Department of our institute for each patient undergoing HSCR surgery. During the period 1984–2004, 695 patients with intestinal dysganglionoses were diagnosed histochemically in our Department of Pediatric Surgery. Of these patients, 480 (69%) had classic forms of HSCR according to the 1976 classification of Bettex [26], 31 (4.5%) had ultralong forms, 18 had hypoganglionosis, 5 were unclassifiable and 161 had intestinal neuronal dysplasia (IND). In all patients with HSCR, it was possible to demonstrate a hypoganglionic segment of variable length proximal to the aganglionic bowel and extending into the dilated colon (Fig. 25.6). In our institute, the whole hypoganglionic segment has always been resected along with the aganglionic one.

Out of 480 patients with classic HSCR, 427 were treated using Soave's procedure. This group of patients (group II in Table 25.1) were characterized by a truly radical treatment and an improved perioperative care. The results achieved are summarized in Table 25.1 (only those with classic HSCR treated using Soave's procedure are included). Early complications occurred in 17 of the 427 patients (3.9%) who underwent Soave's procedure. Among them, three presented with intestinal obstruction due to adhesions of the small bowel (0.7%). A 2-month-old male patient presented with early rectal stenosis related to an incorrect mucocolonic anastomosis, which was successfully treated with repeated dilatations. One patient with rectal cylinder abscess presented with fever on postoperative day 5 and the diagnosis of rectal cuff abscess was made by CT evaluation. The patient was scanned from the base of the bladder to the perineum at intervals of 5 to 10 mm, parallel to the pubococcygeal line (Fig. 25.7) [27]. The most frequently observed early complication was postoperative enterocolitis which was seen in 11 patients (2.6%). Six patients (1.4%) complained of persistent enterocolitis as a late complication. Mild constipation was experienced by 2.6% of the patients and was generally related to incomplete endorectal mucosal dissection which resulted in a too-high anastomosis. These patients were successfully treated by repeated anorectal dilatations. It is important to observe that in neither group I nor group II have the complications of actual fecal incontinence, bladder dysfunction or erection or ejaculation problems been seen following Soave's procedure. In fact, these late complications are not justifiable after a correctly performed endorectal pull-through.

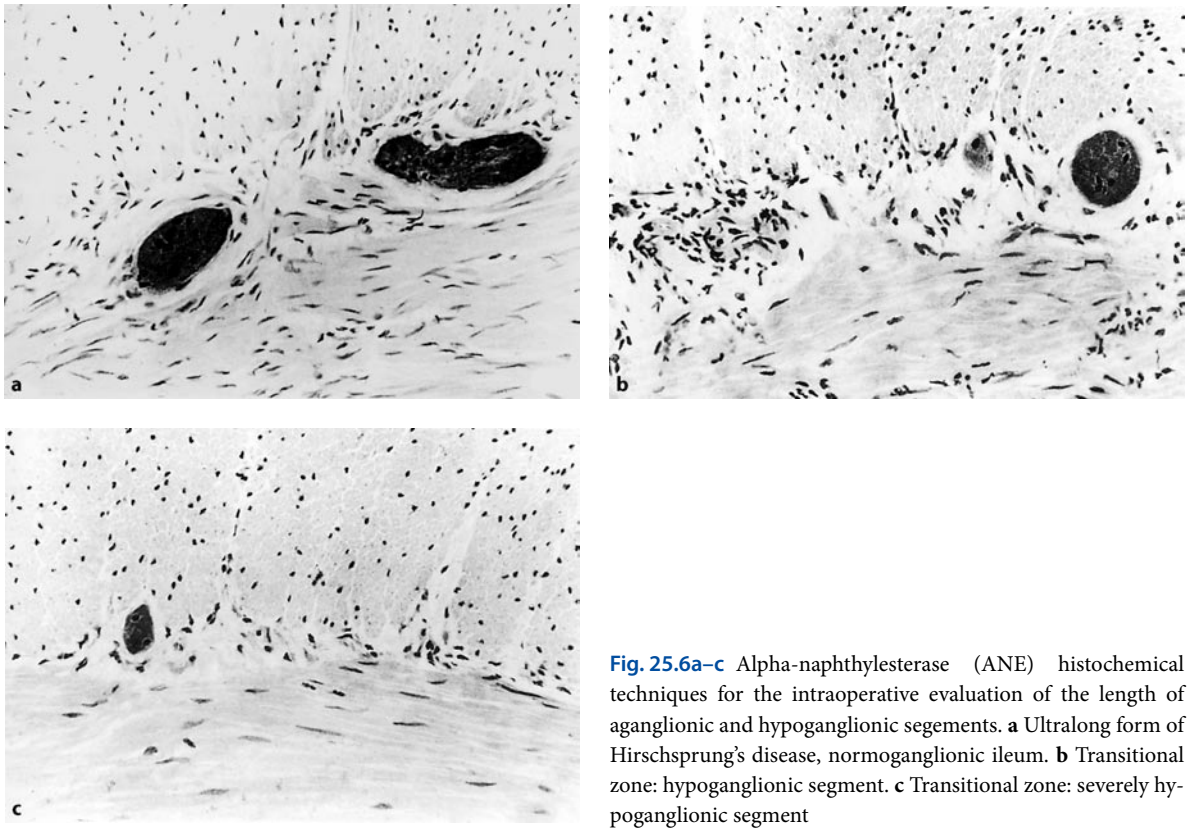

Fig. 25.6a–c Alpha-naphthylesterase (ANE) histochemical techniques for the intraoperative evaluation of the length of aganglionic and hypoganglionic segements. **a** Ultralong form of Hirschsprung's disease, normoganglionic ileum. **b** Transitional zone: hypoganglionic segment. **c** Transitional zone: severely hypoganglionic segment

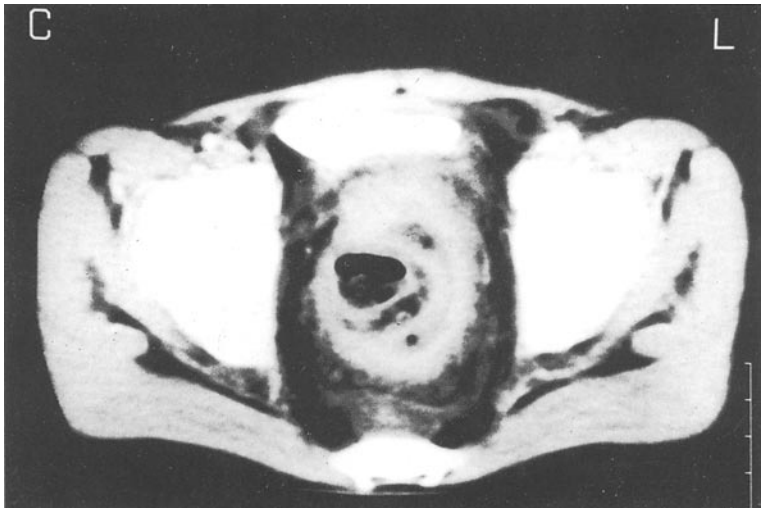

Fig. 25.7 Postoperative pelvic CT shows a rectal cylinder abscess between the muscular rectal sleeve and the pulled-through colon in Hirschsprung's disease operated on with Soave's procedure. The space between the two cylinders shows air and liquid content

Table 25.1 Complications in two groups of HSCR patients undergoing Soave's procedure at the Department of Pediatric Surgery of Gaslini Children's Hospital

	Group I (1961–1983)	Group II (1984–2004)
Patients treated		
Total	271	427
Age less than 3 months	0 (0%)	73 (15.2%)
Early complications	50/271 (18%)	17/427 (3,9%)
Intraoperative death	5 (1.8%)	0 (0%)
Early postoperative death	7 (2.6%)	0 (0%)
Rectal stenosis	5 (1.8%)	2 (0.5%)
Rectal cylinder abscess	5 (1.8%)	1 (0.2%)
Peritonitis	4 (1.5%)	0 (0%)
Intestinal obstruction	3 (1.1%)	3 (0.7%)
Persistent constipation	4 (1.5%)	0 (0%)
Persistent enterocolitis	4 (1.5%)	11 (2.6%)
Evisceration	3 (1.1%)	0 (0%)
Retraction of the stump	3 (1.1%)	0 (0%)
Necrosis of the pulled-through bowel	2 (0.7%)	0 (0%)
Pelvic abscess	5 (1.8%)	0 (0%)
Late complications		
Chronic enterocolitis	6%	6 (1.4%)
Constipation	5.6%	13 (3%)
Soiling	5.6%	0 (0%)
Downright fecal incontinence	0%	0 (0%)
Bladder dysfunction	0%	0 (0%)
Erection and ejaculation problems	0%	0 (0%)

25.6.3 Interpretation of Persistent Chronic Enterocolitis

Chronic enterocolitis can present as a late complication of Soave's procedure and also of other types of radical surgery. The causes of persistent enterocolitis are multifactorial and controversial. The most frequent cause is probably incomplete surgery with persistence of aganglionic or hypoganglionic pulled-through bowel. This condition predisposes to fecal stasis, chronic bowel mucosal inflammation and secondary enterocolitis. This accounts for the different frequencies of chronic enterocolitis in group I (6%) and group II (1.4%) (see Table 25.1). Another controversial cause of persistent enterocolitis is the possibility of associated IND [28]. In fact, this dysplastic innervative pattern is frequently observed in the bowel segment proximal to the hypoganglionic one [29]. In most cases the transitional zone is characterized by a distal aganglionic segment, intermediate hypoganglionic and proximal dysplastic one. Usually, the dysplastic segment is relatively short and seems to be a histochemical feature typical of the transition zone. In rare, different cases, the intraoperative histochemical study can show a very different condition characterized by an IND dysplastic segment even longer than the aganglionic one.

The clinical impact of HSCR with associated IND was investigated by Hanimann et al. [30] in 1991. The Authors considered two different groups of HSCR patients, those without IND and those with associated IND. There were no significant differences in early and late complications and in the results between the two groups, suggesting that the association HSCR-IND is a distinct disease from isolated IND and that the IND-involved segment may be retained without increased risk of morbidity. In order to identify a possible IND pattern in the aganglionic segment, we decided to use intraoperative combined histochemistry: rapid-AChE [18], recently developed at Gaslini Children's Hospital in Genoa, and ANE [14, 16, 19, 20]. The association of these techniques made it possible to recognize both mature ganglion cells and their distribution within the myenteric plexus, and the acetylcholinesterase-positive nerve fibers in the muscle layer.

In group II (1984–2004), patients with postoperative chronic constipation repeated rectal suction biopsy of the neorectum did not show persistent aganglionosis or an associated IND pattern, but only a slight increase in cholinergic fibers in the lamina propria without other Borchard criteria [31]. In contrast, the histomorphological study showed a chronic follicular colitis, with a mean of three lymphatic follicles per section. The lymphatic infiltration of the lamina propria was massive. According to the previous studies by Immura et al. [32] in 1992 and Puri et al. [33] in 1994, we consider this enterocolitis to represent a complex alteration of the immune response of the mucosa that can be associated with HSCR even after surgery.

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Rehbein's Procedure (Deep Anterior Resection)

26

A. M. Holschneider and R. Rassouli

26.1 Principles

Classical Hirschsprung's disease is characterized by two phenomena: the narrow segment and the decreased opening ability or achalasia of the internal sphincter. Any surgical method of treatment for Hirschsprung's disease must thus fulfill two criteria:

1. Removal of the aganglionic narrow segment, including the dilated sigmoid.
2. Elimination of the achalasia (defective opening) of the internal anal sphincter.

Both criteria can be met by the abdominal resection, which has been in use since 1953 [1]. This method is useful not only for the most common form of Hirschsprung's disease, where a short narrow segment is present in the rectosigmoid, but is also suitable for the forms with a long narrow segment or with aganglionosis of the entire colon. It can also be in those with megarectum (short and ultrashort segment). The method of interposition of small bowel with preservation of the ileocecal valve, reported by Sauer and Fasching in 1993 [2], is also used in patients with total colonic aganglionosis. In the further course of this operation the anastomosis between the ileal and rectal stump is made according to the principle of Rehbein's method.

The following description of the operative technique is limited to the most commonly occurring typical form, in which the narrow aganglionic segment is confined to the rectosigmoid.

26.2 Age at Operation

We have generally performed the abdominal resection at 6 to 9 months of age. We think that an earlier operation cannot be considered sufficiently safe.

26.3 Colostomy: Yes or No?

In newborns on principle and infants in poor overall condition, we do not perform a primary resection, but rather do a colostomy immediately proximal to the aganglionic segment. Our operative technique in creating the two-way colostomy corresponds to that described by Nixon [3, 4] utilizing a triangular skin flap which is laid down as a "bridge" under the pulled-out colon. The stoma is usually established at the hepatic flexure. The narrow segment can be resected 4–6 months after surgery for the colostomy, when the infant has attained a weight of 500–600 g and has continuously thrived for 3–4 months. The colostomy is left in place during the resection of the narrow segment but taken down 2 weeks later after having performed a contrast medium enema to ensure that the anastomosis has healed.

We never performed a primary resection in newborns because the histological and histochemical diagnosis is not safe enough in this age group, the maturation of the

bowel having not yet finished. The only possible histological differentiation in this age between present ganglion cells and aganglionosis does not allow the different types of neuronal intestinal malformations to be identified or the length of the involved segment above the aganglionic segment to be recognized. Some primary resections carried out in newborns will be unnecessary or inadequate. Furthermore, aganglionic, hypoganglionic or neuronal intestinal dysplastic bowel segments may be left in situ and may cause postoperative obstruction. On the other hand, colostomy is a safe, mostly uncomplicated procedure and allows biopsies from the colostomy side in both the oral and aboral directions to obtain more detailed information on the length of the malformed bowel segment and its specific histomorphological structure. However, infants of more than 3 months of age undergo primary resection without colostomy.

26.4 Our Modification of Rehbein's Technique

Our surgical technique differs from that of Rehbein in several points.

We do not puncture the bladder intraoperatively in order to empty it, but place a catheter preoperatively, which is left for about 1 week to avoid pressure from the filling bladder on the anastomosis. Besides, some patients are unable to void immediately after the operation, especially those with a simultaneous bladder enlargement. Before removing the bladder catheter, these children require bladder training with closure of the catheter for increasing times until spontaneous voiding is possible.

The child is placed in a semilithotomy position, the pelvis elevated and the legs abducted so that a gastrointestinal anastomosis (GIA) stapler can easily be introduced transanally into the rectum. After a left transrectal incision, which is usually extended down to the symphysis, we expose the pelvis using a Denis Brown self-retractor. We consider this practice to be gentler than the inconstant pressure of retractors held by assistants. However, the specula of the self-retracting instrument are fixed only at the abdominal wall muscles. The bladder and the peritoneum of the pelvic floor are not pulled into the surgical field with holding sutures as suggested by Rehbein; additional retractors held carefully by the assistants expose them in the depths of the surgical field.

26.5 Mobilization of the Colon and Rectum

The intraabdominal operation begins with mobilization of the left colon once the vascular supply and especially the marginal arcade has been inspected. We always mobilize the left flexure and resect the megacolon after full mobilization, and anastomose the rectal stump to the upper quarter of the descending colon or the splenic flexure.

Four holding sutures are then placed at the lowest point of the resected rectum prior to division and clamped with curved clamps. The anterior aspect of the rectum is opened and held up with further sutures progressively, according to the extent of the division. These sutures are clamped with straight clamps to distinguish them from the others. After initial incision of the rectum, the latter is cleaned with Betaisodona sponges. The narrow segment and megacolon are then resected until nearly congruent lumens of both bowel segments are achieved, so that the anastomosis can be performed without major differences in caliber. The afferent colon is now grasped with a soft, round vascular clamp and 1 cm from the resectional border a pursestring clamp is inserted. The pursestring suture is then made mechanically by introducing a 3-0 prolene suture through the branches of the clamp (Fig. 26.1). The aganglionic segment is resected 2 mm distal to the pursestring clamp. The rectal stump is left open. The severed rectum stump can be exposed nicely when tension is applied to the holding sutures.

We dissect the rectum more extensively than Rehbein, almost down to the levator ani muscle, which means 3–4 cm down from the peritoneal reflection in infants and 4–5 cm in older children. If the anastomosis is stapled it is deeper in the pelvis than if it is hand-sutured. Postoperatively, the anastomosis should be palpable 3–4 cm above the dentate line. Intraoperatively this can be estimated by introducing a Hegar bougie in the anal canal.

26.6 Anastomosis

The anal canal and the anorectal sphincters are now dilated with Hegar bougies up to the size of the stapling devices which will be used for the anastomosis. Dilatation is then continued one size further, and the rectal stump revised. Unfortunately, no pursestring suture instrument small enough to be introduced into the depth of the small pelvis in infants is commercially available. Therefore, the pursestring suture at the rectal resectional border is created by hand as a continuous suture using 3-0 prolene. The stapling device is then introduced into the anal canal and the rectal pursestring suture knotted over its proximal part. After the transmural holding sutures have been removed, the oral bowel segment is pulled over the proximal part of the stapling device and knotted. The two parts of the device are approximated mechanically and the stapler is fired (Fig. 26.2).

Before removing the instrument two or three additional sutures are made at the anterior wall of the anastomosis and under careful tension on these sutures the instrument is removed. The complete anastomosis is now inspected carefully with the rectal holding sutures held under tension. It is checked for impermeability and

Fig. 26.1 Gastrointestinal anastomosis (GIA) stapling device in situ. The mechanical pursestring clamp is closed, the pursestring suture performed and the bowel resected. The pursestring suture at the rectal stump is tied

Fig. 26.2 Both of the pursestring sutures are tied and the stapling device is fired

attention is paid to ensuring that the mucosa has been buried securely. The holding sutures are now removed and the pelvic floor peritoneum is closed after a soft drain has been placed near the anastomosis and brought out of the abdominal wall extraperitoneally. This drain is left in place for 7 days. Additionally, a bowel tube is put transanally over the anastomosis to allow liquid stools and flatus to be easily evacuated. Flatus is usually passed spontaneously on the second or third postoperative day, and stool through the tube on the fourth day. If we have not established a colostomy preoperatively, we put the patient on parenteral nutrition for 6 to 7 days. On day 12 postoperatively a radiographic contrast enema is performed to ensure the sealing of the anastomosis [4]. If a stapling anastomosis is not possible, which might be due to shrinking of the colon after colostomy, we perform the anastomosis with interrupted sutures according to the Herzog's technique, using 5- or 6-0 atraumatic Vicryl in infants and 5- or 4-0 in children. The entire wall of the afferent colon and the rectum is included in the initial puncture, while only the mucosa goes into the returning puncture. At first, the posterior wall sutures are left long; they are tied only after all the sutures are in place. The anterior wall sutures are tied immediately. The holding sutures are removed as soon as the suture line has reached them. The knots lie within the bowel lumen. The last two anterior wall sutures no longer contain mucosa; they are simply Lembert sutures, with their knots on the outside. The anastomosis consists usually of one layer.

26.7 Differences in Caliber of the Rectum and Colon

If there is a large difference in lumen size between the afferent colon and the rectum, when the afferent colon is dilated, a triangular segment is removed from it according to Rehbein's suggestion, so that the afferent bowel segment becomes tapered like a funnel. When the rectal lumen is greatly dilated compared to the afferent colon—which is usually the case in the so-called secondary megacolon—the "back" of the afferent colon is incised according to the Denis Browne technique of end-to-back anastomosis in order to obtain congruent lumina to anastomose. We do not gather the rectum as Rehbein proposed.

26.8 Procedure for Long Aganglionic Segments

If there is a long aganglionic segment which includes the entire left colon or parts of the transverse colon as well, then the anastomosis must be made at the ascending colon or the hepatic flexure. For this, it is necessary to mo-

Table 26.1 Length of the involved specimen (*n*=186)

Short segment	9.7% (*n*=18)
Rectosigmoid colon	52.2% (*n*=97)
Colon descendens	20.4% (*n*=38)
Colon transversum	7.5% (*n*=14)
Colon ascendens	3.7% (*n*=7)
Total colon	4.3% (*n*=8)
Involvement of the small bowel	2.2% (*n*=4)

Table 26.2 Early complications (*n*=191)

Fistula/insufficiency	2.1% (n=4)/6.6% (*n*=13)
Stenosis	9.9% (*n*=19)
Bladder dysfunction	0.5% (*n*=1)
Wound infection	1.1% (*n*=2)
Ileus	5.8% (*n*=11)

bilize the entire right colon and the hepatic flexure and to divide the middle and right colic arteries, so that the bowel is perfused by the ileocolic artery and the marginal arcade of the cecum and ascending colon. The adequacy of perfusion must be tested carefully by trials of clamping. To make the anastomosis, the entire small intestine is displaced into the left half of the belly and the cecum and the ascending colon are turned 180 degrees, so that the cecum and appendix are transferred to the right upper quadrant and the hepatic flexure down to the small pelvis. It is advisable to perform an appendectomy once the anastomosis is finished. With this technique, we have also been able to anastomose the cecum to the rectum. If there is total aganglionosis of the colon, we anastomose the rectum to the distal ileum. At the time of writing we had 11 such patients under treatment, all of whom were doing well.

The advantages of intraabdominal resection in congenital megacolon and allied disorders are obvious: no repositioning of the patient, no position changes for the surgeon and the assistants, and the procedure can be used for any form of megacolon, even resections of too-long residual narrow segments. Anastomosis is always possible, even with large differences in caliber.

26.9 Own Results with Rehbein's Technique

The experience with Rehbein's procedure in the German-speaking countries was collected in a study in 2002 [5]. We were able to gather the data of 200 patients from 22 centers. The data were collected by questionnaire and the children were followed up in the individual participating hospitals for at least 3 years after the procedure, which had been done between 1993 and 1997.

The histological evaluation of the resected specimen showed Hirschsprung's disease with an ultrashort segment in 18 (9.7%) of the patients. A typical Hirschsprung's segment involving the rectosigmoid colon was found in 97 (52.2%) of the patients. The colon descendens was affected in 38 (20.4%) of the children, the colon transversum in 14 (7.5%), and the colon ascendens in 7 (3.7%). Eight (4.3%) of the children suffered from total aganglionosis. The small bowel was involved in 4 (2.2%) of the patients (Table 26.1).

26.9.1 Early Complications

An anastomotic leak was observed in 13 out of 191 patients (6.6%) leading to fistula in 4 patients (2.1%). An anastomotic stricture developed in 19 patients (9.9%). However, in 4 patients with anastomotic leak, a new colostomy had to be established and the leak disappeared spontaneously. In 2 patients the leak was closed again by a new suture. Urinary dysfunction, which was observed in only 1 patient (0.5%), resolved without further treatment after a few weeks. A wound infection was observed in 2 patients (1.1%) and early postoperative ileus in 11 (5.8%) patients (Table 26.2).

26.9.2 Late Complications

Altogether 161 of 191 patients (61.4%) recovered completely from the surgical procedure without further bougienage or other treatment. However, 43 patients (22.8%) suffered from persistent postoperative constipation, which was treated by sphincter dilatations and in 15 of the patients (7.9%) a further resection due to a hypoganglionic segment was necessary. A sphincteromyectomy was performed in 9.4% of the patients (Table 26.3).

Recurrent enterocolitis was observed in 20 (10.6%) of the patients. Stool incontinence occurred in 8 (4.2%) of the patients. These 8 patients suffered from chronic constipation, so that this incontinence must be seen as an overflow incontinence. Two children (1.1%) died, one because of a *Candida* sepsis and one from pneumonia. Other late complications (e.g. ileus, subileus) were found in 18 patients (9.5%) (Table 26.4).

Table 26.3 Reoperations (n=191)

Sphincter dilatation	52.9% (n=101)
Sphincteromyotomy	9.4% (n=18)
Resection	7.9% (n=15)
Others (e.g. adhesiolysis, colostomy)	12.7% (n=24)

Table 26.4 Late complications (n=189)

No late complications	61.4% (n=116)
Enterocolitis	10.6% (n=20)
Constipation	22.8% (n=43)
Incontinence	4.2% (n=8)
Late lethality	1.1% (n=2)
Enuresis	0.5% (n=1)
Others (e.g. Ileus)	9.5% (n=18)

Table 26.5 Anastomotic stricture/anastomotic insufficiency/fistula

Surgical procedure	Reference	Stenosis (%)	Insufficiency/fistula (%)
Swenson	8	7.6	5.6/3.3
Soave	11, 12	0.7/8.2	2.0
Duhamel	9	0.7	2.2
Rehbein	10	8.7	3.4/1.3
Rehbein	5	9.9	6.0/2.1

26.9.3 Comparison of the Different Techniques

The results of the latest German study [5] were compared with the results of large series in the literature, including the series of patients reported by Hoffman-von-Kap-herr and Enger [6], by Holschneider [7] (427 patients) on the different techniques, by Sherman et al. [8] (814 patients) on Swenson's procedure, by Bourdelat et al. [9] (2430 patients) on Duhamel's procedure, by Fuchs and Booss [10] (146 patients) on Rehbein's procedure, and by Jasonni and Martuciello [11] (298 patients) and Teitelbaum et al. [12] (134 patients) on Soave's technique.

Holschneider's series of patients from 1982 [7] is the only series which has been collected by the same team in various hospitals, so that this series of 427 patients is the most objective in the literature. The series of Bourdelat et al. (2430 patients) is based on a questionnaire without a personal follow-up.

In the German study [5] we observed more anastomotic leaks than in the series of Holschneider in 1982 or the series of Fuchs and Booss in 1999. However, the incidence was lower than the incidence of leaks after Swenson's procedure, but as high as the incidence after Duhamel's and Soave's technique. Jasonni and Martuciello [11] and Teitelbaum et al. [12] have reported excellent results with Soave's technique in their latest report (Table 26.5).

26.9.4 Anastomotic Leak

In total the incidence of anastomotic leak after Swenson's procedure varies in the literature between 5.6% and 14.5%, after Duhamel's procedure between 2.2% and 9.5%, after Rehbein's procedure between 2.6% and 3.4% and after Soave's endorectal pull-through between 0.3% and 7.7% (Table 26.6).

26.9.5 Rectal Stricture

The frequency of rectal stricture was 9.9% in the recent German series of patients [5], 8.7% in the series of Fuchs and Booss [10], 7.6% in the series of Sherman et al. [8], 0.7% in the series of Jasonni and Martuciello [11] and 8.2% in the series of Teitelbaum et al. [12]. After performing Rehbein's technique the incidence of rectal stricture varies from 3.3% to 13.1%, after Swenson's technique from 7.6% to 10.5%, after Duhamel's technique from

Table 26.6 Anastomotic leak

Swenson			Duhamel			Rehbein			Soave		
Reference	Total no. of patients	Incidence (%)	Reference	Total no. of patients	Incidence (%)	Reference	Total no. of patients	Incidence (%)	Reference	Total no. of patients	Incidence (%)
6	2319	10.4	6	1108	6.9	6	864	2.6	6	858	5.2
7	76	14.5	6	63	9.5	6	176	2.8	6	105	7.7
8	880	5.6	9	2430	2.2	10	146	3.4	11	298	0.3
									12	134	2
Total		5.6–14.5			2.2–9.5			2.6–3.4			0.3–7.7
5								Leak 6.6, fistula 2.1			

Table 26.7 Rectal stricture

Swenson			Duhamel			Rehbein			Soave		
Reference	Total no. of patients	Incidence (%)	Reference	Total no. of patients	Incidence (%)	Reference	Total no. of patients	Incidence (%)	Reference	Total no. of patients	Incidence (%)
6	2306	8.3	6	970	28.7	6	563	3.3	6	711	11.6
7	76	10.5	7	63	14.3	7	176	13.1	7	105	23.7
8	880	7.6	9	2430	0.7	10	146	8.7	11	298	0.7
									12	134	8.2
Total		7.6–10.5			0.7–28.7			3.3–13.1			0.7–23.7
						5	191	9.9			

0.7% to 28.7%, and after Soave's technique from 0.7% to 23.7% (Table 26.7).

26.9.6 Enterocolitis

Periods of enterocolitis were observed in the latest German study [5] in 10.6% of patients and in the series of Fuchs and Booss [10] in 13.3% of the patients. The average number of patients suffering from enterocolitis was 12.3% in a large collective study performed by Snyder and Ashcraft [13] in 2000.

Comparing the different techniques in Holschneider's series [7], the incidence of enterocolitis was 13.2% after endorectal pull-through, 3.8% after Swenson's procedure, and 4.7% after Duhamel's technique. However, there are huge variations in the incidence of enterocolitis in different reports. The incidence ranges from 3.8% to 33.7% after Swenson's technique, from 4.7% to 13.9% after Duhamel's technique, from 6.3% to 13.4% after Rehbein's anterior resection and from 1.6% to 13.2% after Soave's endorectal pull-through (Table 26.8).

26.9.7 Stool Incontinence

Snyder and Ashcraft reported stool incontinence in 8.2% out of 6019 patients [13]. In the last German study [5] stool incontinence was proven in only 4.2% out of 189 patients. The incidence of stool incontinence was 12.6% in Holschneider's earlier study. It ranged from 4.2% to 12.6% after Rehbein's technique, from 3.1% to 14.3% after Swenson's technique, from 1.2% to 9.4% after Duhamel's technique and from 0.0% to 17.9% after Soave's technique (Table 26.9).

26.9.8 Constipation

The persistent aganglionic segment leads to higher frequency of chronic constipation after Rehbein's procedure. The length of that segment is in the range 5–6 cm. The incidence of chronic constipation was therefore 22.8% in the recent series, 7.9% in Holschneider's [7] earlier study and 15.4% in the series of Fuchs and Booss [10] (Table 26.12). On average chronic constipation occurred in 8.2% of patients after the various procedures according to Snyder and Ashcraft [13]. The frequency of constipation varied from 8% to 9.2% following Duhamel's technique, and from 2.7% to 10.4% following Soave's technique (Table 26.10).

However, regarding chronic constipation one has to take into consideration the time interval from the primary resection to the follow-up. Holschneider [7] observed that shortly after the procedure, 35.8% of patients were constipated but after 8 years only 7.9% of patients still suffered from constipation. The latest investigation following Rehbein's procedure [5] was performed on average of 3.5 years after the resection. Therefore, the incidence of 22.8% of constipated patients is not the final result, and will improve with time (Table 26.11).

In the recent German series [5] sphincter dilatation had to be performed in 52.9% of patients. Sphincter dilatations are a part of Rehbein's procedure. The dilatation starts before the resection and has to be repeated several times after healing of the anastomosis. It can be stopped 3 months after the definitive correction of the megacolon. Sphincteromyectomy had to be performed in 9.4% of patients as a result of recurrent anal sphincter achalasia. Re-resection had to be performed in 7.4% of our patients. The reasons were a hypo- or aganglionic segment left in situ, anomalies of the myenteric plexus or anastomotic stricture.

In the series of Holschneider [7] 49.4% of the children needed dilatations, 13.6% myectomies and 2.8% reoperation. In the study of Fuchs and Booss [10] 14.3% of the patients needed dilatations, 4.7% sphincteromyectomies and 7.4% reoperation (Table 26.12).

26.10 Final Considerations

The main and unsolved problem in Hirschsprung's disease is not the technique but insufficient preoperative diagnosis. The length of the eventual hypoganglionic segment above the aganglionic part of the bowel is hard to establish. Neurological malformations of the plexus myentericus are difficult to detect, therefore, in most patients reoperation is necessary due to a long hypoganglionic segment, malformation of the myenteric plexus, or a very low acetylcholinesterase activity in the nonresected colon or ileum. This problem is common in all operative techniques, and has not been solved by the newer technique of laparoscopically assisted endoanal pull-through [15].

Up to now the results of the transanal one-stage approach of De la Torre-Mondragon and Ortega-Salgado in only five series have been reported with only a very small number of patients and presenting only short-term results. The frequency of complications seem to be similar to those following open surgery, but the patients suffer less discomfort (Table 26.13).

In the techniques of De la Torre-Mondragon and Ortega-Salgado [15] and Georgeson [22], problems develop from anastomosing only the anal mucosa with the full-thickness of the colon. This problem is well known from the Soave-Boley technique. In Duhamel's procedure there might be problems with stapling of the anastomosis and the myectomy of the internal anal sphincter. Possible injury to the extramural nerve supply of the nervi erigen-

Table 26.8 Enterocolitis

Swenson			Duhamel			Rehbein			Soave		
Reference	Total no. of patients	Incidence (%)	Reference	Total no. of patients	Incidence (%)	Reference	Total no. of patients	Incidence (%)	Reference	Total no. of patients	Incidence (%)
2	390	15.6	2	339	5.9				2	280	6.4
7	80	3.8	7	64	4.7	7	176	6.3	7	106	13.2
14	89	33.7	14	713	13.9				14	370	13.8
8	859	22.5	9	2430	6.8	10	146	13.4	11	298	1.6
Total		3.8–33.7			4.7–13.9			1.6–13.2			0.7–23.7
						5	189	10.6			

Table 26.9 Incontinence/soiling

Swenson			Duhamel			Rehbein			Soave		
Reference	Total no. of patients	Incidence (%)	Reference	Total no. of patients	Incidence (%)	Reference	Total no. of patients	Incidence (%)	Reference	Total no. of patients	Incidence (%)
21	390	3.1	21	339	1.2				21	280	0.7
7	77	14.3	7	53	9.4	7	176	12.6	7	106	17.9
			14	713	2.5				14	370	4.3
8	860	12.9	9	2430	5.3	10	146	5.4	11	298	0.0
Total		3.1–12.9			1.2–9.4			4.2–5.4			0.0–17.9
						5	189	4.2			

Table 26.10 Constipation

Swenson			Duhamel			Rehbein			Soave		
Reference	Total no. of patients	Incidence (%)	Reference	Total no. of patients	Incidence (%)	Reference	Total no. of patients	Incidence (%)	Reference	Total no. of patients	Incidence (%)
7	80	10	7	65	9.2	7	176	7.9	7	106	10.4
8	No constipation mentioned		9	2430	8	10	146	15.4	11	298	2.7
Total		10			8–9.2			7.9–15.4			2.7–10.4
						5	189	22.8			

Table 26.11 Constipation (long-term follow-up)

Postoperative results	35.8%
Follow-up (3.5 years)	22.8%
Follow-up (8 years)	7.9%

Table 26.12 Incidence of postoperative sphincter dilatations, myectomies and reoperations

Reference	Dilatation	Myectomy	Reoperation
7	49.4	13.6	3.8
10	40.3	4.7	7.4
5	52.9	9.4	7.9

Table 26.13 Technique of De La Torre-Mondragon and Ortega-Salgado [15] and modifications. Preliminary results in the literature 1998–2001

Reference	Number of patients	Age at operation	Length of resected specimen (cm)	Complications
15	5	24 days to 21 months	11–20	No complications
16	9	3 weeks to 19 months	7.7–22	SID *n*=1, postoperative apneic spells *n*=1, constipation *n*=1, stricture *n*=1, muscle cuff narrowing *n*=1
17	10	Neonates	?	Stenosis *n*=1, enterocolitis *n*=1
18	24[a]	0.25 to 30 months	Rectosigmoid to transverse colon	Enterocolitis *n*=7, wound infection *n*=2, perineal excoriation *n*=11, stricture *n*=12
Höllwarth et al., personal communication	21[b]	?	9–27	Retrorectal abscesses *n*=1, stenosis *n*=1, stool incontinence *n*=1, Bougienage *n*=3
19	33	<1 to >6 months	6–9	Dilatations *n*=26 (79%), stricture *n*=1, enterocolitis *n*=2 (6%), Diarrhea *n*=3, Chronic constipation *n*=2

[a]Transanal Soave with routine laparoscopic visualization, 9; transanal Soave with selective laparoscopy or minilaparotomy, 15.
[b]Three hospitals.

tes to the rectum is an additional problem in Swenson's procedure. The main problem in Rehbein's procedure is the 4–5 cm aganglionic segment left in situ, which could be obstructive. This obstruction could get worse if there is an additional hypoganglionic oral border of the resected segment. Therefore, it is difficult to decide at what height the anastomosis should be placed. Accordingly, it is important to establish the anastomosis 4 to 5 cm above the dentate line to decrease the tone of the internal anal sphincter.

Karanjia et al. [20] in 1992 reported two series of adult patients. In 26 patients a deep anterior resection was performed with the anastomosis 3 cm above the dentate line and in 42 patients the anastomosis was established 6 cm above the dentate line. In those with the anastomosis 3 cm above the dentate line the mean bowel frequency per day, the difficulty in distinguishing feces from flatus, deferring the urge for 15 minutes, and the frequency of soiling were significantly greater postoperatively.

The aim of Rehbein's procedure is therefore to establish the anastomosis at the right level in order to decrease the tone of the internal anal sphincter but avoiding sphincter insufficiency. The effect of such a deep anastomosis is the same as a sphincteromyectomy. Rehbein's procedure is a difficult but very safe and effective method to treat Hirschsprung's disease and allied disorders. However, the technique cannot be performed laparoscopically. Therefore it has lost its importance, but should not be forgotten because it might be helpful as a secondary re-do procedure and anterior rectal resection for other reasons.

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Transanal Pull-Through for Hirschsprung's Disease

S. Somme and J. C. Langer

27

27.1 Introduction

The surgical treatment of Hirschsprung's disease has gone through several changes since Ehrenpreis in 1946 suggested that functional obstruction due to aganglionosis of the distal colon was the cause of the proximal colonic dilatation [1]. Swenson and Bill developed the first operation in 1948 that was successful in removing the aganglionic segment and establishing intestinal continuity [2]. Later, Duhamel developed the retrorectal anastomosis for Hirschsprung's disease [3] and Soave developed the endorectal pull-through [4]. Prior to the development of these operations, the only successful surgical procedure was a diverting colostomy.

Until recently these operations were always performed in two or three stages, the first stage being the placement of a diverting colostomy or ileostomy. "Leveling" colonic biopsies were obtained to determine the location of the transition zone as part of this first operation. The second stage was performed later, usually between 3 months and 1 year of age. This operation involved removing the aganglionic segment and creating an anastomosis between the normally innervated small bowel or colon and the anus. Some surgeons preferred to protect the anastomosis with a proximal loop stoma, and close the stoma at a third operation.

Over the past two decades it has become increasingly recognized that the routine use of a colostomy is unnecessary, and an increasing number of pediatric surgeons perform the reconstruction as a single stage procedure at an early age.

In this chapter, the transanal endorectal pull-through operation is described. The development of this operation was the result of a number of advances in the treatment of Hirschsprung's disease, including earlier diagnosis, better preoperative, perioperative and postoperative care, and the development and popularization of minimal access techniques in pediatric surgery.

27.2 Primary Pull-Through

In 1980 So et al. described the use of the Soave endorectal procedure as a single stage pull-through without a preliminary colostomy [5]. These and several other authors did the operation at several months age, managing the children with total parenteral nutrition (TPN) or elemental feeding and colonic irrigations to prevent retention of stool and secondary enterocolitis [5–7]. Since these initial reports there have been many single and multi-institution studies published that demonstrate the safety of single-stage repairs using each of the commonly performed operations [8–10]. In addition to minimizing the rate of complications due to the presence of a stoma [11] and decreasing the number of hospitalizations and cost [12], the avoidance of a colostomy has dramatically improved the quality of care to children with Hirschsprung's disease in developing countries, where multiple visits to the hospital may be impractical and the presence of a stoma represents a significant social stigma [13].

27.3 Development of the Transanal Pull-Through

With the rapid development of laparoscopic techniques in the early 1990s, pediatric surgeons began to move toward less invasive surgical procedures for a wide variety of conditions. Georgeson et al. reported a technique utilizing laparoscopic dissection of the rectum combined with an anal mucosal dissection in 1995 [14] and subsequently laparoscopic approaches to both the Duhamel and Swenson procedures were described [15, 16]. These are discussed elsewhere in this volume.

The transanal pull-through was an extension of the procedure of Georgeson et al., but without the laparoscopic intraabdominal dissection. This approach was concurrently described by De la Torre-Mondragon and Ortega-Salgado [17] and by Langer et al. [18] in 1998 and 1999. Since then the technique has evolved and a number of variations have been described. Despite the fact that this technique has now been widely adopted by surgeons all over the world, there remain a number of controversies regarding the optimal approach. The technique is described in this chapter, the data supporting its use is reviewed, and the ongoing controversies in its use are outlined.

27.4 Surgical Technique

The technique is illustrated in Fig. 27.1.

27.4.1 Preoperative Preparation

The diagnosis is confirmed by rectal biopsy. Prior to surgery, the colon must be decompressed and enterocolitis, if present, controlled. Nutritional status must also be evaluated and optimized. It has been shown that even patients presenting with intestinal obstruction or enterocolitis may respond well to aggressive nonsurgical management with antibiotics, decompression, and support [19]. In an older child with severe enterocolitis or massive colonic distension, a defunctioning stoma should be considered. We have found that routine preoperative mechanical bowel preparation is unnecessary and can cause significant distension and vomiting. Mechanical irrigation of the bowel can be accomplished with equal effectiveness from below once the child has undergone anesthesia. Intravenous prophylactic broad spectrum antibiotics are used in all patients.

27.4.2 Anesthesia

The operation is done under general anesthesia. In addition, a caudal block done at the beginning and at the end of the procedure provides excellent intraoperative anesthesia and postoperative analgesia.

27.4.3 Positioning

The patient is placed in the lithotomy position either transversely or longitudinally at the end of the operating table. The transverse position is particularly advantageous if laparoscopy is to be used for preliminary biopsies. The rectum and sigmoid colon are irrigated from below until clear. A urinary catheter is optional. We tend not to use one, choosing to intermittently empty the bladder with a Crede maneuver during the procedure. Some surgeons prefer the prone jackknife position for the transanal pull-through. Although this position provides excellent visualization, it makes concurrent use of laparotomy or laparoscopy, for biopsies or mobilization, impossible.

27.4.4 Submucosal Dissection

An anal retractor or retraction sutures are placed to expose the anus and distal rectal mucosa. Some authors recommend submucosal injection of a dilute epinephrine solution or air to enhance development of the submucosal plane [20]. The rectal mucosa is circumferentially incised using cautery approximately 3–5 mm from the dentate line, depending on the size of the child. Multiple fine sutures are placed in the proximal cut edge of the mucosal cuff, and traction is applied while the endorectal submucosal dissection is carried proximally. The optimal length of the submucosal dissection is a subject of controversy, and is addressed below.

27.4.5 Mobilization of the Rectum

When the submucosal dissection has been completed, the rectal muscle is divided circumferentially. Dissection then continues proximally, dividing all vessels as they enter the rectum, staying right on the rectal wall. When the peritoneal reflection is reached, the sigmoid is then mobilized in the same fashion and the rectum and sigmoid are delivered through the anus. Throughout this dissection, blood vessels are divided using cautery or ligated, depending on their size.

27.4.6 Anastomosis

The colonic dissection is completed when the transition zone is reached. The controversy about whether the pathological transition zone should be identified before the anal dissection or during mobilization of the rectum and sigmoid colon is addressed below. The colon is divided at least 2 cm above the most distal normal biopsy to prevent the possibility of a transition zone pull-through [21, 22]. The rectal muscular cuff is then split longitudinally, ei-

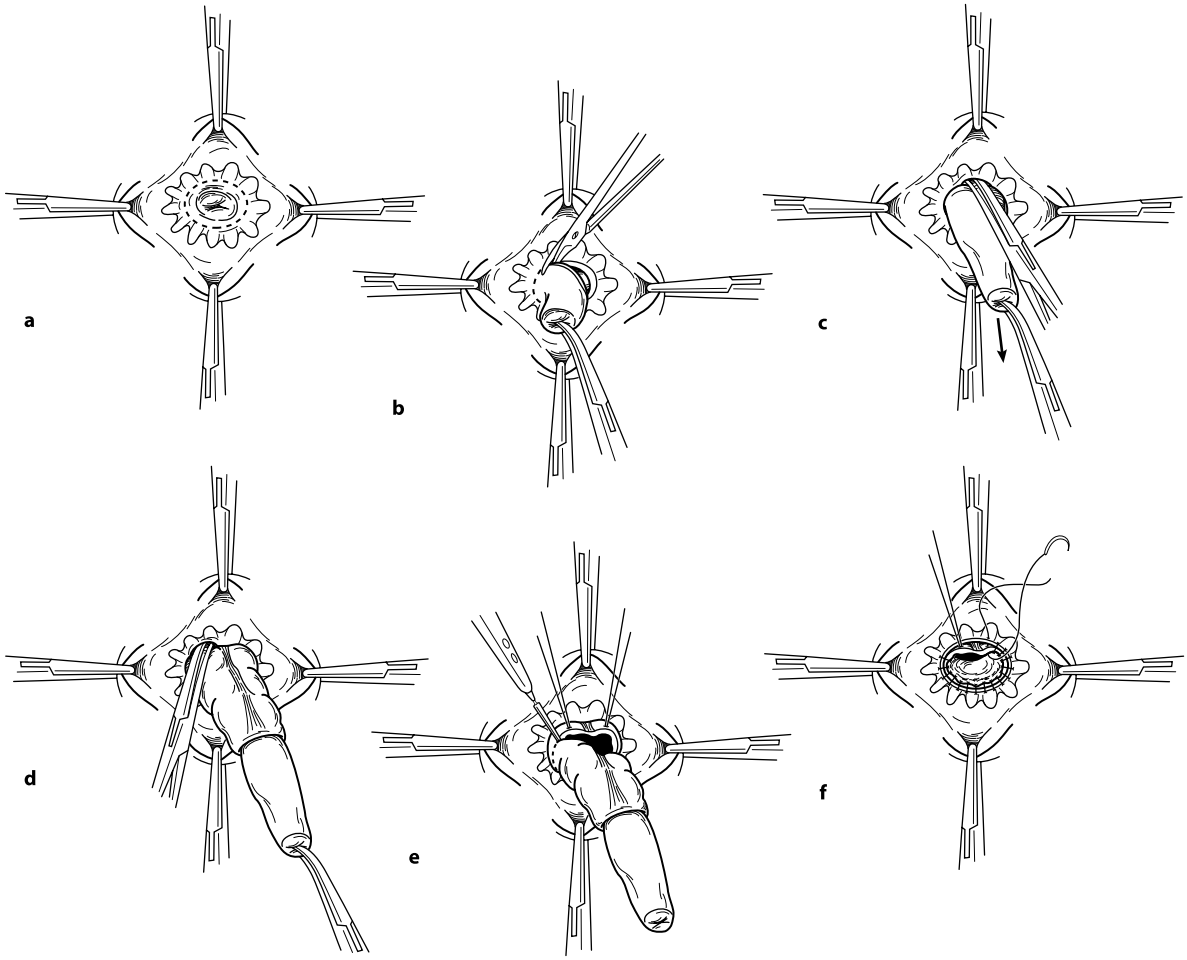

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Fig. 27.1 **a** The anal retractor is placed and the mucosa is incised with the needle-tip cautery approximately 3–5 mm from the dentate line. **b** Traction sutures are placed and the submucosal dissection is carried proximally. **c** The muscular cuff is incised circumferentially. **d** Rectal and sigmoid vessels are divided as they enter the bowel. **e** The biopsy site is reached, and the colon is divided several centimeters proximal to it. **f** The anastomosis is performed

ther anteriorly or posteriorly to avoid constriction of the pulled through bowel. A standard Soave-Boley anastomosis is then performed. Drains are rarely used.

27.4.7 Postoperative Care

If a caudal block is used, acetaminophen alone is given for pain. Narcotics are reserved for the few patients whose pain is not controlled with acetaminophen. Feeds are started immediately postoperatively. The patient is discharged once stooling has started and feeds are tolerated. Parents are instructed regarding perianal skin care and are educated to recognize the symptoms and signs of postoperative enterocolitis.

The anus is calibrated with a finger or dilator between 7 and 14 days after the operation, and then weekly for the next 4–6 weeks. Routine daily dilatations by the parents are not prescribed unless there is a stricture or cuff narrowing detected at the weekly visits.

27.5 Results of the Transanal Pull-Through

There are now numerous papers from a wide variety of countries reporting outcomes in children undergoing the

transanal pull-through [17, 18, 23–31]. However, there are only a few series comparing the transanal pull-through to a control group undergoing an open approach. These series suggest that the transanal approach is associated with less pain, shorter time to recovery, shorter hospital stay, and lower cost [32–34]. Long-term outcome data after transanal pull-through are not available. In particular, the incidence of adhesive small-bowel obstruction, which one would expect to be lower after transanal pull-through than laparotomy, is not yet known.

In a recent extensive multicenter review [23], we reported that about half of the patients underwent surgery in the first month of life, with the mean age at the time of surgery being 5 months. None of the children needed blood transfusions, the average blood loss was 16 ml, the average time to full feeding was 36 hours, and the mean hospital stay was 3.4 days. The rate of complications was low: 6% developed enterocolitis, and 4% stricture. None of the patients died of complications related to the surgery. These results concur with those from many other centers.

27.6 Ongoing Controversies

27.6.1 Laparoscopic Versus Transanal Pull-Through

The theoretical advantage of the pure transanal approach compared to the laparoscopic operation is the lack of intraabdominal dissection of the rectum, which may be complicated by mechanical or thermal injury to abdominal or pelvic structures. In addition, the transanal approach does not require laparoscopic equipment or skills, which makes it far more accessible for surgeons in developing countries. No studies have thus far compared the results of the laparoscopic and transanal operations.

27.6.2 Intraabdominal Colonic Biopsies

Once the anal dissection has been started in a child with Hirschsprung's disease, the surgeon is committed to a Soave or Swenson reconstruction. This becomes problematic if the pathological transition zone is significantly higher than the radiological transition zone, for two reasons. Firstly, many surgeons prefer a Duhamel procedure for long-segment disease [35], and secondly many surgeons prefer to use an ileostomy for these children and perform the final reconstruction only when the ileostomy output has thickened. Approximately 20% of neonates with Hirschsprung's disease have no radiographic transition zone, and in 8% of children with a rectosigmoid transition zone on contrast enema, the pathological transition zone is more proximal [23, 36]. We therefore believe that it is more prudent to establish the pathological transition zone prior to starting the anal dissection.

There are two ways to achieve this goal: laparoscopy or the use of an umbilical incision [37]. The evidence suggests that the use of a preliminary biopsy using either technique does not have a negative impact on postoperative outcome [23]. If an experienced pathologist is available to verify the presence of ganglion cells on frozen sections, and the transition zone is in the left side of the colon, the operation will be completed in one session. In cases where the pathologist is not able to verify the presence of ganglion cells, the pathological transition zone is in the right colon or small bowel, or the surgeon is not confident in the pathological expertise available [38], the repair should be postponed until final analysis has been performed on formalin-fixed material, or a stoma should be fashioned.

27.6.3 Length of the Muscular Cuff

In the original descriptions of the transanal endorectal pull-through, the submucosal dissection was extended above the peritoneal reflection or about 5–6 cm [17, 18]. However, we have seen some patients in whom the long muscular cuff "rolled down" and created a tight constricting band around the pulled-through bowel, despite longitudinal division of the cuff prior to the pull-through. As we have gained experience and confidence with the operation, we have increasingly shortened the muscular cuff to approximately 1–2 cm. Excellent results using a short cuff have been reported by Rintala [28]. Some authors have taken this further and omit the submucosal dissection entirely, performing what is essentially a transanal Swenson procedure. Despite the theoretical risk of injury to the prostate or bladder, preliminary reports of this approach appear promising [29].

27.6.4 Use of a Stoma

Despite the trend among pediatric surgeons to avoid routine colostomies, there are still some situations in which a colostomy or ileostomy is indicated. These include the very sick child with enterocolitis, the neonate with free air, the older child with massive megacolon, uncomplicated Hirschsprung's disease without access to an experienced pathologist, long-segment disease, and the child with trisomy 21 and developmental delay. The transanal approach can be used for reconstruction in a child with a pre-existing colostomy without the need for a full laparotomy.

27.7 Conclusions

The transanal pull-through procedure for Hirschsprung's disease is safe and effective in most situations. It can be performed at an early age, including the neonatal period.

A one-stage transanal pull-through is associated with a similar range of complications as any of the open operations, and results in less pain, shorter hospitalization, minimal or no intraabdominal dissection, and a superior cosmetic result. The operation can be done by any pediatric surgeon, and does not require the technology and skills necessary for laparoscopic surgery. Long-term follow up studies are needed to accurately assess the functional outcome of this procedure.

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Duhamel's Procedure

B. M. Ure and M. L. Metzelder

28

28.1 General Aspects

Since Duhamel introduced a new operative technique for Hirschsprung's disease in infants in 1956 [1, 2], his procedure has gained wide acceptance worldwide. A recent British Association of Pediatric Surgeons' survey confirmed that 62% of consultants prefer Duhamel's technique for children with Hirschsprung's disease [3]. The principle of Duhamel's technique is to exclude the rectum instead of removing it. The proximal normally innervated colon is pulled through a simple cleavage of the rectal space. The colon then appears at the posterior wall of the anal canal. A large enterotomy connects the excluded rectum with the pulled-through colon and the newly created rectum has an anterior aganglionic and a posterior ganglionic bowel.

There has been extensive discussion on whether to initially perform a colostomy with subsequent pull-through and final closure of the stoma, or whether to perform a one-stage procedure. Other points of discussion were appropriate modifications, including the use of laparoscopy, the use of Duhamel's technique for extended aganglionosis and for re-do pull-through. These aspects are discussed in this chapter after presentation of the details of the operative technique.

28.2 Operative Technique

Initially, biopsies are taken at appropriate sites for frozen section before mobilizing the sigmoid colon and rectum. The final decision on the extent of resection upwards is made according to the results of histological examination.

28.2.1 Mobilization of the Upper Colon and Closure of the Rectum

The sigmoid colon and upper rectum are mobilized after opening the lateral peritoneum. The proximal colon is closed with a pursestring suture and the rectum is divided just above or at the peritoneal floor, similar to Hartmann's operation. One or two layers of resorbable sutures are used to oversew the distal portion of the rectum.

Resection of the upper colon is performed as necessary. The narrowed aganglionic zone and the megacolic segment are mobilized simultaneously. Most of the dilated portion of the colon should preferably be resected in order to allow an easier anastomosis with the almost normal caliber of the rectum. After the appropriate mobilization and resection, vascular divisions are performed without regard to the classical anatomical schemes of vascularization.

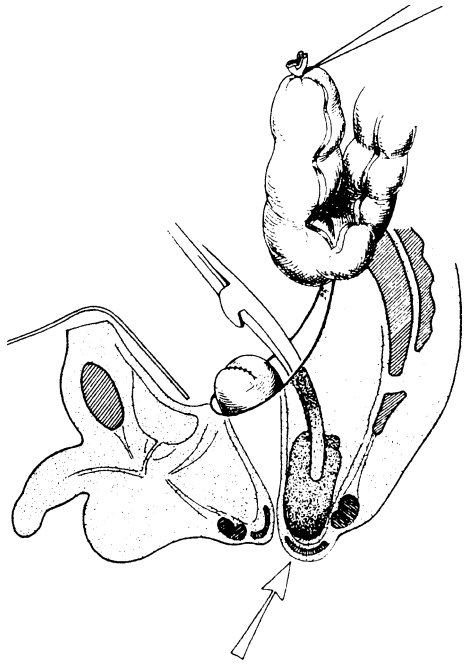

Fig. 28.1 The long curved forceps fitted with a small sponge is introduced into the retrorectal space for dissection down to the pelvic floor and the eventually dilated anal sphincter

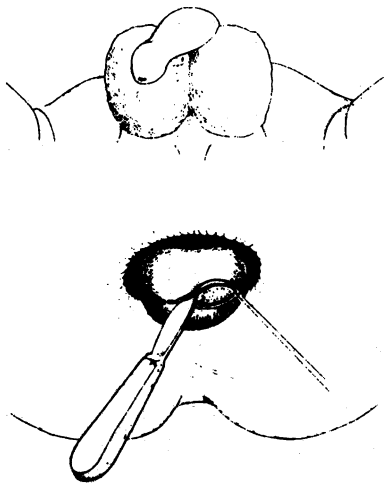

Fig. 28.2 Semicircular incision 1–1.5 cm above the anal margin

28.2.2 Preparation of the Rectal Space

The opening of the mesorectum provides good access to the retrorectal space. This space is cleaved down to the pelvic floor between the preserved sacrogenitopubic laminae. A curved forceps fitted with a small sponge is pushed down to the posterior wall of the anal canal and is diverted through the anus, which may be dilated previously (Fig. 28.1).

28.2.3 Endoanal Incision

A posterior semicircular incision is made about 1–1.5 cm above the anal margin, just above the dentate line (Fig. 28.2). The mucosa and the internal sphincter are opened to the retrorectal space, which is filled with the sponge introduced abdominally. The sponge and the tip of the clamp are protruded through the anus and provisional sutures are introduced at the two angles of the anal incision.

28.2.4 Retrorectal Pull-Though Procedure

Using the sponge as a guide, another forceps is drawn in a retrograde direction through the retrorectal space into the peritoneal cavity (Fig. 28.3). The proximal portion of the colon is then grasped and pulled downwards into the retrorectal space and through the posterior anal incision. Attention should be paid to avoiding torsion or forced stretching. After securing vitality and hemostasis of the pulled-through colon, the anastomosis is performed with interrupted resorbable sutures by attaching the posterior part of the pulled though colon to the lower lip of the anal incision. Thereafter, the anterior part of the pulled through colon is attached to the upper part of the incision, creating an end-to-side colorectal anastomosis.

In Duhamel's original description [1, 2], the side-to-side anastomosis of the anterior aganglionic rectum and posterior ganglionated colon is created by crushing the septum using two Kocher clamps, which are introduced to meet in an inverted V at the apex of the rectal pouch (Fig. 28.4). The position of the clamps is checked intraabdominally by palpation. The clamps fall off after 4 to 10 days (Fig. 28.5). An increasing number of pediatric surgeons have, in recent years, performed the rectocolic anastomosis with a stapling device [4, 5] (Fig. 28.6).

28.3 Modifications of the Duhamel Procedure

28.3.1 Level of the Anal Anastomosis

Duhamel described the anal transverse incision at the anocutaneous junction in 1956 [1, 2]. He modified his technique in 1963 in order to avoid soiling related to the entire division of the internal sphincter [5] and proposed

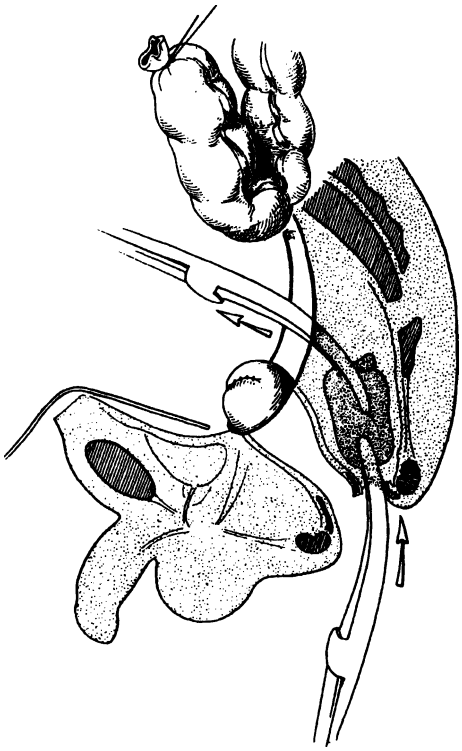

Fig. 28.3 Pull-through: perianal insertion of a forceps through the semicircular incision. The forceps is introduced into the peritoneal cavity after grasping the sponge. The colon is pulled through the endoanal incision and an end-to-side colorectal anastomosis is accomplished

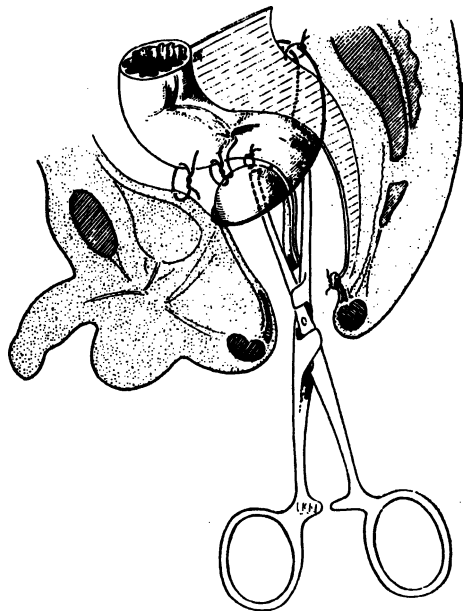

Fig. 28.4 Anastomotic technique as originally described by Duhamel using two Kocher clamps, which are introduced to meet in an inverted V at the apex of the rectal pouch, checked intraabdominally by palpation. In recent years most pediatric surgeons have performed the rectocolic anastomosis with a stapling device

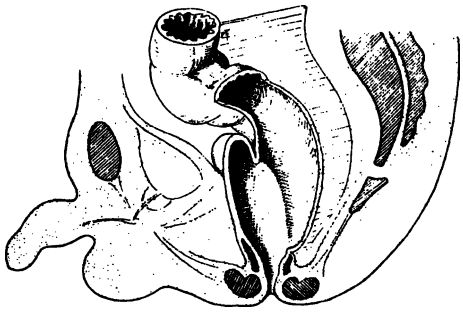

Fig. 28.5 Schematic illustration of completed Duhamel's procedure: the anterior portion of the new rectum consists of the aganglionic anterior wall and the posterior portion of the normally innervated posterior colon. The dorsal portion of the anal sphincter is reduced

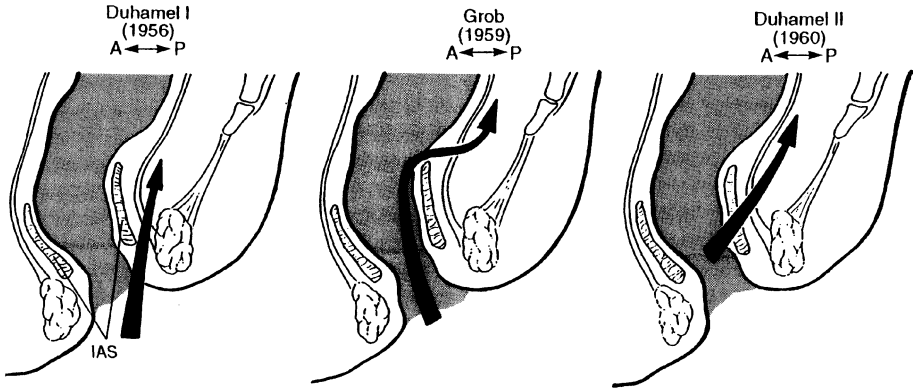

Fig. 28.6 Modified Duhamel's procedures using a stapling device described by Duhamel et al. [5]

incising the posterior half of the circumference of the anal canal 1 cm above the anal margin, thereby preserving the distal part of the internal sphincter. However, this occasionally caused prolapse of the colonic mucosa and anal incontinence. Grob [6] incised the posterior anal wall 1.5 to 2.5 cm above the mucocutaneous junction in order to preserve the internal sphincter. This technique preserved continence, but occasional fecal impaction of the rectal pouch with obstruction of the colon, producing constipation and urinary tract infections, remained problematic. In a recent survey [7] on 1525 Duhamel procedures, the level of endoanal incision was stated to be "medium" in 93%, low in 7%, and high in less than 1%. However, there was no clear definition of the levels, and in only 13% was the internal anal sphincter identified during the operation.

28.3.2 Size of the Rectal Stump

Numerous surgeons have suggested dissecting and closing the rectum as far down as possible [6, 8, 9]. It was postulated that minimization of the size of the rectal stump reduces the occurrence of fecal stasis in the blind pouch. However, the pelvic dissection deprives Duhamel's operation of its main advantage, which is the limited perirectal dissection.

28.3.3 Complete Division of the Colorectal Septum

In Duhamel's original description, the anterior wall of the pulled through normal bowel and the posterior wall of the anal canal and rectum are crushed between the blades of two Kocher clamps. Numerous other devices based on pressure necrosis have been proposed [10–15]. In addition, it was suggested that the entire colorectal septum be divided between the anal and the proximal anastomosis, performing an end-to-end anastomosis of the upper end of the anterior wall of the rectum and of the incised anterior wall of the colon. This modification, initially proposed by Martin and Caudill in 1967 [16], was also used by Ikeda et al. [17] and by Soper and Miller [18]. The proximal and distal anastomoses were made with sutures and the colorectal septum was divided by crushing clamps.

The use of a gastrointestinal anastomosis device (GIA) was suggested by Steichen et al. in 1968 [19]. The authors left the upper end of the rectum open to ensure that the entire length of the device was within rectum and colon. The side-to-side anastomosis was accomplished by using the GIA and the proximal anterior/lateral circumference of the rectal stump was sutured to the superior lip of the opening in the colon subsequently. Duhamel's operation was entirely performed using mechanical sutures by Steichen et al. in 1987 [20]. The EEA-stapler was used for the anal and the proximal anastomosis and the GIA was used for the side-to-side colorectal anastomosis. Today, surgeons generally use staplers for the colorectal anastomosis [4, 21, 22].

28.3.4 Extended Duhamel's Procedure for Extensive Aganglionic Segments

Martin [23, 24] introduced a long side-to-side anastomosis between the normal ileum and aganglionic rectum and colon for patients with total colonic aganglionosis. The principle is similar to the Duhamel's procedure, but the anastomosis is somewhat longer. The aim of the Martin modification was to allow resorption of fluid and electrolytes by the long aganglionic segment which was confirmed by recent reports [25]. It was suggested that

the operation be performed at the age of 1 year and some modifications, including the use of stapling devices to create a long anastomosis between the aganglionic colon and ganglionic ileum, were introduced.

However, the reported results after Martin's modification were not favorable. Enterocolitis, frequent liquid stools, excoriated perineum, and incontinence occurred in up to 60% of patients [26]. Many surgeons have abandoned Martin's technique in recent years due to lack of evidence on advantages compared to ileorectal anastomosis using the classical Duhamel technique [27, 28].

28.4 Complications and Results of Duhamel's Procedure

The incidence of complications during the immediate period after Duhamel pull-through has been extensively reviewed by Bourdelat et al. [7]. The survey of 2430 patients who had undergone Duhamel's procedure or its modifications in 31 institutions revealed anastomotic leakage in 2.2%, necrosis in 0.09% and stricture in 0.7%, and a mortality rate of 1.6%. Other authors have confirmed a comparatively low incidence of immediate postoperative complications after Duhamel's procedure [29–31].

The majority of postoperative deaths are related to enterocolitis [7]. Symptoms to define Hirschsprung-associated enterocolitis include diarrhea, abdominal distension, colicky pain, sepsis, and bloodstained stool [32, 33]. Postoperative onset of enterocolitis may be related to anastomotic leakage, stricture or stenosis [34]. However, any postoperative hospital admission due to diarrhea, distension and abdominal pain may be regarded as enterocolitis. The reported incidence of postoperative enterocolitis varies from 5% to 35% depending on the pull-through technique and the definition of enterocolitis [29, 34–36]. After Duhamel's procedure, the incidence of enterocolitis was 5% to 26% [7, 36–39], and has been reported to be somewhat lower than after Swenson's [30] and Soave's technique [40].

Incontinence after Duhamel's pull-through has been reported in 0% to 20% of patients, depending on the definition of the condition and the follow-up technique [36, 39, 41]. There was no evidence for different incontinence rates between the common pull-through techniques in the detailed analyses of Holschneider [42] and Snyder and Ashcraft [43]. Heij et al. [41] performed a systematic follow-up after Duhamel's pull-through and confirmed a considerably higher incidence than derived from most series. Of 63 patients, 17 suffered some degree of incontinence, and 22 soiling and/or constipation. Mattioli et al. [31] noted symptoms of incontinence in 42% of 65 infants, and confirmed an increased risk of incontinence in children with total colonic aganglionosis after ileal pull-through [31].

Constipation and fecal impaction represent a particular problem after Duhamel's procedure due to a larger capacity reservoir partially consisting of aganglionic bowel. Constipation was previously reported in 5% to 8% of patients after Duhamel's pull-through [7, 44, 45]. However, a more recent analysis by Rescorla et al. [46] showed normal bowel habits in only 65% of 103 patients; 27% used enemas or stool softeners, and 8% had severe constipation. Jung [38] reported fecaloma formation in 3 out of 77 patients.

Boemers et al. [47] have particularly addressed the effect of Duhamel's operation on lower urinary tract function. Only 3 out of 11 children had normal urodynamic findings 6 months postoperatively. Postoperative residuals were 156% higher than preoperatively which suggests partial detrusor denervation. The authors recommend routine urodynamic investigation of children with postoperative voiding problems.

28.4.1 Primary Versus Staged Duhamel's Procedure

Pull-through operation for Hirschsprung's disease has traditionally been performed in three stages. The first stage included placement of a colostomy or ileostomy. The second stage was the pull-through procedure at 3 to 12 months of age. Most surgeons protected the anastomosis with the initially placed stoma or, in patients with more extended resection, with a proximally placed loop enterostomy. The third stage included closure of the stoma.

The first experience with one-stage repair of Hirschsprung's disease was reported in 1980 by So et al. [48]. Since then, all techniques commonly used for Hirschsprung's disease have been performed in one stage [48–52]. Several publications deal with results of various pull-through techniques, including some patients with Duhamel's procedure [53, 54] and show that the length of postoperative stay and the incidence of enterocolitis following one-stage techniques are comparable to those following staged pull-through [49]. The number of hospitalizations, the total number of days in hospital, the incidence of stoma-related complications [51, 55], and cost [56] were significantly reduced.

The number of reports on series using exclusively Duhamel's one-stage procedures remains limited [39, 57–60]. Van der Zee and Bax [60] reported on 22 children with a mean postoperative stay of 8 days. At follow-up after more than 5 years, 15 children had normal spontaneous defecation, 8 displayed irregular soiling and 6 needed laxatives or rectal irrigations. Jung [38] performed primary Duhamel pull-through in 30 children. Postoperative intestinal obstruction was seen in three, wound disruption in four, and fecaloma formation in three. Mir et al. [57] operated on ten children, of whom two developed enterocolitis during a follow-up of 3 years. Pierro et al. [58]

analyzed a series of 124 children and found no difference in postoperative complications between primary versus staged Duhamel's procedure, and less satisfactory results after primary Swenson's than after primary Duhamel's procedure.

28.5 Laparoscopic Duhamel's Procedure

Laparoscopic techniques have been mainly suggested for Swenson's procedure [61–64], and less frequently for Duhamel's operation [65–68]. Briefly, laparoscopically assisted Duhamel's operation is performed via four or five cannulae and basically includes the steps originally described by Duhamel. Details of the operation have extensively been reported [66–68]. Bowel biopsies to determine the extent of aganglionosis before ablation are taken laparoscopically [69]. Further steps include laparoscopic mobilization of the sigmoid and descending colon close to the bowel wall, dissection and closure of the rectal stump, and laparoscopically controlled pull-through.

Reports on the experience with laparoscopic Duhamel's technique remain scarce. Fewer than 100 patients have been reported so far (Table 28.1). The feasibility was excellent and the rate of complications, such as leakage, stenosis, and enterocolitis compares favorably with data derived from conventionally operated series (Table 28.1). The postulated advantages include excellent visualization, atraumatic dissection causing less damage to pelvic nerves, less postoperative pain, and faster recovery, but prospective trials are lacking. Bufo et al. [65] showed that the length of hospitalization was 2.5 days after one-stage laparoscopic versus 10.6 days after traditional Duhamel's resection, and the costs were significantly lower. Bonnard et al. [70] reported on five patients with extended or total colonic aganglionosis, who underwent successful laparoscopic resection and subsequent posterior ileoanal anastomosis. The authors stated that there was no postoperative soiling, incontinence or constipation. Further series of 30 patients were reported by de Lagausie et al. [71] and 10 patients by Moog et al. [72].

28.6 Duhamel's Technique for Re-Do Pull-Through Procedure

Some patients with severe constipation or enterocolitis after pull-through do not respond to conservative management. In particular, a re-do pull-through may be indicated in patients with recurrent fecaloma formation or episodes of enterocolitis [73]. Further indications include retained aganglionosis, especially after transition zone pull-through, intestinal neuronal dysplasia, strictures, and segmental bowel dysfunction with bowel dilatation [74–77].

Numerous authors prefer Duhamel's technique for re-do pull-through, in particular after former Swenson's and Rehbein's procedure, due to difficulties in pelvic dissection [75–78]. The outcome was satisfactory in several series of patients, most including fewer than ten patients [75, 76, 78, 79]. Wilcox and Kiely [77] achieved excellent results in 19 children, of whom 14 were continent and had regular bowel movements. Antao et al. [80] recently reported on 17 patients who underwent a modified open re-do Duhamel's pull-through procedure with a short rectal pouch and low anastomosis. All of them were continent, and all had spontaneous regular bowel habits.

Weber et al. [76] postulated the advantages of re-do Duhamel's procedure compared to Soave's procedure. The latter required more difficult and extensive dissection, with several patients requiring more than one re-do operation. Van Leeuwen et al. [79] used the endorectal pull-through technique for re-do operation after initial endorectal resection in patients with adequate rectal cuff. Otherwise Duhamel's technique was preferred. Aggarwal et al. [81] suggested combining abdominal and posterior sagittal approach for re-do pull-through and reported an excellent exposure and results in four children after failed Swenson's operation.

Table 28.1 Publications on experience with laparoscopic Duhamel's procedure

Reference	No. of patients	Leakage	Abscess/fistula	Stenosis	Enterocolitis	Incontinence	Constipation
66	5	None	Not recorded	Not recorded	Not recorded	Not recorded	Not recorded
71	30	1 (5%)	1 (5%)	None	None	1 (5%)	3 (10%)
67	19	None	Not recorded	Not recorded	Not recorded	Not recorded	Not recorded
72	10	1 (10%)	Not recorded	Not recorded	Not recorded	Not recorded	Not recorded
70	5	None	None	None	None	None	None

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29 Early and Late Complications Following Operative Repair of Hirschsprung's Disease

D. C. Little and C. L. Snyder

29.1 Overview

Since Harald Hirschsprung's classic description in 1886, over 100 papers on complications following repair of Hirschsprung's disease have been published. Original works by Swenson (1948), Rehbein (1953), Duhamel (1956), and Soave (1964) and their predecessors emphasized large single-institution or even single-surgeon experiences rendering comparative outcome analysis difficult. Contemporary surgical management has evolved from the traditional three-stage approach to the more recent introduction of minimally invasive laparoscopic techniques [1, 2] and neonatal one-stage reconstruction [3, 4]. Initial results of these procedures have been limited to single-center or small multicenter series with relatively short follow-up. Although multiple studies have suggested that the popular endorectal technique is safe and efficacious [5–7], the influence on the incidence of late complications is yet to be fully determined. Many of the techniques can also be done in an open or laparoscopically assisted manner. Different risks and benefits are attendant with each of these choices.

The majority of children with Hirschsprung's disease have satisfactory results following definitive pull-through reconstruction. Complications occurring after the surgical repair of Hirschsprung's disease can be temporally categorized into early and late complications. However, there is significant overlap in regard to the time period during which these may occur. Some complications (e.g., wound infection, bleeding, stricture, bowel obstruction, dehiscence, stomal complications) are not unique to Hirschsprung's disease, and are discussed only briefly. Overall, most children with HD do not develop complications within the first 30 days postoperatively. The most commonly encountered late complications are chronic constipation, enterocolitis, and encopresis. Most will present within the first few postoperative months, and symptoms will gradually improve with time. Other complications such as fistulae, and genitourinary and sexual dysfunction, will infrequently be encountered.

29.2 Early Complications

29.2.1 Wound Infection

By definition, surgical repairs of Hirschsprung's disease are classified as clean-contaminated cases. The risk of infection should be low in most cases. Skinner reviewed

over 2500 operative cases and documented a 1.7–19.2% incidence of wound infection for all four primary repairs [8]. Factors contributing to the incidence of wound infections include adequate preoperative bowel preparation, perioperative antibiotics, adequate preoperative nutrition, meticulous hemostasis, length of operation and sterile surgical technique.

29.2.2 Bleeding

Significant postoperative bleeding after definitive repair of Hirschsprung's disease is rare. Obviously, preexisting coagulopathy, sepsis, inadequate intraoperative hemostasis all are potential contributing factors. Hematoma in the early postoperative period may increase the risk of infection and anastomotic complications. With careful technique, this complication should be avoided.

29.2.3 Anastomotic Complications

29.2.3.1 Leak

Anastomotic leak is the most serious of the early postoperative complications. Factors increasing the risk of this complication include: tension, ischemia, technical (inadequate repair), poor nutritional status and other general wound-healing problems (steroids, etc), residual aganglionosis, and distal obstruction. Down's syndrome may be associated with an increased leak rate. One study suggests that the risk of anastomotic leak is independent of patient age or the length of aganglionic bowel [9]. Postoperative rectal manipulation (temperature, examination, or medications) or examination in the early postoperative period may lead to anastomotic problems. A sign should posted at the bedside prohibiting such manipulations. Suspected leaks are usually evaluated with water-soluble contrast enemas (Figs. 29.1 and 29.2).

The incidence of anastomotic failure varies from 1% to 10%. Leaks may be subclinical, resulting in stricture formation. Some studies suggest that a large percentage of strictures result from a small anastomotic leak. Major anastomotic leaks can lead to localized abscess formation or free peritoneal leakage and sepsis. Obviously, more severe leakage may require percutaneous drainage, surgical exploration, diverting proximal colostomy, and eventual anastomotic revision.

29.2.3.2 Pelvic Abscess

The overall incidence should be less than 5%. The same factors resulting in leak are also implicated in this complication. CT scan is the diagnostic procedure of choice. Diagnosis of pelvic abscesses requires a high index of suspicion and subsequent prompt intervention to avoid further morbidity including extension of the infection, systemic sepsis, and necrosis of the pull-through segment. Treatments may range from percutaneous drainage to stomal diversion.

29.2.3.3 Cuff Abscess

The incidence is usually under 7% [10–12]. This complication may occur after the Soave-Boley operation. An abscess is located between the rectal muscularis and the colonic pull-through segment. Factors leading to an increased risk for this complication include: ischemia, retained rectal mucosa, bleeding, pelvic contamination, and tension. Some authors feel that transabdominal peritoneal drainage for the first two to three postoperative days reduces the risk of cuff abscess [13]. Treatment varies from simple broad-spectrum antibiotic coverage (with or without percutaneous drainage) to diversion. Small fistulas or sinus tracts may resolve spontaneously.

29.2.4 Dehiscence

Wound dehiscence occurs in less than 3% of children undergoing definitive repair [11]. Attention to technique, adequate hemostasis, good nutrition, and avoidance of ischemia, tension and infection are preventative.

29.2.5 Retraction of the Pull-Through Segment

The incidence of retraction is less than 10% [10, 14, 15], usually occurring in the early postoperative period. If retraction is suspected, examination under anesthesia will confirm the diagnosis. If very minimal, transanal repair may be attempted. Incomplete retraction can be managed with a proximal diverting colostomy and delayed revision in several months.

29.2.6 Stomal Complications

Stomal problems such as retraction, stenosis, parastomal hernia, skin breakdown, and prolapse can occur. However, the incidence is no different for patients with Hirschsprung's disease than for other diseases. Stomal therapists, working under the direction of a pediatric surgeon, often will detect problems at earlier stages and thus provide for earlier intervention. Avoidance of stomal complications is one of the arguments used by advocates of primary one-stage repair.

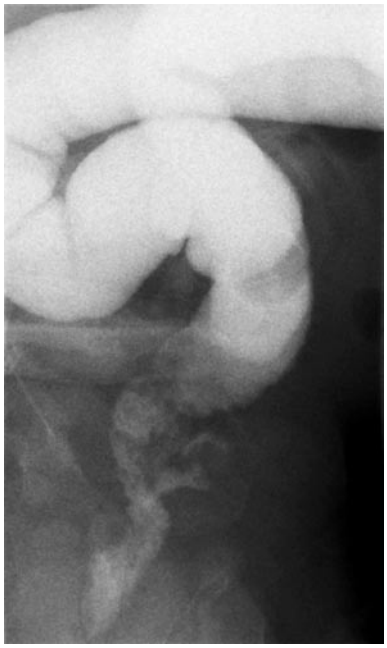

Fig. 29.1 A 1-year-old patient with anastomotic leak following Soave's procedure

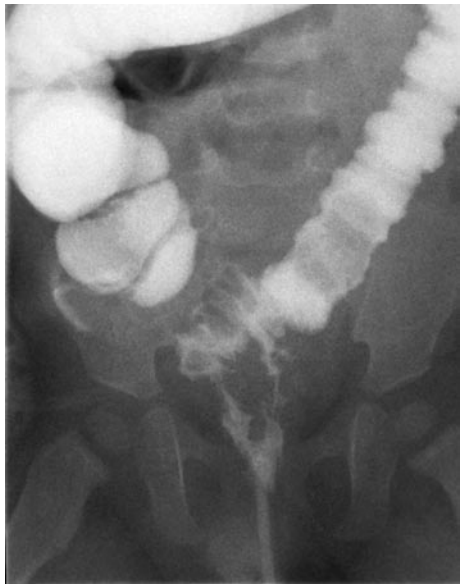

Fig. 29.2 Same patient as in Fig. 29.1 2 months later. A fistula from the distal to proximal rectum has developed

29.3 Late Complications

29.3.1 Enterocolitis

Hirschsprung's enterocolitis is the most serious and potentially life-threatening complication of Hirschsprung's disease. It may occur before or after definitive repair. Enterocolitis of Hirschsprung's disease was first recognized by Hirschsprung in 1886 [16] and Swenson and Fisher in 1956. Bill and Chapman are credited with the first detailed description in 1962 [17].

Despite significant advances in elucidating the genetic etiology of Hirschsprung's disease [18] and improved surgical techniques, little progress has been noted in discovering the etiology or prevention of Hirschsprung-associated enterocolitis (HAEC) (Figs. 29.3 and 29.4). Many theories have been proposed including mechanical dilatation and fecal stasis, alterations of mucin components, increased prostaglandin activity, *Clostridium difficile* infection [19], rotavirus infection, and impaired mucosal immune defense mechanism. Unfortunately, the pathogenesis of enterocolitis is poorly understood. The presence of stasis or relative obstruction may be causative in some patients (residual aganglionic colon, stricture, pelvic inflammation, sphincter achalasia). This perplexing problem includes a wide range of clinical presentations including abdominal distension, explosive diarrhea, vomiting, fever, lethargy, rectal bleeding, and shock [20]. The cost of caring for an infant with HAEC is more than 2.5 times that of an infant with Hirschsprung's disease and no enterocolitis [21].

Historically, a younger age at diagnosis and repair has implied an increased risk of HAEC. Teitelbaum et al. noted a significantly increased incidence of low-grade enterocolitis in infants undergoing a primary endorectal pull-through [6]. These patients are felt to have a more severe disease process. Furthermore, infants who experience enterocolitis before operation have an increased risk of occurrence of HAEC following operation [17]. The older child that 'escapes' the newborn period without detectable disease may have a milder variant. These older children show a different pattern of presentation and a consistently shorter transition zone compared with neonatal disease. A recent report noted that Hirschsprung's disease in the older child did not portend a worse outcome compared with younger children [22]. Enterocolitis is also more common in children with long-segment disease (two- to threefold increase). There is no racial predilection, but it may be more common in boys than girls.

Reports of enterocolitis following operative repair of Hirschsprung's disease varies by publication and operative procedure. Duhamel's repair is probably associated with the lowest rate of enterocolitis. In 4000 cases, Duhamel patients were noted to have a 7.1% incidence of enterocolitis. A Japanese study of 1628 patients noted a considerably higher incidence with 35% following Sw-

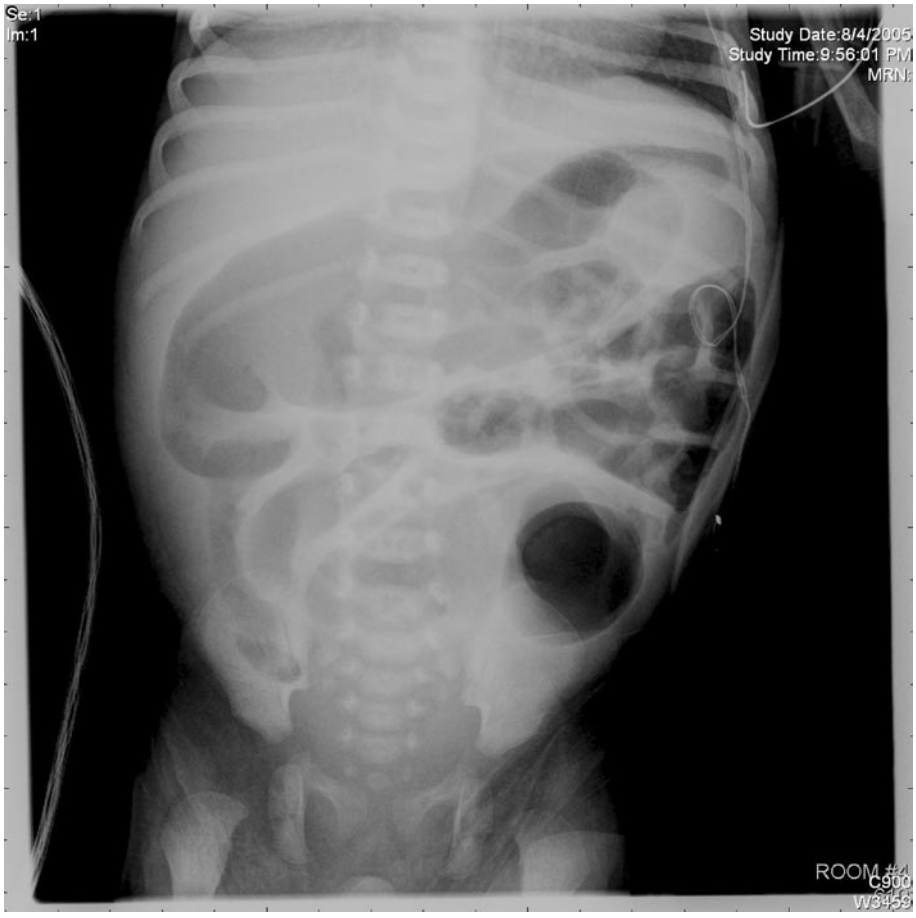

Fig. 29.3 Anteroposterior radiograph demonstrates classic findings of enterocolitis including moderate distension of bowel lumen and edema of bowel wall

enson, 14% following Duhamel, 20% following Soave, and 12% following Boley's procedure [23]. In a survey from the surgical section of the American Academy of Pediatrics, enterocolitis was noted to occur in 16% of children undergoing a rectosigmoidectomy procedure such as Swenson or Rehbein. However, this same group of surgeons reported a 6% incidence following Duhamel pull-through and 25% following the Soave-Boley procedure [15]. Hackam et al. noted a 32% incidence of postoperative enterocolitis in their review of 105 consecutive patients from the Hospital for Sick Children [24]. This incidence correlated with patients having anastomotic complications and intestinal obstruction. Moore et al. note that the incidence of enterocolitis is higher for patients with total colonic aganglionosis than for those with short-segment disease [25]. Patients with trisomy 21 may have a higher risk of HAEC [21], felt to be related to humoral and cellular immune deficiency [26]. In one study, almost 45% of infants with trisomy 21 developed HAEC [21]. Associated anomalies and difficulty in diagnosis may impact the severity of the enterocolitis.

The multicenter analysis of Teitelbaum et al. compared primary endorectal pull-through with a two-stage approach and noted a trend towards a higher incidence of enterocolitis in the primary endorectal pull-through group (42%) compared with those with a two-stage approach (22%) [6]. These authors note that a lower threshold in diagnosing enterocolitis in the more recent years may explain the difference between the two procedures.

The incidence of enterocolitis depends on the type of repair, presence or absence of predisposing factors, and institutional diagnostic criteria for enterocolitis. The reported incidence varies widely in the literature. These factors limit comparative analysis. Table 29.1 shows the incidence of enterocolitis in collected series.

Early recognition with prompt treatment are important for successful outcome. In 1956 Swenson and Fisher advocated rectal tube decompression for the initial treatment of enterocolitis [27]. Rectal decompression and irrigations are still advocated by many in the absence of signs of necrosis or peritonitis. Aggressive fluid resuscitation, bowel rest, and administration of broad-spectrum antibiotics are administered, and resection with diversion is necessary if peritonitis or clinical worsening occurs.

If repeated bouts of enterocolitis persist after definitive pull-through, investigation into mechanical causes should

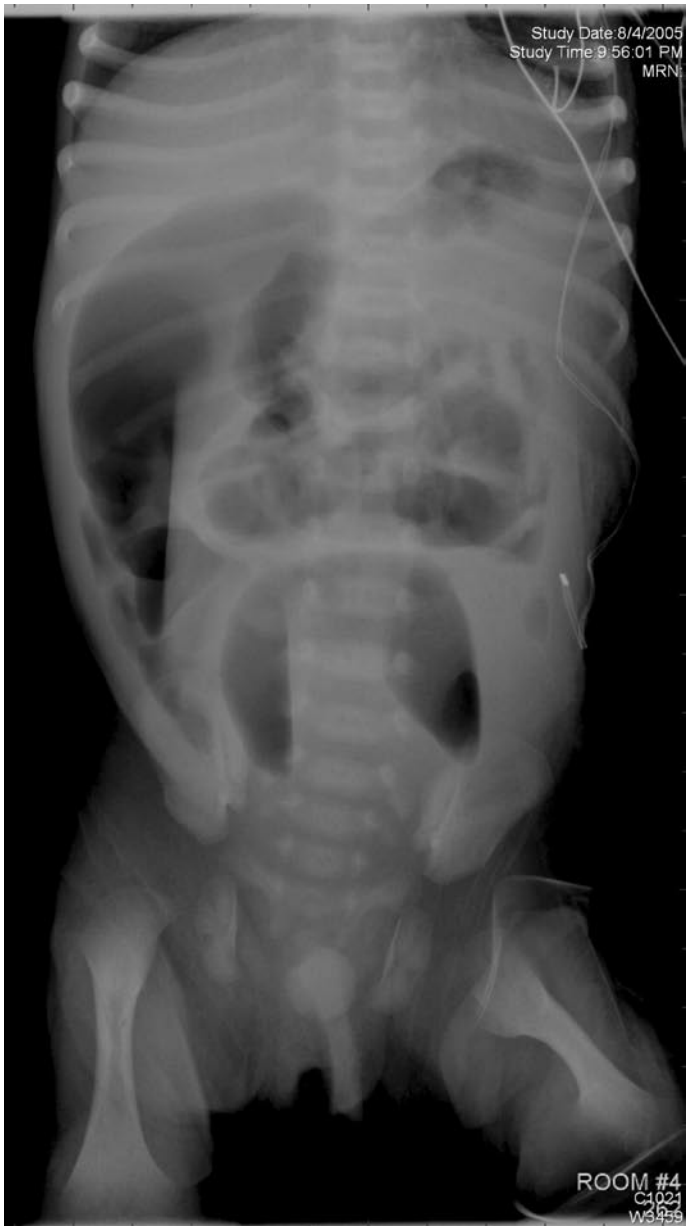

Fig. 29.4 Lateral radiograph demonstrates significant air-fluid levels in a patient with enterocolitis

be considered. Contrast enema, manometry, and rectal biopsy may be necessary. Most patients with enterocolitis will improve over time. Polley et al. and Marty et al. have recommended internal sphincterotomy for those who have persistent enterocolitis despite appropriate investigation [28, 29]. Children with enterocolitis secondary to obstruction may be treated either temporarily by botulinum toxin injection or more permanently with sphincterotomy. In Swenson's series of 880 patients, sphincterotomy was eventually necessary in 6.8% of children [30].

The incidence of enterocolitis directly correlates with mortality. Several series have noted that approximately 50% of deaths are directly related to an enterocolitis episode [12, 15, 29]. In a survey of members of the AAP concerning 1196 patients with Hirschsprung's disease, enterocolitis occurred at the time of diagnosis in 168 patients (14%) with an alarming 30% mortality [15]. In Swenson's series of 880 patients, death after discharge from enterocolitis occurred in about 1% [30].

29.3.2 Constipation

Constipation is probably the most common complaint following surgery (Fig. 29.5). The assessment of severity is highly subjective. The actual rates of constipation may be

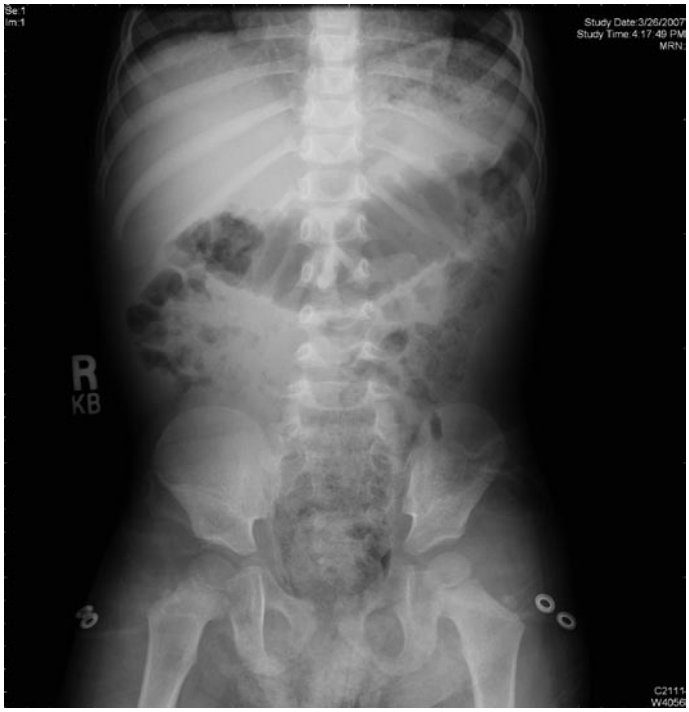

Fig. 29.5 Moderate constipation following Soave's procedure is noted throughout the ascending and descending colon in this 2-year-old patient

underestimated given that many patients are maintained on stool softeners and/or enemas. Rates of constipation between the Swenson, Duhamel, and Soave procedures are roughly equivalent. However, the Rehbein procedure showed a higher rate of constipation necessitating treatment with sphincter dilatation, further resection, or sphincteromyectomy [31]. An increased rate of constipation is not surprising following the Rehbein procedure given that there is a 4–5 cm aganglionic segment left in situ which can become obstructive. A decreased rate of sphincter insufficiency is balanced with increased rates of constipation.

Constipation may result from incomplete resection, sphincter achalasia, stricture formation, fecaloma, neuropathic ganglionic bowel, acquired proximal aganglionosis or may be "functional". Table 29.1 demonstrates the incidence of constipation in collected series. In a pooled sample of almost 8000 patients, the overall incidence of constipation was 7.9%.

Incomplete resection is more likely when frozen sections are relied upon to determine the level of proximal innervation for definitive repair. Accurate interpretation of seromuscular frozen biopsies is paramount in determining the success of the pull-through segment. Frozen sections are prone to sampling and interpretation error. Furthermore, the circumferential distribution of the transition zone is uneven creating a leading edge of ganglion cells extending into the aganglionic distal bowel [32]. Occasionally, these factors result in the use of transitional zone colon for the pull-through. The use of the transition zone for the pull-through segment is associated with an increased risk of enterocolitis, 61% in one series [33]. Treatment options include rectal myectomy and revision of the pull-through [34, 35]. Fecaloma is the presence of a large stool bolus in the aganglionic anterior segment of bowel. It may present with constipation. Alternatively, the obstruction may only allow more liquid material to pass in the form of diarrhea. It is usually associated with Duhamel's procedure, as a result of the partially functional reservoir that has been surgically created. The elimination of blind-ending aganglionic pouches/diverticulum has diminished this complication. The advent of laparoscopically stapled Duhamel procedures could potentially lead to this complication, unless steps are taken to eliminate the blind pouch. The pathogenesis of acquired aganglionosis remains obscure. Etiologies include vascular compromise of the pull-through with subsequent neuronal ischemia, viral infection with neuronal loss, or abnormally innervated proximal bowel. Cohen et al. described five patients (3% incidence) of acquired aganglionosis most of whom were treated successfully with myectomy [36].

Extensive evaluation of mild postoperative constipation is usually not indicated. For those failing a bowel regimen, a more detailed work-up is indicated. Contrast studies will identify pronounced rectal dilation and stricture. Repeat biopsy should be obtained to verify the presence of normal ganglion cells. Manometric analysis to rule out sphincter achalasia or other dysmotility should be obtained. Constipation may be caused by high anal

Table 29.1 Reported long-term complications of combined series: a review of the literature published from 1967 to 2004 (*ERPT* trans-anal endorectal pull-through procedure, *n* total number of patients in the series, † insufficient data)

Complication	Swenson		Duhamel		Soave		Rehbein		ERPT		All[a]	
	n	%	*n*	%	*n*	%	*n*	%	*n*	%	*n*	%
Enterocolitis	3531	13.4	4042	7.1	1268	4.5	440	8.2	†	†	10381	10.6
Constipation	2600	10.3	3567	7.0	571	3.7	367	15.5	149	4.1	7981	7.9
Bowel obstruction	1369	8.3	1288	7.6	1025	5.9	†	†	†	†	4012	7.5
Incontinence	2953	10.8	4010	4.7	1216	4.9	367	8.2	†	†	9063	7.1
Stricture	2188	7.1	3180	2.2	781	6.1	337	9.5	290	4.5	7198	5.0
Mortality	1373	2.8	3591	1.5	902	2.3	191	2.0	149	2.0	6532	2.0

[a]Includes combined series

resting pressure and a weak rectal peristalsis as noted on anorectal manometry and intestinal transit studies with imaging of the anal sphincter complex [37]. Colonic transit studies may provide further insight into the mechanism.

Constipation may be expected to improve over time. Rescorla et al. noted that 88% of patients had difficulty passing stools within the first five postoperative years [12]. However, these symptoms improved with longer follow-up. All patients had satisfactory stooling after 15 years [12]. Lifschitz and Bloss noted that 33% suffered from constipation after the initial operation but only 9% reported persistent constipation after an average of 5 years [38]. Patients with trisomy 21 reportedly have poorer bowel function [39]. Enemas are often necessary to control constipation or soiling after Hirschsprung's disease. Antegrade enemas via button cecostomy or appendicostomy are used in selected patients.

29.3.3 Bowel Obstruction

29.3.3.1 Adhesive

Violation of the peritoneal cavity leads to the formation of intraabdominal adhesions and the possibility of future bowel obstruction. Factors increasing the risk of adhesive obstruction include: prior operation, bleeding, leak, intraoperative contamination, and dehiscence. Early reports noted the incidence of postoperative bowel obstruction to be as high as 18% [23]. A combined review of over 4000 postoperative patients noted an incidence of adhesive bowel obstruction of 7.5% (Table 29.1). With many surgeons now favoring laparoscopically assisted procedures or complete endorectal procedures, the risk of postoperative bowel obstruction may decrease [40]. Fortunately, most bowel obstructions will respond to bowel decompression. In one study, only 20% of patients diagnosed with postoperative bowel obstruction required operative management [41].

29.3.3.2 Internal Hernia/Other

The incidence of internal hernia in most series is <2%. It is important to secure the mesentery of the pulled-through segment to the retroperitoneum in order to prevent this complication. Postoperative intussusception can occur after any operation. Patients with Hirschsprung's disease do not appear to be at any increased risk. If suspected, sonography is currently the diagnostic modality of choice. Another rare cause of early bowel obstruction is a twist of the pulled-through segment. When severe, vascular compromise may arise followed by anastomotic dehiscence.

29.3.4 Continence

Fecal soiling has the greatest negative impact on the quality of life in children with Hirschsprung's disease [42]. Soiling is physically, emotionally, and psychologically disabling [43]. Social withdrawal and poor academic performance are often the end result. Precise assessment of continence is difficult because of the retrospective nature of many of the published reports and lack of objective assessment of children's stooling pattern. Distinctions between occasional soiling and significant incontinence are difficult. Although uncommon, the surgeon should consider the possibility of retained aganglionosis. Additional work-up may include barium enema, manometry, and rectal biopsy. MRI of the pelvis and lower back can be useful in selected patients.

Most children will achieve satisfactory continence with time. Occasional soiling appears to improve over time. Rescorla et al. noted that 12% of their patients less than 5 years of age had some degree of soiling; however, be-

tween 10 and 15 years of age, the incidence declined to 6%. No patient older than 15 years suffered incontinence [12]. Another study found that fecal incontinence was more common in patients less than 15 years of age, but once the child reached late adolescence, bowel control improved significantly with only 8% having fair to poor continence [44]. A review of 880 patients undergoing the Swenson procedure noted that the incidence of soiling decreased from 8% at 5 years' follow-up to less than 2% at 20 years' follow-up [9]. Finally, a review of 2430 postoperative Duhamel patients noted that only 5.3% showed evidence of soiling [45]. Table 29.1 demonstrates the incidence of incontinence in collected series. The combined incidence was 7.1% in nine thousand patients.

Current literature clearly supports gradual improvement in stool continence. Surgeons should maximize medical treatment including the implementation of dietary modifications and bulking agents as the first line of therapy, prior to considering surgical intervention.

29.3.5 Strictures

The incidence of stricture is 8–24% historically, and is more common after Soave and Swenson repairs. Two multicenter reviews of endorectal procedures noted a 4.2–4.8% incidence of stricture [5, 46]. The etiology of anastomotic stricture can be multifactorial, including a narrow muscular cuff, technical complications, compromised blood supply, sequelae following an anastomotic leak, or failure to adhere to a dilation program. Constipation will usually result. Identification is facilitated by digital rectal or proctoscopic examination. Strictures not responding to conservative management may require stricturoplasty or a re-do pull-through procedure. In severe cases, formation of a colostomy and mucous fistula with antegrade dilation over a string may be useful [47].

Additional complications may result from strictures. Rectal dilation may lead to leak, increased constipation, stasis, bacterial overgrowth, dehiscence from tension, or colon retraction. Rectal spasm and colonic inertia can also cause similar problems. Historically, many strictures have responded to conservative outpatient treatment with rectal dilatations with Hegar dilators. Dilations should be avoided for at least 3–4 weeks after the pull-through. As many as one-third to one-half of all clinically significant strictures require surgical intervention [48]. In a review of 7000 patients, the overall incidence of strictures was 5%, with Duhamel procedures having the lowest stricture rates (Table 29.1).

29.3.6 Perianal Excoriation

This is very common after definitive repair and stomal takedown, but usually resolves within 2–3 months. The use of barrier creams beginning on postoperative day 1 may help to limit the severity of this problem. With resolution of postoperative diarrhea, the perianal skin will heal. The incidence of this complication can be expected to decrease with the trend towards neonatal primary repair. Coordinated care with a stomal therapist can be quite valuable in preventing or treating perianal excoriation.

29.3.7 Sphincter Achalasia

Sphincter achalasia is defined as failure of the internal sphincter to relax. Children may present with a myriad of symptoms including chronic constipation or overflow incontinence. Furthermore, children may have difficulty discriminating between solid, liquid and gaseous stools. The use of anal manometry is helpful in evaluating disorders of the internal anal sphincter. A review of patients undergoing postoperative manometry by Harrison et al. noted that persistent loss of normal relaxation of the internal anal sphincter with distension is common, regardless of the technique used [49]. Although many may be asymptomatic, those who are clinically symptomatic may benefit from repeated dilatations or lateral sphincterotomy [50]. Botulinum toxin injections into the internal anal sphincter may be used to asses the potential benefits of later myectomy [51], since the effect of the toxin is transient, usually less than 6 months [52].

29.3.8 Voiding and Sexual Dysfunction

Any operation requiring pelvic dissection places a child at risk for injury to nerves affecting bladder and sexual function. Duhamel's and Soave's modifications were designed to reduce the risk of injury to the delicate pelvic structures. Theoretically, the endorectal procedure should completely avoid injury to pelvic vessels and nerves while protecting the internal sphincter.

The etiology of voiding dysfunction is multifactorial and includes damage to the pelvic splanchnic nerves, the hypogastric nerves, or the pelvic nerve plexus. Parasympathetic denervation to the pelvic splanchnic nerves will lead to a flaccid bladder whereas sympathetic denervation to the hypogastric nerves may result in loss of bladder compliance and incompetence of the bladder neck and posterior urethra [43].

Postoperative enuresis for the different surgical techniques averages 9.5% [53]. Data for individual procedures are as follows: Rehbein 5.4%, Swenson 10.4%, Soave 15.3%, and Duhamel 14.3%. Endorectal follow-up studies of Elhalaby et al. [46] and Langer et al. [5] on a combined 290 patients do not specifically mention urological or sexual dysfunction.

Routine preoperative urodynamic screening is not recommended since children with Hirschsprung's disease are not at increased risk of urological problems. However, a large rectal reservoir may lead to outflow obstruction.

Patients with postoperative urinary complaints should be evaluated, usually initially with sonography and voiding cystourethrography. Urodynamic studies may be needed. Long-term voiding dysfunction is rare.

Discovery of sexual dysfunction requires extensive long-term follow-up. Moore et al. reported sexual dysfunction in 9% following Duhamel's operation and 10% following Swenson's operation [54], with a significantly lower incidence of sexual dysfunction and micturition disturbance following Soave's procedure. The main sexual difficulties identified in female patients were dyspareunia and primary infertility. Male patients voiced concern over poor erections, low sperm counts, or psychosexual problems. A review of 282 patients noted that 101 men with a prior Swenson's procedure had gone through puberty and none had developed impotence. Of these men, 80 were married with a total of 146 children [30]. Another study found a 2.4% incidence of ejaculatory dysfunction in 84 patients after Swenson's procedure [55]. Similar to the data concerning urinary dysfunction, overall assessment of complications demonstrated a significantly ($p<0.01$) lower incidence of sexual dysfunction and micturition disturbance following Soave's procedure when compared to Duhamel's and Swenson's procedures [54].

29.3.9 Mortality

Mortality is low (under 2%) with operative and early deaths being quite rare. Apart from children who die of associated cardiac anomalies or other major anomalies, toxic enterocolitis remains the most common cause of disease-related postoperative death. Additional etiologies include sepsis, abscess, hemorrhage, pneumonia, and embolism [45]. A significant reduction in mortality has occurred over the past 40 years. This may be attributed to improved resuscitation and management of comorbidities, use of parenteral nutrition, earlier detection and prevention of enterocolitis, and improved operative and perioperative care. Table 29.1 demonstrates the incidence of mortality in collected series.

29.3.10 Neonatal Reconstruction and Late Complications

Considerable controversy still exists concerning the efficacy of one-stage neonatal reconstruction. Complications from multistage procedures are well-known. Reliable data concerning the incidence of continence, sexual dysfunction, and constipation for one-stage procedures will require more time, since the procedures are relatively new. Also, as these patients mature they will be better able to voice concerns and thus physicians may more readily identify complications. A comparison of one- and two-stage procedures found a 63% complication rate with the one-stage open technique having a 30% incidence of postoperative enterocolitis [56]. Other studies have shown equivalent rates of complications between the one-stage and multistage approaches. One-stage enterocolitis rates have ranged from 10% to 14%. Another author noted an 8–10% incidence of postoperative enterocolitis [57]. Langer and Winthrop compared one-stage and two-stage Soave's procedures, and found a lower incidence of enterocolitis in babies weighing less than 4 kg [47]. Another study of the laparoscopic approach with one-stage neonatal reconstruction showed a very low incidence of adhesive bowel obstruction. Strictures were rare, and enterocolitis did not occur in this series [2].

29.4 Conclusion

Hirschsprung's disease is a neurogenic intestinal obstruction with potential for chronic illness. A wide spectrum of complications has been reported following definitive repair of Hirschsprung's disease. Enterocolitis remains the most serious late complication following definitive repair. Continued advances in our understanding of the disturbances in bowel motility and the immunological and neurohormonal forces involved in this disorder will result in an improving prognosis.

Traditional multistage procedures still have a role, especially in the very small, critically ill child. The long-term complications of one-stage and laparoscopically assisted procedures are currently not clearly known. Fortunately, the majority of patients with Hirschsprung's disease do quite well following definitive operation regardless of the technique employed. The great majority (94%) of children will become well-adjusted members of society [25]. Early development milestone deficiencies appear to improve over time. Appropriate preoperative conference with family members must include a candid discussion of the importance of realistic expectations and the need for close parental surveillance for late complications.

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Long-Term Results and Quality of Life After Treatment of Hirschsprung's Disease and Allied Disorders

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30

30.1 Introduction

The late follow-up of children with Hirschsprung's disease gives one the best opportunity to critically evaluate the efficacy and results of a particular surgical procedure. Unfortunately, most reviews of Hirschsprung's disease are hampered by the fact that follow-up periods are rather short and may not adequately reflect a patient's long-term outcome and lifestyle. In general, the most commonly encountered problems include constipation, incontinence, enterocolitis and the overall impact of the disease on lifestyle. Other complications, such as fistulae, obstructions and impotence are quite rare and are discussed toward the end of the chapter. Each of these problems is covered in the context of the four most commonly performed pull-through procedures: the Swenson, Duhamel and Soave (endorectal pull-through, ERPT, including the transanal route). The most recent technical modifications and literature reviews are included.

30.2 Continence

Probably no other complication has a greater impact on the overall quality of life than incontinence. An accurate assessment of continence is often difficult to obtain because many earlier series were based on retrospective reviews and often lacked a careful assessment of the child's stooling pattern. Table 30.1 provides relative rates of incontinence from several large series. Unfortunately, incontinence rates are often not reported in some of the larger series. Other series fail to make a distinction between occasional soiling and significant incontinence. Overall, a few generalities can be stated regarding continence rates. First, several authors have noted higher rates of incontinence in patients with trisomy 21 or other syndromes related to mental retardation [1–5]. In some series, however, such an association was not noted. The typical incidence of incontinence ranges from 3% to 8%; however, several series from the past decade have noted a higher percentage of soiling [6–11]. Catto-Smith et al. noted that 27% of their patients had severe incontinence with no improvement over time [7]. A high incontinence rate was also noted by Heij et al., with 17% of their patients with standard length Hirschsprung's disease having severe incontinence [6]. Bai et al. and Diseth et al. note that fecal incontinence had the greatest detrimental effect on the overall quality of life [8, 11] (see below).

It is difficult to attribute a cause to these high incontinence rates; however, technique and overall experience with each pull-through and the details of how the stooling history was obtained vary widely between series. Because of the popularity of the transanal route for ERPT, many have questioned whether further compromise of the anal sphincter will adversely affect future continence. Several reports, although containing only moderate numbers of long-term follow-up patients, suggest that this is not the case [12–14]. Further, manometric measurements of anal sphincter tone are similar in those undergoing an endorectal dissection via the transanal versus the transabdominal route [15].

An important overall trend noted in several series is a gradual improvement in incontinence rates over time. Polley et al. noted that no patient more than 3 years of age was incontinent in their 10-year review of patients undergoing an ERPT procedure at our hospital [16]. Several additional patients in their series were referred for symptoms of incontinence after a Swenson repair, and were

Table 30.1 Incidence of incontinence after pull-through for Hirschsprung's disease

Reference	Type of pull-through	Incidence of incontinence (%)
21	ERPT	10
18	Swenson	3.2
20	Swenson	8
66	Rehbein	0
67	Duhamel	7.2
	ERPT	5.1
	Swenson	19.5
39	Soave	2.1
	ERPT	1.1
70	ERPT	0
	Duhamel	0
	Swenson	0
54	Mix, Duhamel predominating	9[a]
2	Mix	6–12.6[b]
7	Mix	27
6	Duhamel	17–27[c]
17	Duhamel	0–12[d]
68	ERPT, primary	0
14	Transanal ERPT	0
69	ERPT, primary	21
46	ERPT, primary	1
58	Transanal ERPT	17[e]

[a]Of these patients, three were mentally retarded and three suffered from a leak after pull-through.
[b]The lower of the two numbers excludes those with total colonic aganglionosis and those with trisomy 21.
[c]The lower of the two numbers are those for standard length aganglionosis only.
[d]The 12% represents stooling within the first 3 years after the pull-through and the 0% is the incidence of incontinence after long-term (>10 years) follow-up.
[e]Follow-up of children over 3 years of age in this series is limited to less than half of the patients.

successfully treated with an enema program. Rescorla et al. noted that 12% of patients under 5 years of age had some degree of soilage and incontinence [17]; whereas, only 6% had these symptoms by 10 and 15 years of age, and no patient complained of incontinence after 15 years of age. A similar experience was noted in Swenson's 1975 series, in which 15.3% of patients had temporary soiling; however, this figure declined to 3.2% as patients were followed long-term, and no patient over 11 years of age complained of incontinence [18]. Heikkinen et al. [3], Baillie et al. [19], and Yanchar and Soucy [9] also noted a strong correlation between improvement in soiling and adolescence. These results strongly suggest that the pediatric surgeon dealing with a patient with incontinence should work closely and aggressively using medical treatment, dietary modifications and constipative agents prior to considering any form of surgical therapy.

30.3 Stooling Frequency and Constipation

Stooling frequencies, unfortunately, are rarely documented in most large reports of Hirschsprung's disease. Liem et al. noted normal stooling frequency in 94% of their patients who underwent the Swenson procedure [20]. Erdek and Wilt accurately documented the number of bowel movements in each of their patients who were followed long-term after an ERPT procedure [21]. Five of their 29 patients had three or more bowel movements per day. Overall these authors noted a gradual decline in the number of bowel movements as these patients were followed for longer periods of time.

Figure 30.1 shows the number of bowel movements in our own group of 23 infants who had undergone an ERPT for Hirschsprung's disease as a primary procedure [22]. The frequency of stooling has a rather steep decline

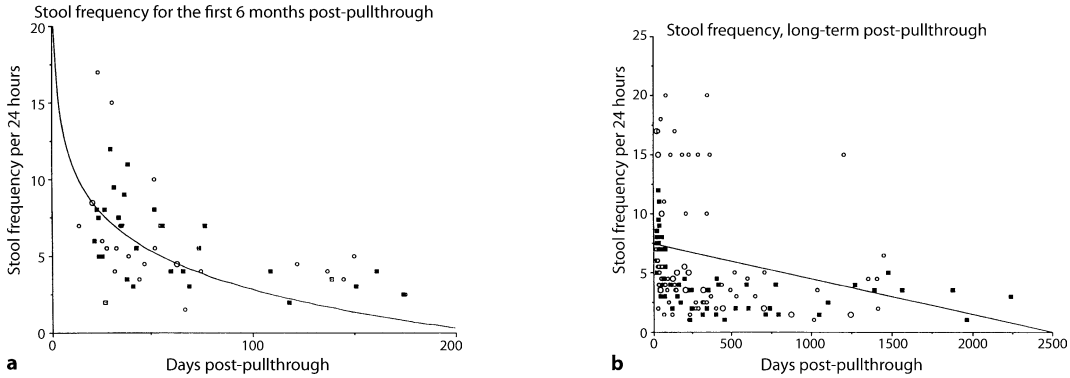

Fig. 30.1a,b Stooling frequency of 23 neonates and young infants cared for at our hospital with a primary endorectal pull-through without an interval leveling colostomy. Children who developed enterocolitis are represented by an *open symbol* and those without enterocolitis are shown by a *closed symbol*. **a** Stooling frequency within the first 6 months after the pull-through. The regression line shown has an r^2 value 1.0. **b** Stooling frequency after 6 months. The *regression line* shown has an r^2 value of 0.79

within the first 3 months after the pull-through procedure. Patients initially started with five to eight bowel movements per day, and this frequency declined to approximately three to five bowel movements per day over this time period (Fig. 30.1a). A second slope was then noted between 3 months and 1 year of age when the number of bowel movements declined from three to five per day to two to three per day (Fig. 30.1b). This trend then remained fairly stable with a slight decline to approximately two bowel movements per day over the next year. Both Tariq et al. [23] and Wang et al. [24] noted that stooling frequency was high following a pull-through; however, as with our study, a gradual improvement was noted, so that with long-term follow-up frequency was only slightly higher than that described for normal children [25, 26].

Constipation is a frequent complaint of children after a pull-through procedure for Hirschsprung's disease (Table 30.2). It may well be that patients with Hirschsprung's disease continue to have prolonged colonic transit time as the cause of their constipation [19]. The evaluation of a child with constipative symptoms after an apparently successful pull-through procedure requires a thorough history, physical examination and diagnostic work-up. Very often the history of constipation may be short in duration and often improves with time. Physical examination will rule out the development of a postoperative stricture (see below), as well as the formation of a spur in an improperly performed Duhamel procedure. Finally, in the absence of a mechanical obstruction, a complete diagnostic evaluation will be required. In a review of this problem by Moore et al. in 1994, the most informative work-up was obtained by performing a rectal biopsy [27]. Of the 178 children in their series cared for with Hirschsprung's disease, 16 still had constipative or obstructive symptoms at 4 or more years of age. An extensive work-up showed that 13 of the 16 patients had abnormal findings including 4 with a retained aganglionic segment and 9 with evidence of intestinal neuronal dysplasia (IND). Manometric work-up in their series was not predictive of a poor outcome and did not help in the overall assessment of the patient. However, manometry may be beneficial in the work-up of some patients. Anal manometry, however, may provide useful information regarding persistent spasticity of the internal sphincter, and may help to understand the cause of either constipation or incontinence [28].

Most forms of constipation are without a histopathological correlate and can readily be managed without such an extensive evaluation. Rates of constipation range from 6% to 11% in most series. Overall, the Swenson procedure has the highest incidence of constipation. In Soave's series, 14.4% of children undergoing the Swenson procedure were constipated [29]. Puri and Nixon found that 34.5% of children were constipated after a Swenson procedure [30]. Soave noted that the Duhamel procedure had the lowest rate of constipation at 6.7%. Importantly, however, these rates may well underestimate the overall number of patients who require medication to normalize their stooling. As an example, Rescorla et al. reported that 8% of their patients had severe constipation; however, 27% of all of their patients had some constipative complaints which required medication [17]. A similar rate of 26% requiring medication to normalize their bowel habits is noted by Marty et al. despite their reporting only a 7.5% constipation rate [2]. Occasionally, patients with severe constipative symptoms may require a posterior myotomy or myectomy. The results of these studies in properly selected patients may be quite satisfactory. Based on two recent reviews of patients with persistent stooling problems, a detailed schematic of the work-up that should be initiated was developed (Fig. 30.2) [31, 32].

Table 30.2 Incidence of constipation after pull-through for Hirschsprung's disease

Reference	Type of pull-through	Incidence of constipation (%)
21	ERPT	21
65	Rehbein	11
29	ERPT	9.5
	Duhamel	6.7
	Swenson	14.4
	Rehbein	12.6
69	ERPT	0
	Duhamel	0
	Swenson	3
20	Swenson	6
3	Mix, Duhamel predominating	56
2	Mix	7.5
6	Duhamel	32
67	ERPT, primary	13
14	Transanal ERPT	0
17	Duhamel	8
45	ERPT, primary	28[a]

[a]Defined as more frequent than every 3 months

30.4 Enterocolitis

Enterocolitis is a fairly frequent complication which may trouble patients after a pull-through procedure [33–36]. Rates of enterocolitis after pull-through vary widely among series. This variance more than likely relates to differences in the definition of enterocolitis as well as its differentiation from other processes, such as gastroenteritis or spasm of the internal sphincter. Reported rates are noted in Table 30.3 and range from 2% to 43%. In two of the largest series of Hirschsprung's disease, a significantly higher incidence of enterocolitis was noted in those patients who had undergone Swenson's pull-through [37, 38]. This high incidence of enterocolitis was also noted in Swenson's own review from 1975, and this may be due to the inclusion of several patients who underwent this pull-through in the earlier years of the Swenson procedure, prior to it being modified to a lower anastomosis with a partial inclusion of the internal sphincter on the posterior aspect [39]. Postoperative enterocolitis has been associated with a number of deaths in several series. In fact, when examining those deaths due directly to Hirschsprung's disease, in several series approximately 50% of the deaths were due to complications directly related to an enterocolitic episode [17, 18, 38, 40]. The majority of these deaths, importantly, occurred within the first year following the pull-through [41].

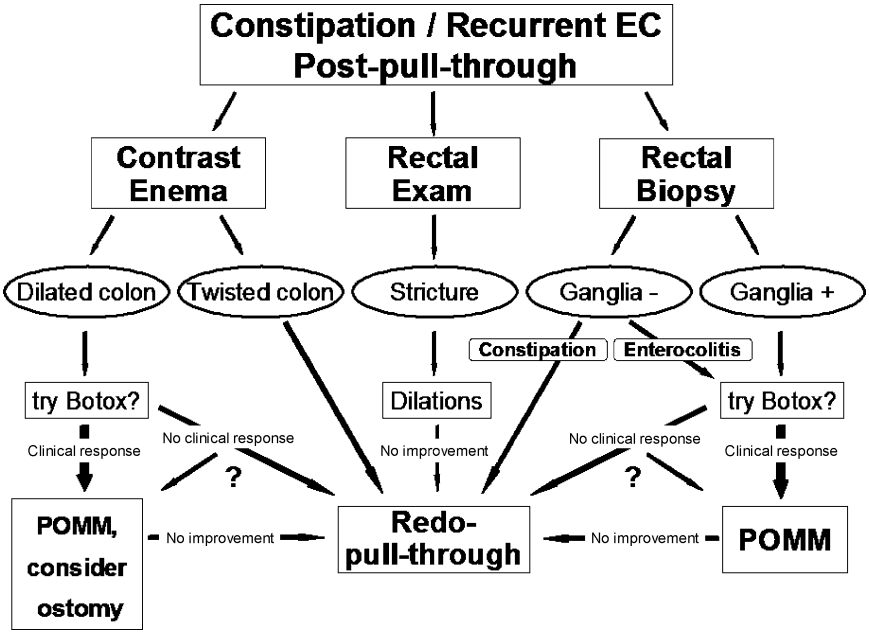

Fig. 30.2 Flow chart for the workup and treatment of patients with chronic constipation or recurrent enterocolitis (EC) after a pull-through operation for Hirschsprung's disease. Note that a botulinum toxin (Botox) injection may be used before proceeding to myectomy. If the patient derives long-term benefit from Botox, no further treatment is needed. If the patient develops transient improvement with Botox, a myectomy should be considered. Abbreviation: POMM, posterior myotomy or myectomy

Table 30.3 Incidence of enterocolitis after pull-through for Hirschsprung's disease (*NS* not stated)

Reference	Type of pull-through	Incidence of enterocolitis (%)	Enterocolitis requiring surgery (%)	Deaths due to enterocolitis after pull-through (%)
38	ERPT	2	NS	75
	Duhamel	6		
	Swenson	16		
65	Rehbein	0	NS	NS
34	Duhamel	6.3	60	0
65	ERPT	7.4		NS
70	Swenson	21		46
20	Swenson	3		
2	Mix	27	22	71
16	ERPT	16	38	0
37	ERPT	12	NS	NS
	Duhamel	14		
	Swenson	34		
33	ERPT	21.4	16	0
45	ERPT	5	42	NS
12	Transanal	NS	6	NS
57	Transanal	NS	17	NS

Most patients with enterocolitis have been treated conservatively, as noted by Swenson in 1975. The majority of these episodes occur within the first 2 years after the pull-through. Although some authors have noted an association between enterocolitis before pull-through and an increased incidence of enterocolitis after pull-through [42], this trend has not been seen by other authors [36, 43]. In our own series of patients with Hirschsprung's disease, a lack of correlation between enterocolitis before and after pull-through was also seen [33]. However, evidence of enterocolitis seen pathologically on the rectal biopsy or pull-through specimen was highly correlated with the development of enterocolitis after pull-through [33], and this may denote a high risk group of patients for subsequent enterocolitis. Enterocolitis after pull-through is managed conservatively with washouts and oral antibiotics. Several authors have described the performance of an internal sphincterotomy in patients with persistent enterocolitis after pull-through. Marty et al. performed 8 in 37 patients with enterocolitis after pull-through [2]. A more recent report comprising the largest series of posterior myectomy or myotomy in 32 children suggests excellent results in controlling enterocolitis [31]. It is important to note that we would advocate a significant period of conservative therapy, since most patients with enterocolitis after pull-through will improve over time.

Patients undergoing a primary pull-through appear to have a high incidence of enterocolitis (ranging from 8% to 42%). Depending on the series, this is either comparable to or higher than historical controls undergoing a staged approach [44–47].

The transanal approach, which has become the most popular approach to Hirschsprung's disease, has been associated with varying rates of enterocolitis. In a review by Langer et al. of multicenter experience, a 6% incidence was noted [12]. Whereas, Minford et al. noted a much higher incidence (35%) of enterocolitis [48].

30.5 Total Colonic Aganglionosis

The long-term outcome of patients with total colonic aganglionosis (TCA) needs to be examined separately, because the overall results in this particular group of patients are significantly worse when compared to standard or even long-segment Hirschsprung's disease. Analysis of outcome is hampered by a lack of separation of this particular group of patients in many series, as well as the overall small number of patients with TCA who may be treated in one particular institution. In the series of Ikeda and Goto there were 67 patients (4%) with TCA [37]. In the patients in that report, the Duhamel procedure was the predominant pull-through, and there was an associated mortality rate of 13%. In the review of Kleinhaus et al. for the American Academy of Pediatrics, the majority of surgeons performed either

a Duhamel or Swenson procedure, with an associated morbidity of 62% and 70%, respectively, and mortality rates of 25% and 30% [38]. Good results were noted in both of the above series with the ERPT procedure with a 0% morbidity and 0% mortality; however, only nine patients underwent this procedure including patients from both series. Overall stooling frequency in either of these two series was not mentioned. Stooling frequency and incontinence appeared quite high in the series of Marty et al., who noted that 40% of their patients with TCA had severe soiling [2]. Heij et al. also noted that six of their seven patients with TCA were incontinent [6]. The majority of patients in these latter series had undergone a Duhamel or Martin procedure. This is in contrast to the series of Endo et al., who noted that despite a history of loose stools in virtually all nine of their patients (one to five loose stools per day), the frequency of bowel movements improved steadily over time and often had decreased to two to five times a day by 2–3 years of age. All of the patients with TCA cared for by Endo et al. underwent a Kimura patch of aganglionic colon just proximal to their ileoanal anastomosis [49]. Endo et al. did note a significantly higher frequency of stooling in those patients with more proximal and extensive aganglionosis. A comparison of procedures by Hoehner et al. found poor stooling in those children undergoing a Martin modification, and moderately good outcomes in those patients who had either a standard Duhamel or ERPT [50].

A definitive statement with regard to the optimal procedure for TCA cannot be made because of the small numbers of patients in each group. There are a limited number of reports of long-term outcomes, but results have improved in the past two decades [51]. Baillie et al. found that children with TCA had the worst functional outcome of all of their Hirschsprung's disease patients [19]. In our own series of 25 patients with TCA who primarily underwent an ERPT, long-term stooling outcome was found to be good in 83%, with the best stooling scores found in those with the longest follow-up [52].

30.6 Stricture Formation After Definitive Pull-Through Procedure

The incidence of stricture formation varies widely among reported series, from 0% [20, 21] to as high as 20% in the series of Tariq et al. [23] (Table 30.4). Clearly, the development of many of these strictures depends on the clinician's definition of stricture. Most strictures respond to conservative treatment with rectal dilatations. Approximately one-third to one-half of all clinically significant strictures appear to require surgical intervention. No specific type of pull-through was noted to be associated with a higher incidence of stricture. In one series the Swenson procedure was associated with a 0% incidence of stricture [20], but in the series of Kleinhaus et al. [38] the Swenson procedure was associated with the highest incidence of stricture (9.5%). Heikkinen et al. stated that the highest incidence of strictures was in those patients who developed an early postoperative leak [53]. However, Swenson stated that only 3 of his 23 patients who developed a late stricture had a history of an early postoperative leak, and he could find no obvious predisposing factor [18]. It is possible that some of these strictures could have been because the anastomosis was of the circular type performed several years ago, which has been speculated to predispose to a higher rate of stricture formation, as opposed to the current oblique type. More recently the stricture formation has been closely associated with the development of enterocolitis after pull-through [36, 43, 45].

30.7 Impotence and Urinary Dysfunction

The performance of any operative procedure in the pelvis can result in nerve injury. Clearly, many of the operative procedures for Hirschsprung's disease have been designed to specifically avoid these structures. Overall, the incidence of these complications is quite low. The Swenson procedure intuitively appears to be the one which, because of design, would have the highest risk of nerve injury. However, in a review of 282 patients by Swenson, 101 men had gone through puberty and none had developed impotence, 80 were married and had a total of 146 children [18]. Puri and Nixon found that 2 of 84 patients who had undergone a Swenson procedure were unable to ejaculate despite a normal erection [30]. The majority of recent reviews have not identified this to be a problem.

Late urological disturbances are also a very rare complication. It is felt that those procedures with the least amount of pelvic dissection are associated with the lowest incidence of problems with urination. In Puri and Nixon's review in 1977, 7 of 52 patients who had undergone a Duhamel procedure had nocturnal enuresis and 2 had daytime urinary dribbling [30]. Ehrenpreis noted 3 of 30 children, all younger than 3 years of age, with urinary incontinence [54]. Again, although one might anticipate a higher incidence of urinary problems with the Swenson procedure, the incidence is fairly low in most series. Nielsen and Madsen did report 5 of 71 patients (7%) with urinary incontinence, but none of these problems persisted long-term [55]. In Swenson's own series, no patients had urinary problems and only one patient (2%) in the series of Liem et al. had stress incontinence following a Swenson pull-through [18, 20]. Importantly, several more recent studies of patients following various pull-through procedures have failed to identify any patients with these significant urological symptoms [12, 46, 56, 57].

Table 30.4 Incidence of stricture after pull-through for Hirschsprung's disease (*NS* not stated)

Reference	Type of pull-through	Incidence of stricture	Percent needing surgery
37	ERPT	2.8	0.3
	Swenson	6.7	2.2
70	Swenson	NS	3.2
20	Swenson	0	0
38	ERPT	9.4	4.2
	Duhamel	5.5	2.6
	Swenson	9.5	4.3
2	Mix	5.1	NS
23	ERPT	20	NS

30.8 Late Mortality

Table 30.5 shows the incidence of late mortality in several recent series. In most reports, differentiation between early and late deaths is lacking. Furthermore, the exact cause of death is often not mentioned. As noted in the enterocolitis section, a large number of late deaths are related to the development of enterocolitis after pull-through. In some series enterocolitis comprised almost 50% of all deaths; whereas in other reports, no deaths due to enterocolitis after pull-through were noted. Other causes of death include bowel obstruction, pneumonia and other nonrelated medical disorders. No specific pull-through procedure was associated with a higher rate of late deaths.

30.9 Long-term Outcome in Patients With Intestinal Neuronal Dysplasia

Assessment of long-term outcome in patients with IND is difficult because of the relative rarity of this disease. In addition, because the diagnosis of IND has only recently become popularized, long-term follow-up of many patients is lacking. In fact, the majority of patients with IND will not need surgery and should do reasonably well over time [58]. The outcome in patients with more symptomatic disease may not be as optimistic. Ure et al. reviewed 203 patients with IND who were followed long-term [59]. Late results are available in 119 of these patients who underwent surgical therapy between the years of 1963 and 1988. In those patients with a combination of IND type A and B, 'achalasia' of the bowel was seen in one patient and persistent enterocolitis was seen in another. One patient required a subsequent re-resection because of obstructive symptoms. In those patients with only IND type B, two patients had persistent constipation and one patient had 'achalasia' of the bowel. Overall, seven patients with IND required subsequent surgical therapy and four required an internal sphincterotomy for recurrent enterocolitis. The majority of children responded to these secondary procedures and their overall quality of life was felt to be fairly good. In a follow-up study by these authors, it was noted that the best predictor of a successful outcome was the associated finding of aganglionosis [60]; outcomes in other patients were less successful. In another long-term follow up study, a similar finding was noted. In this study, the outcome in patients with IND with or without a more distal aganglionic segment were much worse than a comparative group of patients with Hirschsprung's disease [61]. These authors suggest a much more aggressive level of resection to prevent such poor outcomes.

30.10 Overall Quality of Life

The assessment of overall quality of life is a much more difficult question. In general quality of life issues have been poorly described in most reviews. In one of the more complete works on this subject, Moore et al. [10] states:

Quality of life remains a difficult concept to assess and is influenced by the physical, psychological, spiritual, functional and social well-being of the individual … Functional results are central to quality of life.

Overall quality of life was described as quite good, with 94% of children becoming well-adjusted members of society. Although patients had deficiencies of weight for age, this was generally corrected with time. Additionally, developmental milestones and school performance were satisfactory in most patients (95% and 82%, respectively). Clearly, patients with poor functional outcomes will have a greater tendency to have more psychosocial problems. In general, the review by Moore et al. of quality of life issues was quite positive in children after an ERPT. Factors which were predictive of a poorer quality of life were incontinence (6.1%) and poor family support (percent not stated). Those with incontinence had significant psychosocial maladjustment.

In another evaluation of quality of life issues, a spectrum of neonatal surgical pathologies were examined. In this series, Takayanagi and Suruga identified Hirschsprung's disease as being an indicator of low quality of life [62]. The scoring system used by this group ranged from excellent (100%) to poor (0%). This review used a survey which was done by the physicians who performed the surgery on these children and the children's clinical nurses. Patients with Hirschsprung's disease received grades ranging from 68% to 52% by these two groups, respectively. Grading for Hirschsprung's disease surprisingly ranked on a par with imperforate anus and below that of diaphragmatic hernia and exomphalos.

Table 30.5 Incidence of late deaths after pull-through for Hirschsprung's disease (*NS* not stated)

Reference	Type of pull-through	Mortality (%)	Mortality due to enterocolitis (%)
38	Swenson	1.0	NS
	Duhamel	1.5	
	ERPT	1.1	
71	Duhamel	3.4	NS
20	Swenson	3.0	100
18	Swenson	1.2	50[a]
72	ERPT	4.8	
21	ERPT	3.3	NS
2	Mix	5.1	71
37	Swenson	2.2	NS
	Duhamel	1.5	
	ERPT	2.1	
30	Swenson	0	
41	ERPT	0.6	100
45	ERPT, primary	0	
12	Transanal	0.7	0

[a]No patient had developed enterocolitis by 36 months after pull-through.

Clearly, rating by each individual is subjective and those nurses with less than 15 years experience ranked patients with Hirschsprung's disease with the lowest scores.

Over the past decade several other very good evaluations of the quality of life of patients with Hirschsprung's disease have been done. In a study of children aged 8 to 16 years following Swenson pull-throughs, 87% were graded as having a good to fair quality of life; however, their score was significantly lower than that of age-matched controls [8]. This lower quality of life may be due to a very high rate of fecal soiling (37%) compared to many series [8]. A similar finding was noted in a study by Diseth et al. [11]. These authors found that psychosocial disorders were not different from those in control adolescents. However, the greatest impact on outcome was the high rate of incontinence. Results were somewhat different in a survey of 342 adult patients with either anorectal malformations or Hirschsprung's disease. This study found that the quality of life was comparable between these two groups of disorders, but significantly lower than in healthy adults – with both groups encountering overall physical health problems [63]. Only those patients with anorectal malformations, however, had additional complaints of pain and limitations in role functioning due to their physical problems. Psychosocial functioning had the greatest negative impact on quality of life for both groups.

A multidisciplinary approach can have a positive impact on these psychosocial disorders. Van Kuyk et al. described a multidisciplinary treatment carried out by a child psychologist, a pediatric physiotherapist and a pediatric surgeon comprising a stepwise program to address these problems [64]. These authors found that the majority of children will demonstrate a significant improvement in these disorders. The treatment was as effective in young children (2–5 years of age) as in older children (5–14 years of age).

30.11 Conclusions

As with most operations, complications arising even years after surgery can be attributed to problems occurring during the operation itself. The majority of patients with Hirschsprung's disease have a satisfactory or excellent long-term outcome after their pull-through procedure, provided the procedure is performed by a competent and well-trained pediatric surgeon. However, following even the best operation, problems may persist in many of these children. Therefore, close and long-term follow-up of these children is necessary. Often, a conservative, nonoperative approach can lead to a successful outcome in most patients. As concluded in the last edition of the text by Orvar Swenson:

Resection of the aganglionic colon … is a difficult operation. Yet, if a well-trained surgeon has an opportunity to observe the technical details of the operation and then perseveres, good results can be obtained.

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