Neuronal anomalies and normal muscle morphology at the hypomotile ileocecocolonic region of patients affected by idiopathic chronic constipation

M.-S. Faussone-Pellegrini[1], A. Infantino[2], P. Matini[1], A. Masin[2], B. Mayer[3] and M. Lise[2]

[1]Department of Human Anatomy and Histology, University of Florence, Florence, Italy, [2]Institute of Clinic Surgery II, University of Padua, Padua, Italy and [3]Institute of Pharmacology and Toxicology, University of Graz, Graz, Austria

Summary. Patients suffering from idiopathic slow-transit chronic constipation have a delayed colonic transit referable to a decrease or loss of propagating contractions. Myogenic and/or neural mechanisms have been implicated in the pathophysiology of this dysfunction and neuronal abnormalities have been described at the ascending, descending and sigmoid colon. The morphology and motile behaviour of the ileocecocolonic region, which in healthy subjects regulates cecum filling and emptying, have never been investigated in such disease. Therefore, we endoscopically ascertained whether a motility impairment was present at these junctional areas and neither spontaneous nor provoked occlusive contractions were found at the cecocolonic junction. Light and electron microscope examination of the entire colon revealed apparently normal features of neurons, smooth muscle cells and interstitial cells of Cajal, while immunohisto-chemistry and quantitative analysis demonstrated neuronal anomalies at the junctional areas. These anomalies consisted of low total neuron density and significantly few VIP-immunoreactive neurons at the two enteric plexuses, significantly few NOS-immuno-reactive neurons at the myenteric plexus and significantly more NOS-immunoreactive neurons at the submucous plexus. These findings exclude a myopathy and demonstrate the existence of a neuropathy. In particular, the presence at the ileocecocolonic region of few VIP- and NO-producing neurons suggests that there might be a reduced VIP and NO production which may result in a compromised relaxation and/or onset of propagating contractions, slowing down bolus transit. The presence at the proximal colon of such an abnormality might explain why left colectomy and/or cecorectal anastomosis are unsuccessful in patients with this disease.

Offprint requests to: Prof. M.S. Faussone-Pellegrini, Department of Human Anatomy and Histology, Section of Histology, Viale G. Pieraccini 6, I-50134, Florence, Italy. Fax: 0039. 55. 4271385. e-mail: s_faussone@cesit1.unifi.it

Introduction

Idiopathic slow-transit chronic constipation is a motility disorder of the entire colon. Patients have a delayed colonic transit probably referrable to a decrease or loss of propagating contractions (colonic inertia) and myogenic and/or neural mechanisms have also been implicated in the pathophysiology of this dysfunction (Bassotti et al., 1979; Preston et al., 1983; Preston and Lennard-Jones, 1986). Morphological studies on the ascending, descending and sigmoid colon have demonstrated a reduction in number of the myenteric plexus neurons (Krishnamurthy et al., 1985; Krishnamurthy and Schuffler, 1987; Koch et al., 1988; Cortesini et al., 1995), a decrease of both VIP-content and VIP-producing neurons (Koch et al., 1988; Milner et al., 1990; Van der Sijp et al., 1993; Cortesini et al., 1995) and an increased number of nitric oxide (NO)-producing neurons (Cortesini et al., 1995), suggesting that this disease is a neuropathy and that colonic inertia might be due to an excess of NO production.

The more proximal region of the large intestine should also be affected in this disease, since a markedly short transit time, supported by radioisotope studies and scintigraphic measurements, has been found at this level (Stivland et al., 1991; Van der Sijp et al., 1993; Bassotti et al., 1994). In agreement with the hypothesis that the entire colon is affected, patients responded poorly to surgery both when left colectomy alone (Preston et al., 1984) and a cecorectal anastomosis (Yoshoka and Keighley, 1989) were performed, while a total colectomy followed by ileorectal anastomosis gave fairly good results at follow-up and ensured a good quality of life. It has also been demonstrated that cecopexy considerably shortens the colonic transit time in such patients (Padron et al., 1995), suggesting an important role of the cecal region in regulating the fecal transit,

and that after cecopexy the VIP content might be restored (Padron and Ania, 1995).

Recently, three colonic regions, the right colon, the left colon and the cecocolonic junction, have been distinguished in man since they share region-specific circular muscle morphology (Faussone-Pellegrini et al., 1990a, 1993b), inhibitory innervation pattern (Faussone-Pellegrini et al., 1993a, 1994; Matini and Faussone-Pellegrini, 1994; Matini et al., 1995) and motility (Sarna, 1991a,b). It has also been reported that the cecocolonic junction (CCJ) has a muscular and innervational pattern similar to that of the ileocecal junction (ICJ) and that it undergoes either spontaneous or evoked spasms (Faussone-Pellegrini et al., 1995). Thus, the CCJ has been proposed as a sphincteric area (Faussone-Pellegrini et al., 1995) and as the site from whence colonic propagating contractions initiate (Faussone-Pellegrini et al., 1993b). The entire ileo-ceco-colonic region might, therefore, play an important role in the regulation of the colonic motile activities, ileal flow accomodation and cecum filling and emptying. Consequently, neural and/or muscular anomalies at the CCJ, and at the ICJ as well, might be present in motility disorders of the colon or even cause them. However, the morphology and motile behaviour of these junctions have never been investigated in such diseases.

In agreement with the above-mentioned data and with those reported for those affections of the human sphincters where a defect in relaxation is a prominent feature (Vanderwinden et al., 1992, 1993; Mearin et al., 1993; Hirakawa et al., 1995), in the chronically constipated subjects abnormal numbers of VIP and NO-producing neurons might also be present at the ileocecocolonic sphincters. A possible involvement of the pacemaker region (smooth muscle cells of the inner circular muscle layer plus interstitial cells of Cajal (Couturier at al., 1969; Faussone-Pellegrini and Cortesini, 1984; Faussone-Pellegrini et al., 1990a, 1993b; Rumessen et al., 1993)), however, cannot be ruled out, either at these sphincters and/or throughout the entire colon.

The purpose of this study was to gain an insight into the morphology and motile behaviour of the CCJ and ICJ in idiopathic chronic constipation. The CCJ behaviour was endoscopically evaluated prior to surgery by using the same procedure previously followed in healthy subjects by some of us (Faussone-Pellegrini et al., 1995). Then, in specimens surgically obtained from the selected patients, the ultrastructural features along the entire colon of the smooth muscle and interstitial cells of Cajal were studied in order to ascertain - or exclude - the presence of a myopathy and those of neurons in order to confirm or exclude the presence of a neuropathy. Secondly, a quantitative analysis of the total neuron density and of the density and percentage of immunostained VIP- and NO-producing neurons was performed in order to ascertain whether, similarly to the ascending, descending and sigmoid colon (Cortesini et al., 1995), a neural impairment morphologically

undetectable was also present at the CCJ and ICJ.

Materials and methods

Patients

Seven females (range 21-53 years old, mean 34.22 years) with idiopathic slow-transit chronic constipation underwent total colectomy and ileorectal anastomosis. All patients gave written informed consent. The Italian Law and the ethical guidelines of the Italian National Medical Council were followed throughout the clinical and laboratory procedures.

Clinical data

All patients had symptoms of idiopathic chronic constipation with spontaneous bowel frequency of less than once per week, difficulty in expelling hard feces and excessive staining; these symptoms were of several years duration. The constipation was resistant to conservative therapy with a high fiber diet and laxatives. Organic alterations were ruled out by barium enema. All the colons were normal in size and none of them had melanosis coli. After surgery, all patients evacuated without any problem in fecal continence.

Preoperative assessment consisted of intestinal transit time with radiopaque markers, anorectal manometry, anal sphincter EMG, defecography, colonic and small bowel manometry and scintigraphic transit time study. Plain radiographs of the abdomen were taken four-six days after ingestion of 30 pills (Infantino et al., 1990). The intestinal transit time with markers showed that small bowel transit was normal in all patients, ranging from 12 to 14 hours. Overall colonic transit time was delayed in all patients, with retention of markers at the 5th day in the transverse colon in four patient and in the left colon in three. At anorectal manometry no differences were found with respect to the normal control group. Anal sphincter EMG (Infantino et al., 1995) revealed partial denervation in one patient and paradoxical contraction of the pubo-rectalis muscle in four patients; this alteration resolved following preoperative biofeedback or surgery alone. Defeco-graphy findings ruled out the presence of rectal intussusception, prolapse and incomplete anal emptying. Colonic manometry revealed a reduction in basal activity in all patients, with the absence of HAPC in six; stimulation with standard meal evoked an abnormal reduction in activity in three cases, while stimulation with Bisacodyle evoked espulsion of the catheter or mass movements.

At small bowel manometry, which was performed in five patients, findings were normal in three cases and minor alterations were found in two. Four patients underwent intestinal scintigraphy with Indium[111]; gastric emptying was normal in all patients and at the 5th day "center of mass" (maximum radioactivity) was located in the transverse colon in three cases and in the

descending colon in one.

Five subjects with normal colonic transit time (females, range 43-60 years old, mean 57.8 years) submitted to elective either right or left colectomy for non-obstructive neoplasms represented the control group.

Endoscopy

Pancolonoscopies were performed on four patients (range 23-53 years old, mean 32.5 years) following the same procedure used for controls (Faussone-Pellegrini et al., 1995). Pharmacological treatment, such as sedation, analgesia, antispasmodics, was avoided. In two cases, the right colon could not be reached by the endoscopist because of pain of the patients. In the other two cases (23 and 31 years old), the cecum and the ileocecal area could be scanned and then observed for two minutes without pushing or pulling the endoscope, and without inflating or aspirating the colon. Mechanical stimulation of the ileocecal fold and the intestinal wall around and on the opposite side of the fold was performed by pushing two or three times with biopsy forceps.

Histology

Before immersion in the fixative liquid, full-thickness specimens of the CCJ were cut near, far and opposite to the ICJ opening and those of the ICJ were cut from both the superior and inferior ICJ lips, and further processed for both light and fluorescence microscope examination. Specimens obtained from the colon of the last two patients (23 and 53 years old) were also processed for electron microscopy.

Light and fluorescence microscopy

Specimens of CCJ and ICJ were fixed in 4% paraformaldehyde-buffered solution (PBS 0.1M, pH 7.4) for 6 hours and then paraffin embedded. Serial sections 6 μm thick were picked up on slides pre-coated with poly-L-lysine (Sigma, Milan, Italy, 0.1% solution in distilled water) and oven-dried overnight at +37 °C. Sections were de-paraffinized and brought through graded alcohols to water. Those used for bright field examination were either hematoxylin-eosin (HE) or PAS stained, without any previous or further treatment. For mast cell identification at fluorescence microscopy, sections were stained with tetramethylrhodamine isothiocyanate-labeled avidin (Immunotech, Marseille, France) diluted 1:400 in 1% albumin. For immunocyto-chemistry, sections were stained with an indirect immunohistochemical method in order to reveal the presence of nitric oxide synthase (NOS) and Vasoactive Intestinal Polypeptide (VIP). Non-specific attachment of primary antibodies to the tissue sections was prevented by incubating each of them for 15 minutes, at room temperature, with bovine serum albumin (BSA) 0.1%, in PBS 0.01M, pH 7.4.

For NOS-immunostaining, sections were incubated in a humid chamber with polyclonal antibodies anti-cerebellar NOS (neuronal NOS type I antiserum), raised in rabbit by one of us, B. M. (Mayer et al., 1990) against the purified neuronal enzyme extracted from porcine cerebellum, diluted 1:100, and applied overnight at room temperature. The specificity of the immunostaining for the NOS-antiserum was checked by preabsorption with the purified enzyme (50-100 mg/ml antiserum). This antibody does not recognise the inducible or endothelial isoforms (Mayer et al., 1990).

For VIP-immunostaining, primary rabbit polyclonal antibodies anti-VIP (Amersham, Buckinghamshire, UK), were applied on the sections diluted 1:1000 and incubated at +4 °C overnight. The antiserum to VIP was raised in rabbits using natural porcine VIP conjugated to keyhole limpet haemocyanin, using glutaraldehyde as a coupling agent.

All primary antibodies were diluted with Tris-buffered saline 0.01M pH 7.4; 3% normal serum was added to anti-VIP and anti-NOS antibodies; 0.3% (v/v) Triton X-100 (BDH, Poole, England) was added to anti-NOS antibodies. After incubating with primary antibody, sections were rinsed in 3 changes of PBS of 5 minutes each. Sections were then incubated for two hours with anti-rabbit IgG (whole molecule)-fluorescein-isothio-cyanate (FITC) conjugated (Sigma, Milan, Italy), raised in goat, diluted 1:60 in PBS 0.01M, pH 7.4. Fluoro-chrome-stained sections were rinsed in 3 changes of PBS, pH 7.2-7.6, of 5 minutes each, mounted in glycerine buffer (9:1), examined with a Zeiss Axioskop microscope equipped for epifluorescence. Immuno-positive neurons were counted and photographed in order to determine VIP-positive and NOS-positive neuron densities and percentages. After fluorescence microscope examination, the immunostained sections were further processed for bright field microscopy staining them with HE to determine the total neuron density.

Electron microscopy

Immediately after surgery, specimens of CCJ, ICJ, ascending, transverse and descending colon were cut in strips of about 1 mm thickness, immersed in a fixative solution of 2% cacodylate-buffered glutaraldehyde pH 7.4 and kept in this solution for 6 hours. Then, specimens were rinsed in a buffered solution of saccharose, post-fixed with 1% phosphate-buffered OsO_4, pH 7.4, dehydrated with acetone and embedded in Epon, using flat moulds in order to obtain transverse or longitudinal sections. The semi-thin sections, obtained with a Porter-Blum MT1 ultramicrotome, were stained with a solution of toluidine blue and photographed under the light microscope. Ultra-thin sections of the selected areas were obtained with a LKB NOVA ultramicrotome using a sapphire knife and stained with an alcoholic solution of uranyl acetate, followed by a solution of concentrated bismuth subnitrate. These sections were

Morphometry and statistics

Neurons were counted by two of us (M.-S. F.-P. and P.M.), both blinded as to the source of the sections. Two consecutive sections of each specimen were used, the former to count the NOS-positive neurons and the latter to count the VIP-positive neurons. The counts were separate for the submucous plexus (SMP) and the myenteric plexus (MP). These sections were then stained with HE in order to obtain the total number of neurons/mm section length. In HE-stained sections, neurons were identified on the basis of their distinctive morphology, such as basophilic cytoplasm, vesicular and nucleolated nucleus. In the immunohistochemically-stained sections, neurons were recognized by the presence of immunoreactivity in their cytoplasm. The term "neuronal section" was used to indicate all neuron profiles counted, i.e. both neurons sectioned at the level of the nucleus and those not containing it. Counts were made separately for each available specimen. The data from each patient were then pooled for each junction and plexus. For each patient, the number of neuronal sections was analyzed for each region and plexus. In each specimen, the length of the submucosa and muscle coat on sections was measured along lines parallel to and midway between the inner and outer boundary of the submucosa and muscle coat, respectively. Hence, the number of NOS-positive, VIP-positive and HE-stained neurons per unit section length (mm) of the submucosa and muscle coat was computed for each specimen. The percentage of NOS- and VIP-positive neurons was evaluated as number of immunoreactive neurons/total HE-stained neurons.

Specimens of the ICJ were at least two for each patient, obtained from the superior and the inferior lip, respectively, and comprehensive of the entire wall of the lip and of part of the neighbouring areas (ileum, cecum, ascending colon). The blocks were embedded in flat moulds with the cutting surface parallel to the longitudinal axis of the small bowel since the ICJ wall can be recognized from that of the neighbouring areas in full-thickness, longitudinal sections only, due to the presence at the ICJ of a peculiar inner circular muscle layer (Faussone-Pellegrini et al., 1995). Series of full-thickness sections (a minimum of 8 sections for each case) were cut from the two opposite surfaces of these blocks. The number of the blocks obtained from the CCJ varied among patients from one to three. Similarly to the ICJ, series of full-thickness sections (9-12 sections for each case) were obtained from the two opposite surfaces of each block. To ensure the recognition of the CCJ, the sections were cut parallely to the longitudinal axis of the colon. Only this type of section, in fact, allows the recognition of the CCJ from the neighbouring cecum and ascending colon due to the presence at this junctional area of a peculiar inner circular muscle layer (Faussone-

Pellegrini et al., 1990a, 1993b, 1995).

Control subjects

Altogether, in the HE stained sections, we analyzed a total of 1664 neuronal sections in the ICJ and 1764 in the CCJ for the MP, and a total of 1344 neuronal sections in the ICJ and 1328 in the CCJ for the SMP. In the sections stained for NOS, 796 neuronal sections were counted in the ICJ and 660 in the CCJ for the MP, and 160 neuronal sections were counted in the ICJ and 120 in the CCJ for the SMP. In the sections stained for VIP, we analyzed a total of 200 neuronal sections in the ICJ and 330 in the CCJ for the MP and a total of 712 neuronal sections in the ICJ and 402 in the CCJ for the SMP. A total of 112 mm of MP and 320 mm of SMP were scanned in the ICJ (range for each segment and each layer of each patient from 3 to 11 mm and from 3 to 30 mm for the MP and SMP, respectively). A total of 216 mm of MP and 342 mm of SMP were scanned in the CCJ (range for each segment and each layer of each patient from 5 to 21 mm for the MP and from 5 to 36 mm for the SMP, respectively).

Constipated subjects

Altogether, in the HE-stained sections, we analyzed a total of 1928 neuronal sections in the ICJ and 1737 in the CCJ for the MP and a total of 1504 neuronal sections in the ICJ and 921 in the CCJ for the SMP. In the sections stained for NOS, 744 neuronal sections were counted in the ICJ and 753 in the CCJ for the MP and 348 neuronal sections were counted in the ICJ and 310 in the CCJ for the SMP. In the sections stained for VIP, we analyzed a total of 144 neuronal sections in the ICJ and 183 in the CCJ for the MP and a total of 272 neuronal sections in the ICJ and 60 in the CCJ for the SMP. A total of 244 mm of MP and 440 mm of SMP were scanned in the ICJ (range for each segment and each layer of each patient from 2 to 22 mm and from 4 to 31 mm for the MP and the SMP, respectively). A total of 438 mm of MP and 395 mm of SMP were scanned in the CCJ (range for each segment and each layer of each patient from 3 to 56 mm for the MP and from 5 to 43 mm for the SMP, respectively).

Upon logarithmic transformation of neuron density, to keep the error variance low (Kirk, 1982; Lentner et al., 1982; Bahr and Mikel, 1987; Murray, 1991a,b), these values and those (untransformed) of the percentage of NOS- and VIP-positive neurons (number of positive nerve cells/total HE-stained nerve cells), were checked for homogeneity of variance by Bartlett's test; since no significant inhomogeneity was found (the value of the test was, in each case, lower than that necessary to exclude homogeneity with $p < 0.05$), data were further subjected to split analysis of variance, with two tails, assuming as error variance the one stemming from inter-individual differences. The variance among MP and SMP, that among ICJ and CCJ and the one depending

on the intra-plexus difference between junctions and inter-plexus difference within each junction were determined for control and constipated subjects separately and compared with the error variance to obtain the significance levels of differences. Probability levels that differences were due to chance of less than 5% (p<0.05), 1% (p<0.01) or 0.1% (p<0.001) were recorded and accepted as significant. Mean values and their standard error (S.E.) are presented in the results.

Differences between experimental conditions (controls vs. constipated subjects) were tested. The values for each experimental parameter (HE-neuron density, VIP- and NOS-positive neuron densities and percentages) determined in both the MP and SMP of the ICJ and CCJ were compared with those of each other experimental condition (controls vs. constipated subjects) with Student's t test. All tests were two tails. Probability levels that differences were due to chance of less than 5% were registered and accepted as significant.

Results

Endoscopy

In none of the patients did spontaneous or provoked spasms occurr during endoscopy.

Histology

Light microscopy

At both junctions and all along the other colonic levels (Fig. 5A), muscle layers (Fig. 1A, B, 5A, 6A, 7A) and enteric plexuses (Fig. 1C-F) there was apparently normal architecture. PAS-positivity, however, at the inner circular muscle layer was not so intense as in controls. Neither Schwann cell hyperplasia nor inflammatory reaction were observed at either plexus, but occasional, large-sized neurons could be seen (Fig. 1D, F), and in one case (31 years old) the nerve fibers of the submucous plexus were hypertrophic (Fig. 1E). Connective stroma was poor in inflammatory cells, while mast cells were abundant, especially at the inner portion of the circular muscle layer (Fig. 2A). Blood vessels showed normal caliber.

Electron microscopy

All along the colon and at both junctions, neurons (Fig. 1G) showed apparently normal features. Most of the nerve fibers were normal-sized and nerve endings contained synaptic vesicles (Fig. 3A,B, 4A,B), and only a few of them were large-sized, poor in synaptic vesicles or even empty (Figs. 3A, 4A). The smooth muscle cells of the outer circular muscle layer, of the longitudinal muscle layer and of the muscle sheaths enveloping the MP nerve strands and ganglia (Faussone-Pellegrini et al.,

1990b) were apparently normal (Fig. 3A,B). As in controls, at all colonic levels the smooth muscle cells of the inner circular muscle layer were rich in smooth endoplasmic reticulum and caveolae (Fig. 6B) and had frequent cell-to-cell junctions extended over wide cell surface areas (Fig. 5C). At the CCJ and ICJ (Figs. 6B, 7B,C), however, the glycogen deposits characteristic of these cells in healthy subjects (Faussone-Pellegrini et al., 1993b) could never be observed. The two cell types present at the submucosal border of the circular muscle layer and identified in man as interstitial cells of Cajal and/or as fibroblast-like cells (Faussone-Pellegrini and Cortesini, 1984; Rumessen et al., 1993) shared normal morphology all along the colon (Figs. 4A, 5D) and at both junctions (Figs. 4B, 6B, 7A-C). Well maintained cell-to-cell junctions were present among these cells and between them and the innermost-located smooth muscle cells (Figs. 5D, 6B, 7B,C). Collagen fibers were exceptionally numerous at the submucosal border of the inner circular muscle layer and within it (Figs. 2A, B, 6A, B, 7A-C), both in the young (23 years old) and aged (52 years old) patient. Fibroblasts, however, were rare as were macrophages and other inflammatory cells. Mast cells were filled with granules (Fig. 2B).

Histochemistry

As in controls (Figs. 8A,C, 9A,C), VIP- (Fig. 8B,D) and NOS-positive (Fig. 9B,D,E) neurons, some of which were large-sized (Fig. 9E), were found at the MP and SMP. In full-thickness sections of the submucosa, it could be seen that neither VIP- nor NOS-positive neurons shared a preferential location at one or another of the three subgroups of ganglia (inner, outer and intermediate) that characterize the colonic SMP of man (Hoyle and Burnstock, 1989; Ibba-Manneschi et al., 1995). Intramuscular VIP- and NOS-positive nerve fibers were scarce at all levels. As in controls (Bacci et al., 1995), VIP and NOS antibodies we used labeled within the connective tissue cells provided with an insegmented nucleus and a cytoplasm filled with granules. By using the same procedure followed for controls (Bacci et al., 1995), these cells were identified as mast cells. Among those located at the submucosal border of and within the inner circular muscle layer, few were NOS- (Fig. 10A,B) and many VIP-positive (Fig. 10C,D).

Statistical analysis

In all patients, quantitative analysis revealed that the total neuron density was constantly low at both plexuses of both junctions. The densities and percentages of VIP-positive neurons were significantly few at both plexuses and those of NOS-positive neurons were significantly few at the myenteric plexus and significantly more than in controls at the submucous plexus. The details on the quantitative data we obtained are as follows.
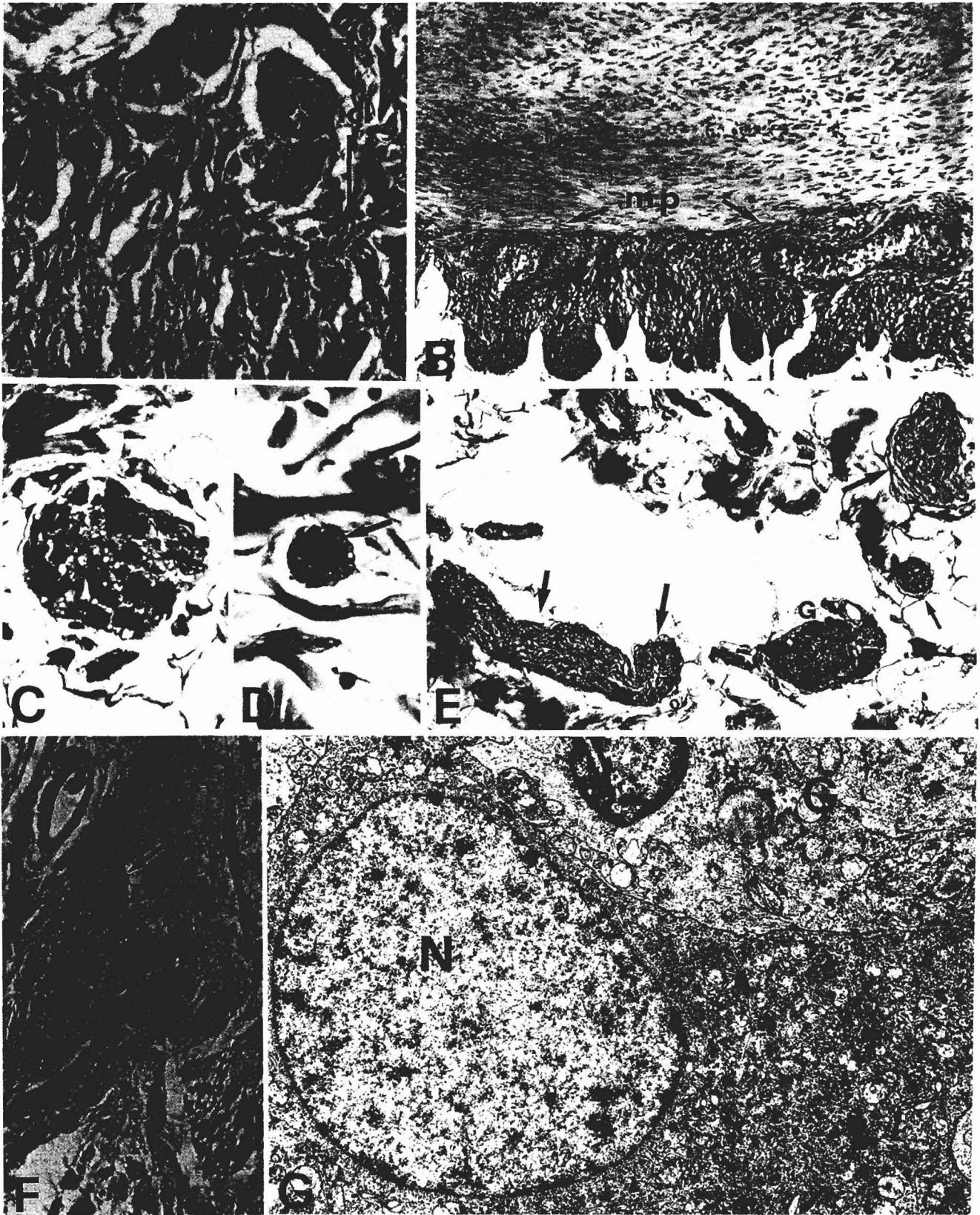
Fig. 1. Ileocecal and cecocolonic junctions in idiopathic chronic constipation. Muscle layers and enteric plexuses.
A-B. Light micrographs, hematoxylin-eosin staining. **A.** The characteristic thick and highly anastomosed inner portion of the circular muscle layer (icl) has normal features. x 150. **B.** Circular and longitudinal muscle layers and myenteric plexus (mp) show normal features. x 150.
C-E. Submucous plexus. **C.** A large ganglion rich in neurons (the same ganglion as in Fig. 9D). **D.** The arrow indicates a large-sized neuron (the same as in Fig. 9E). **E.** Two hypertrophic (thick arrows) and one normal-sized (thin arrow) nerve bundles in the 31-year-old patient. G: ganglion. C and D, x 330; E, x 240. **F and G.** Myenteric plexus. **F.** A ganglion poor in neurons, one of which (arrow) is large-sized. x 240. **G.** Electron micrograph of a ganglion. Both neuronal (N) and glial (G) cells show normal features. x 10,000

Total neuron density (Fig. 11)

Differences between neuron densities in controls and those in constipated subjects were statistically significant at both plexuses of the ICJ (p<0.01), while they were devoid of statistical significance at the CCJ. The differences between plexuses (MP versus SMP) and those between junctions were devoid of statistical significance in such patients, while in controls the differences between plexuses were significant (p<0.05). Moreover, similar to controls (Faussone-Pellegrini et al., 1993b, 1994), the neuronal density in the constipated subjects was found to be lower at the CCJ than at the ICJ and at the SMP than at the MP.

VIP-positive neuron density (Fig. 11) and percentage (Fig. 12)

The differences in VIP-positive neuron densities among controls and constipated subjects were significant (at the ICJ, p<0.0001 for both plexuses; at the CCJ, p<0.05 for the MP and p<0.01 for the SMP). The differences in percentage were significant for the SMP (p<0.05) at both junctions and for the MP (p<0.05) only

at the ICJ. Moreover, contrary to controls, at the CCJ the VIP-positive neuron densities and percentages were lower at the SMP than at the MP; while, as in controls (Faussone-Pellegrini et al., 1993b), VIP-positive neuron densities were found to be lower at the CCJ than at the ICJ and both densities and percentages at the MP of the ICJ were lower than those at the SMP. Furthermore, contrary to controls, the differences in VIP-neuron densities between junctions (ICJ versus CCJ) were found to be significant (p<0.005) in the constipated subjects, while the differences in percentages between plexuses were significant only in controls (p<0.01). No other statistical significance was found for any other difference.

NOS-positive neuron density (Fig. 11) and percentage (Fig. 12)

NOS-positive neuron densities at the MP were found to be lower and those at the SMP higher than in controls. Differences in densities were significant for both plexuses at the ICJ (p<0.01), and only for the SMP at the CCJ (p<0.05). Differences in percentage were significant for both plexuses at both junctions (for the MP, p<0.001
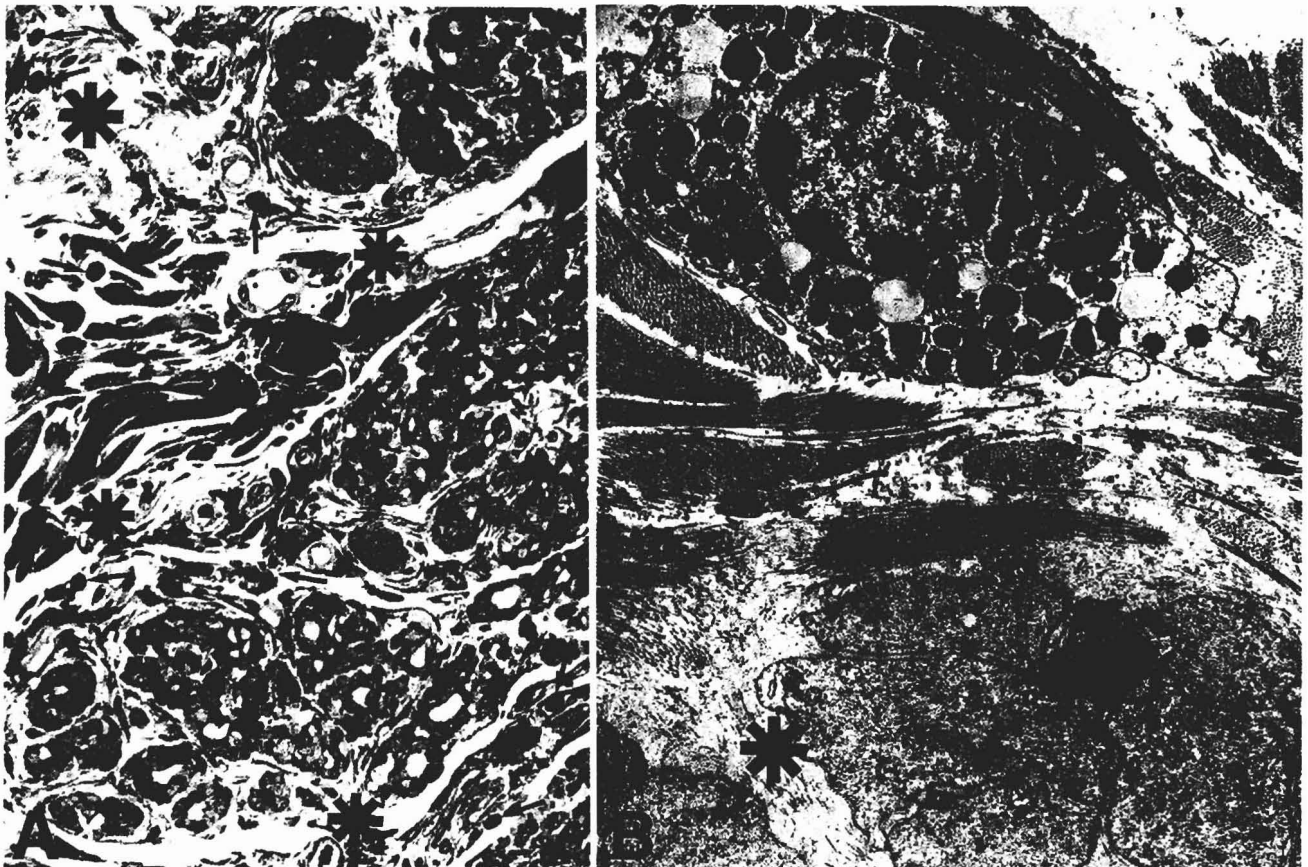

Fig. 2. Ileocecal and cecocolonic junctions in idiopathic chronic constipation. Mast cells. **A.** Light micrograph. Numerous mast cells (arrows) are present at the submucosal border of the inner circular layer (asterisks). Semithin section, toluidine blue. 240. **B.** Electron micrograph of a mast cell (upper side) located near the inner circular layer (asterisk). x 5,000
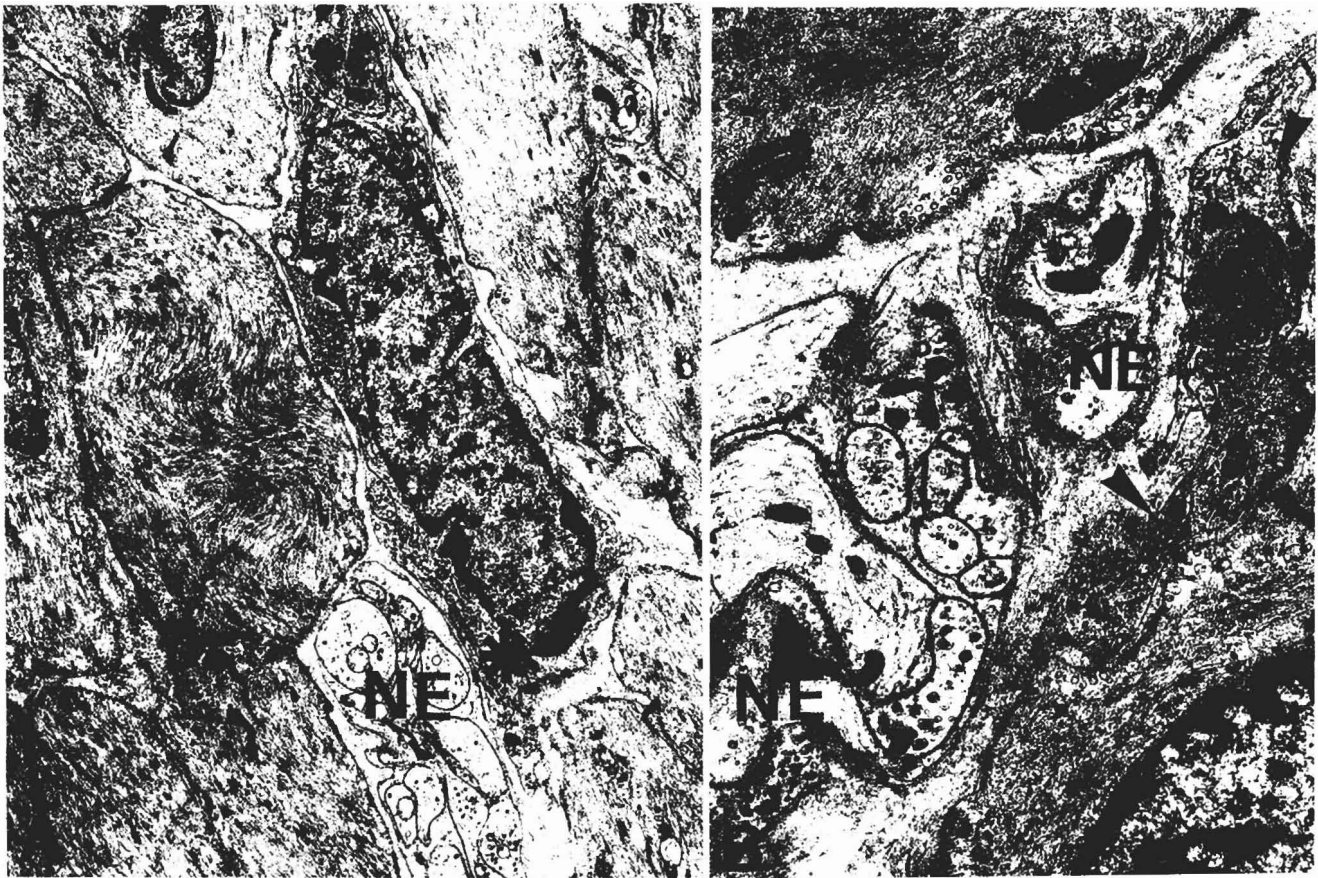
Fig. 3. Ileocecal and cecocolonic junctions in idiopathic chronic constipation. Electron micrographs of intramuscular nerve endings. **A.** Nerve endings (NE), some of which are large-sized, two fibroblasts (asterisks) and smooth muscle cells of the circular muscle layer sharing normal features. x 6,250 **B.** Nerve endings (NE), two interconnected interstitial cells of Cajal (arrowheads) and smooth muscle cells of the muscle sheath surrounding myenteric plexus ganglia. x 20,000
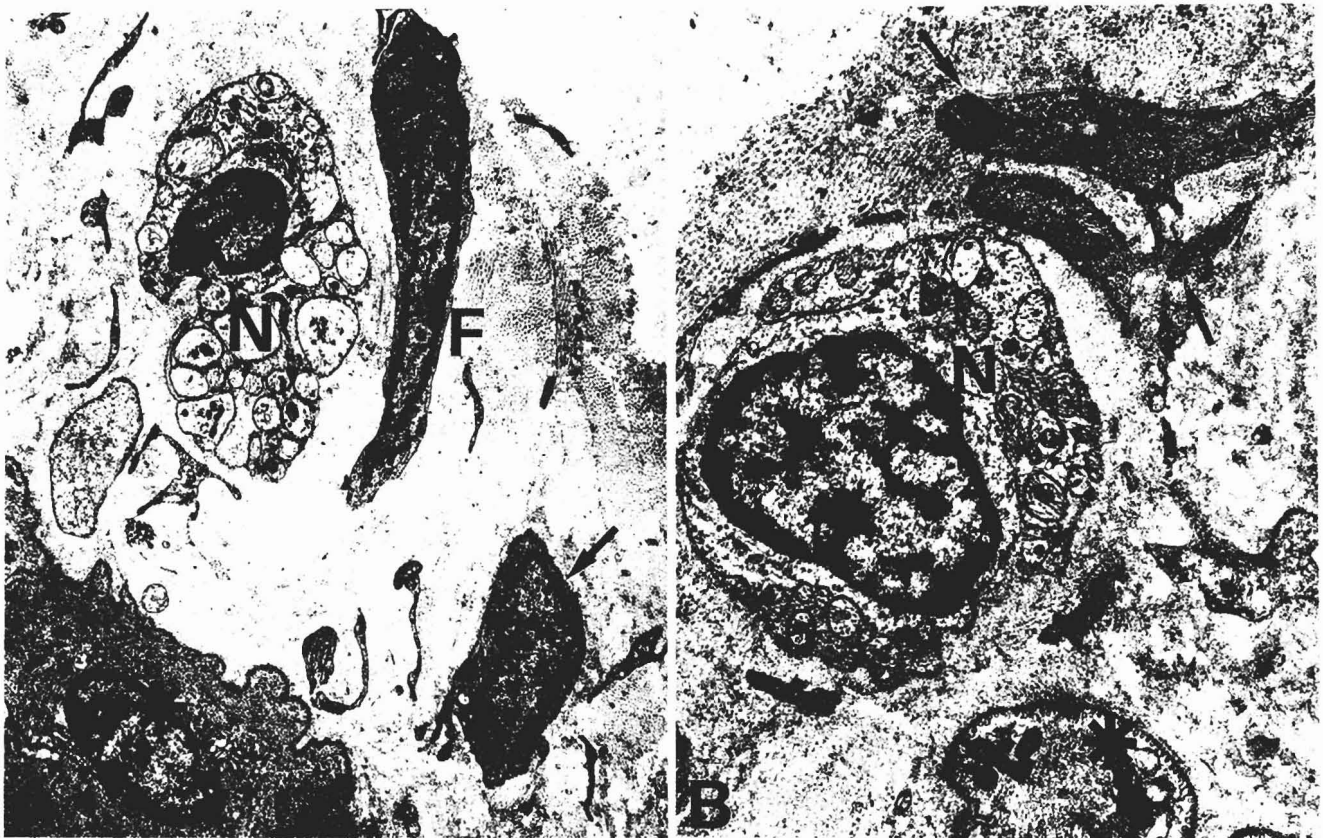

Fig. 4. Right **(A)** and left **(B)** colon in idiopathic chronic constipation. Electron micrographs of the submucosal border of the circular muscle layer. N: Nerve bundles of the outer subdivision of the submucous plexus; F: fibroblast-like cells; arrows: fibroblast-like cells and interstitial cells of Cajal; asterisks: smooth muscle cells of the inner circular layer. A, x 8,000; B, x 14,000
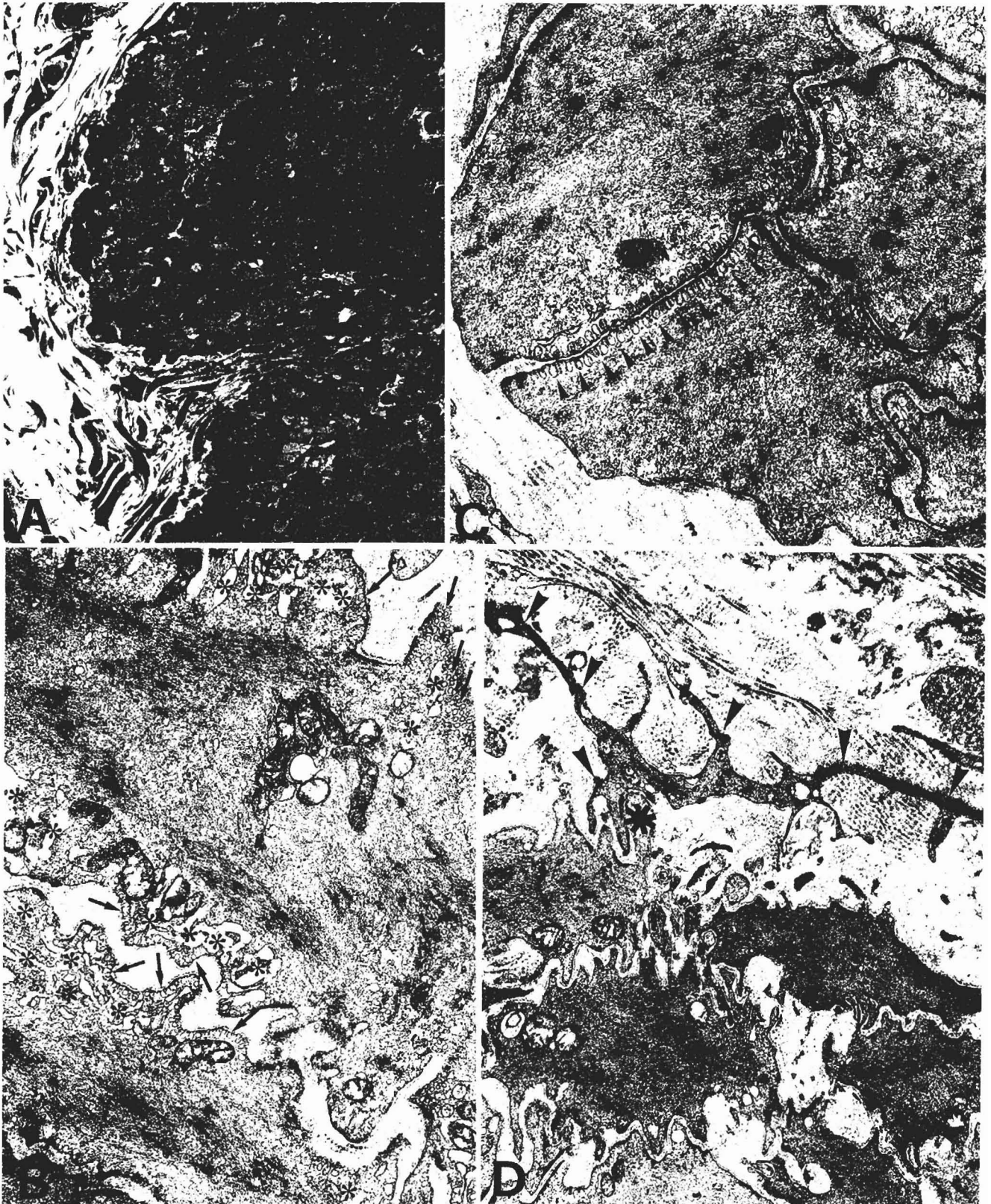
Fig. 5. Right and left colon in idiopathic chronic constipation. Inner circular layer. **A.** Light micrograph of the inner (I) and outer (O) circular muscle layers. Semithin section, toluidine blue. x 480. **B.** Electron microscopy. The smooth muscle cells of the inner circular layer are rich in smooth endoplasmic reticulum (asterisks) and caveolae (arrows) as in normal subjects. x 17,500. **C.** Electron microscopy. All the characteristic cell-to-cell contacts (thin and thick arrows and arrowheads) are present. x 25,000. **D.** Electron microscopy. Thin and ramified branches (arrowheads) of an interstitial cell of Cajal located at the submucosal border of the circular muscle layer. The asterisk indicates a peg-and-socket contact between this cell and one smooth muscle cell. x 15,000

at the ICJ and p<0.01 at the CCJ and for the SMP p<0.01 at both junctions). As in controls (Faussone-Pellegrini et al., 1994), in the constipated subjects NOS-positive neuron densities were lower at the CCJ than at the ICJ and at the SMP than at the MP. No significant differences in the neuron densities were found in constipated patients between plexuses (MP versus SMP) and between junctions (ICJ versus CCJ), while in controls those between plexuses were significant (p<0.005). As in controls, at the CCJ the percentage of NOS-positive neurons was lower at the SMP than at the MP, although without any statistical significance, while in controls a statistically significant difference (p<0.005) was present. At the ICJ, on the contrary, the differences in percentages, but without any statistical significance, were lower at the MP than at the SMP.

No other significant source of experimental variance was found, other than those indicated above.

Discussion

Endoscopy demonstrated that the motility impairment in idiopathic chronic constipated subjects is extended along the entire colon, since, similarly to the other colonic regions, the ileocecal region has also been found hypomotile and neither the spontaneus nor the evoked spams occurring in control subjects at the CCJ (Faussone-Pellegrini et al., 1995) could be seen in these patients.

The presence in this disease of a myopathy (either primary or secondary) could be excluded by examining under light and electron microscopes the muscle wall of the entire colon surgically obtained from these patients. Besides that the muscle layers share the same architecture as in controls, as already reported by others (Park et al., 1995), we could demonstrate by electron microscope examination that also the smooth muscle cells and the putative colonic pacemaker cells - the interstitial cells of Cajal (Faussone-Pellegrini and Cortesini, 1984; Faussone-Pellegrini et al., 1990a, 1993b; Rumessen et al., 1993) - have normal features at all colonic levels. At the ICJ and CCJ, however, the smooth muscle cells of the inner circular muscle layer - which are considered as an important component of the pacemaker tissue present in the human colon (Faussone-Pellegrini and Cortesini, 1984; Faussone-Pellegrini et
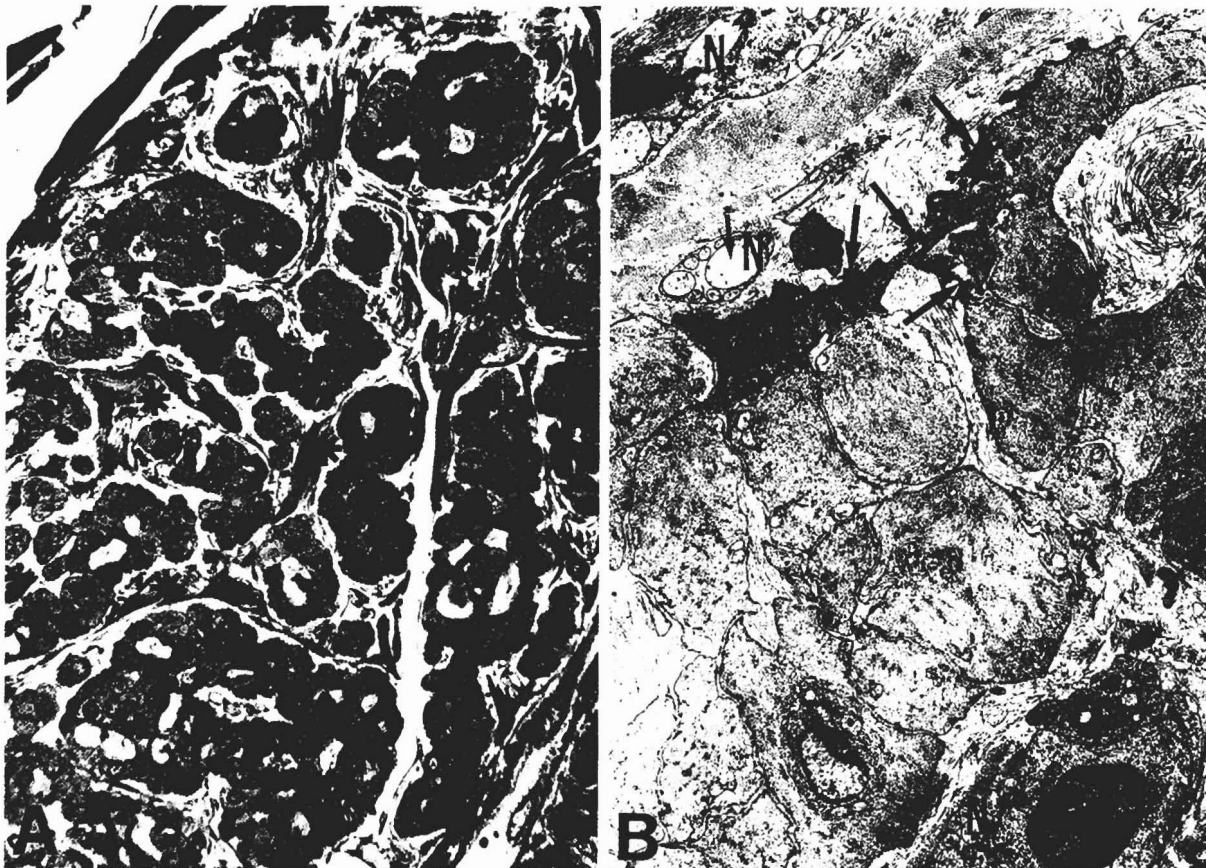

Fig. 6. Ileocecal and cecocolonic junctions in idiopathic chronic constipation. Inner circular layer. **A.** Light micrograph. Large bundles of collagen fibers (asterisks) are present among the smooth muscle cell bundles. Semithin section, toluidine blue. x 480. **B.** Electron micrograph. Nerve bundles (N) in the submucosa and in between the inner and outer (out of the lower side of the micrograph) subdivisions of the circular muscle layer. One nerve ending (thin arrow) is large and empty. Thick arrows indicate a group of interstitial cells of Cajal. The stroma is filled with collagen fibers. x 3,600

al., 1990a, 1993b; Rumessen et al., 1993) - were found devoid of the large glycogen deposits characterizing them in control subjects (Faussone-Pellegrini et al., 1993b), and immersed in a fibrotic stroma. These features were never seen in any of the control subjects (range 43-60 years old), neither are they to be considered as depending on the age of patients, being present both in the youngest (23 years old) and oldest (53 years old) patients. Thus, they should be considered as abnormalities present in constipated subjects. A possible explanation of their presence in such condition might be that they are a consequence of the muscle coat hypofunction and fecal stasis. Their possible pathophysiological significance might be important if it reflects an anomalous behaviour of the inner circular muscle layer of these sphincterial areas. For example, an impairment of the contractile capacity of the inner circular smooth muscle cells, due to the lack of an

important source of energy for muscle contraction (the glycogen) and the excessive richness in collagen fibers, might be suggested.

The quantitative data we obtained at both the CCJ and ICJ on HE-stained, anti-VIP and anti-NOS immunostained sections allowed us to ascertain the presence in idiopathic chronic constipation of a neural impairment at these junctions. Routine light and electron microscope examination enabled us to exclude the existence at their level and throughout the colon of a degenerative neuropathy, since all ganglia showed normal features. Under the electron microscope only some nerve endings, evenly dispersed, showed anomalous features, appearing large-sized and poor in synaptic vesicles. These anomalies, however, have been reported for normal subjects also and presumably are aspecific features due to the duration of surgery.
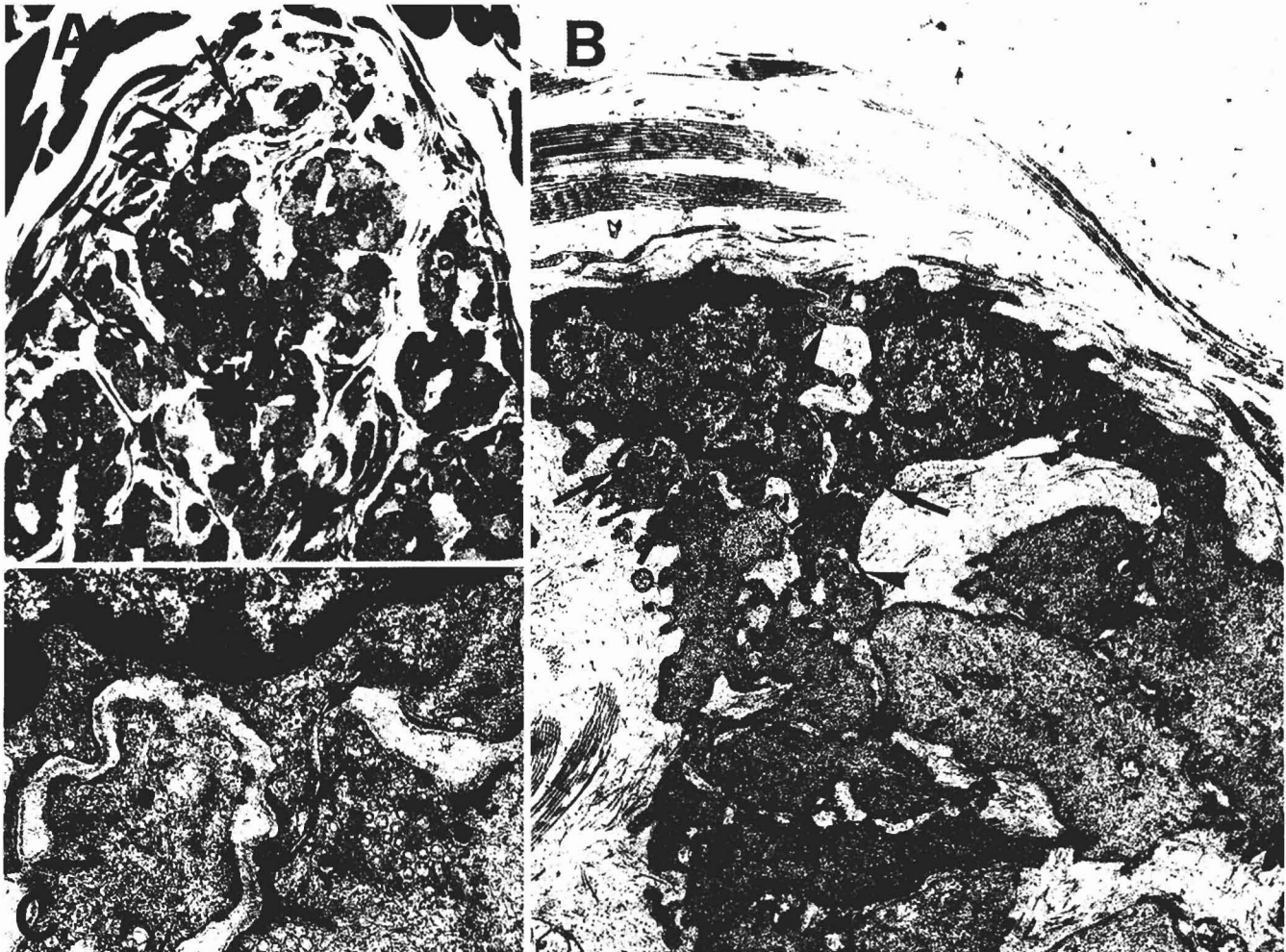
Interestingly, the neural impairment found at the

Fig. 7. Ileocecal and cecocolonic junctions in idiopathic chronic constipation. Inner circular layer and interstitial cells of Cajal. **A.** Light micrograph of presumed interstitial cells of Cajal (arrows) at the submucosal border of the inner circular layer (asterisk). Semithin section, toluidine blue. x 660. **B-C.** Electron micrographs of the submucosal interstitial cells of Cajal. Arrows and arrowheads indicate the cell-to-cell contacts between the interstitial cells and between these cells and the smooth muscle cells of the inner circular layer. B, x 6,250; C, x 25,000

CCJ and ICJ consisted of a constantly low total neuron number (statistically significant at the ICJ) at the MP, similarly to what was reported for the right and left colon of these patients (Cortesini et al., 1995). Parallel to this MP impairment, the VIP- and NOS-positive neuron densities and percentages were significantly different from those found in controls. In particular, at the MP of the CCJ and ICJ the VIP-positive neurons were extremely few and the number of the NOS-positive neurons was markedly reduced. At the right and left colon of these patients a similar reduction in VIP-positive neurons was found, while the number of the NOS-positive neurons was markedly increased (Cortesini et al., 1995). At the ICJ and CCJ, variations in the total neuron number, as well as in those of VIP- and NOS-positive neurons, were also found at the SMP; all variations found at this plexus were similar to those reported for the right and left colon of these same patients (Cortesini et al., 1995). More specifically, a significantly low number of SMP neurons paralleled with a statistically significant reduction of VIP-positive neuron densities and percentages, while NOS-positive neurons were significantly more numerous than in controls. Therefore, it could be concluded that neuronal anomalies affect the entire colon of such patients and

that most of these anomalies are similar between the ileocecocolonic region and the other colonic regions, with the unique exception of the variations in the number of the NOS-positive neurons at the MP.

Possibly, the presence at all colonic levels and at each plexus of an anomalous neuron number, the increase in neural supporting tissue and intramuscular nerve fibers reported by others (Park et al., 1995), as well as the abnormal number of the VIP- and NO-producing neurons might be due to a disturbance which occurred during the ontogenetic period. A similar etiopathogenesis has been proposed for other types of colonic diseases, such as Hirschsprung's disease, and a neural impairment has been found extending to the entire gastrointestinal tract and involving the urogenital apparatus too (Reynolds et al., 1987; Infantino et al., 1994). Some if not most surgeons now advocate testing small bowel motor function so as to ensure that the propulsive defect is confined to the colon. Bowel motor function assessed in five out of seven patients was normal in three and showed minor alterations in two. For this reason, the extension at all gut levels of the neural abnormality seems unlikely in our patients; furthermore radionuclide solid-meal gastric-emptying was normal in all of them and after surgery all patients evacuated
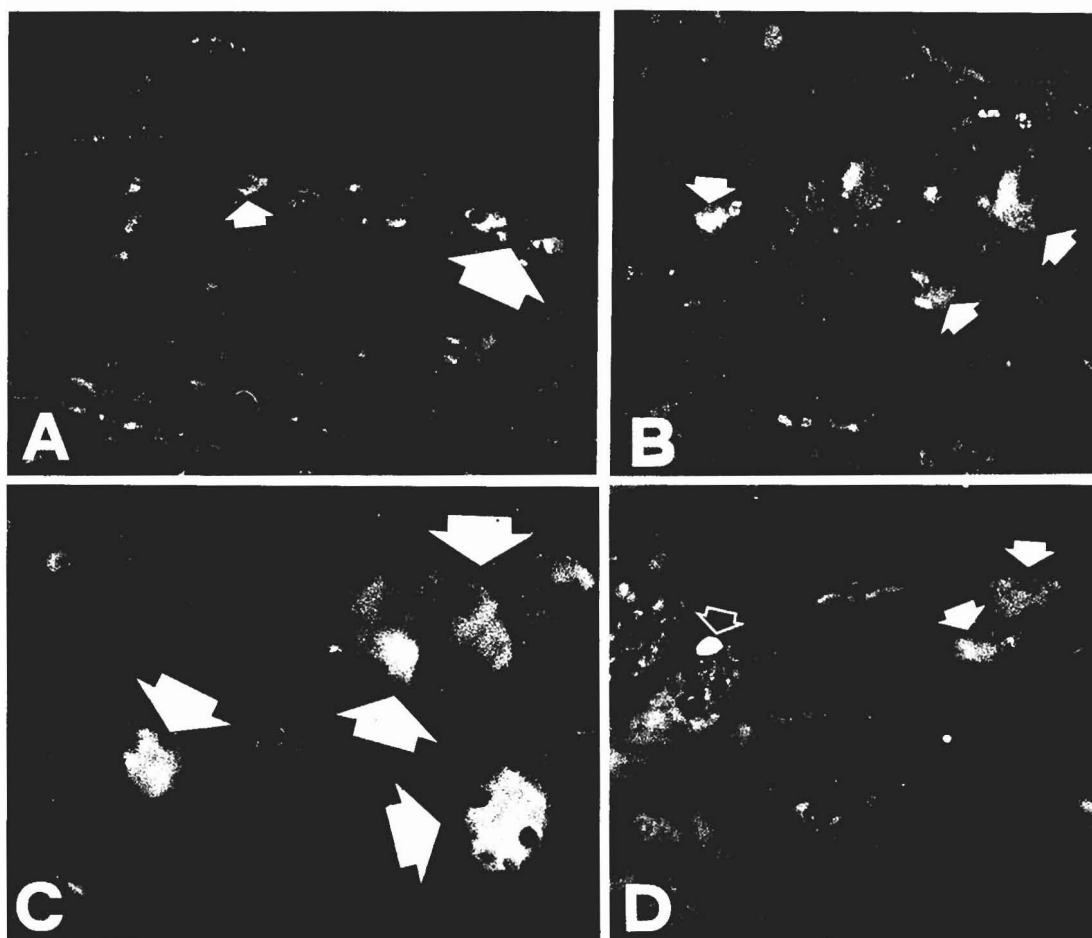

Fig. 8. Ileocecal and cecocolonic junctions. VIP-immunoreactive (IR) neurons.
A and B. Myenteric plexus. VIP-IR neurons (arrows) are few both in A (control subjects) and B (constipated subjects). A, x 200; B, x 400.
C and D. Submucous plexus. VIP-IR neurons (arrows) are numerous and intensely labeled in C (control subjects), while they are few and weakly labeled in D (constipated subjects). Empty arrow indicates one VIP-IR mast cell. C, x 400;

without any problem in fecal continence. It has to be noticed also that all of them still have a good quality of life and no further intestinal disease has taken place.

The presence in idiopathic chronic constipation of an altered inhibitory innervation also at the proximal sphincterial areas of the colon seems to be in agreement with data on the exaggerated reservoir functions of the right colon in this disease (Stivland et al., 1991; Van der Sijp et al., 1993; Bassotti et al., 1994) and may explain why in such patients left colectomy (Preston et al., 1984) and cecorectal anastomosis (Yoshoka and Keighley, 1989) are unsuccessful. A reduction in the VIP-positive innervation in the aganglionic segment of the colon obtained from patients with Hirschsprung's disease (Larsson, 1994) has been correlated to the lack of relaxation of this segment, and the deficiency in the NO-

producing neurons at the pylorus in infantile hyperthrophic pyloric stenosis (Vanderwinden et al., 1992), at the lower esophageal sphincter in achalasia (Mearin et al., 1993; Hirakawa et al., 1995) and at the anal sphincter in Hirschsprung's disease (Vanderwinden et al., 1993), has been correlated with a compromised sphincteric relaxation. In idiopathic chronic constipation, as in the afore-mentioned pathologies, there is a reduction in both VIP and NO at the ICJ and CCJ, specialised areas that in man are believed to play a sphincteric role (Faussone-Pellegrini et al., 1995). This reduction should correlate with a specific functional imbalance of constipated subjects and, presumably, may compromise the relaxation of these areas and, consequently, slow down the bolus transit. In this optique also, the high number of NOS-positive neurons present at the SMP may have a
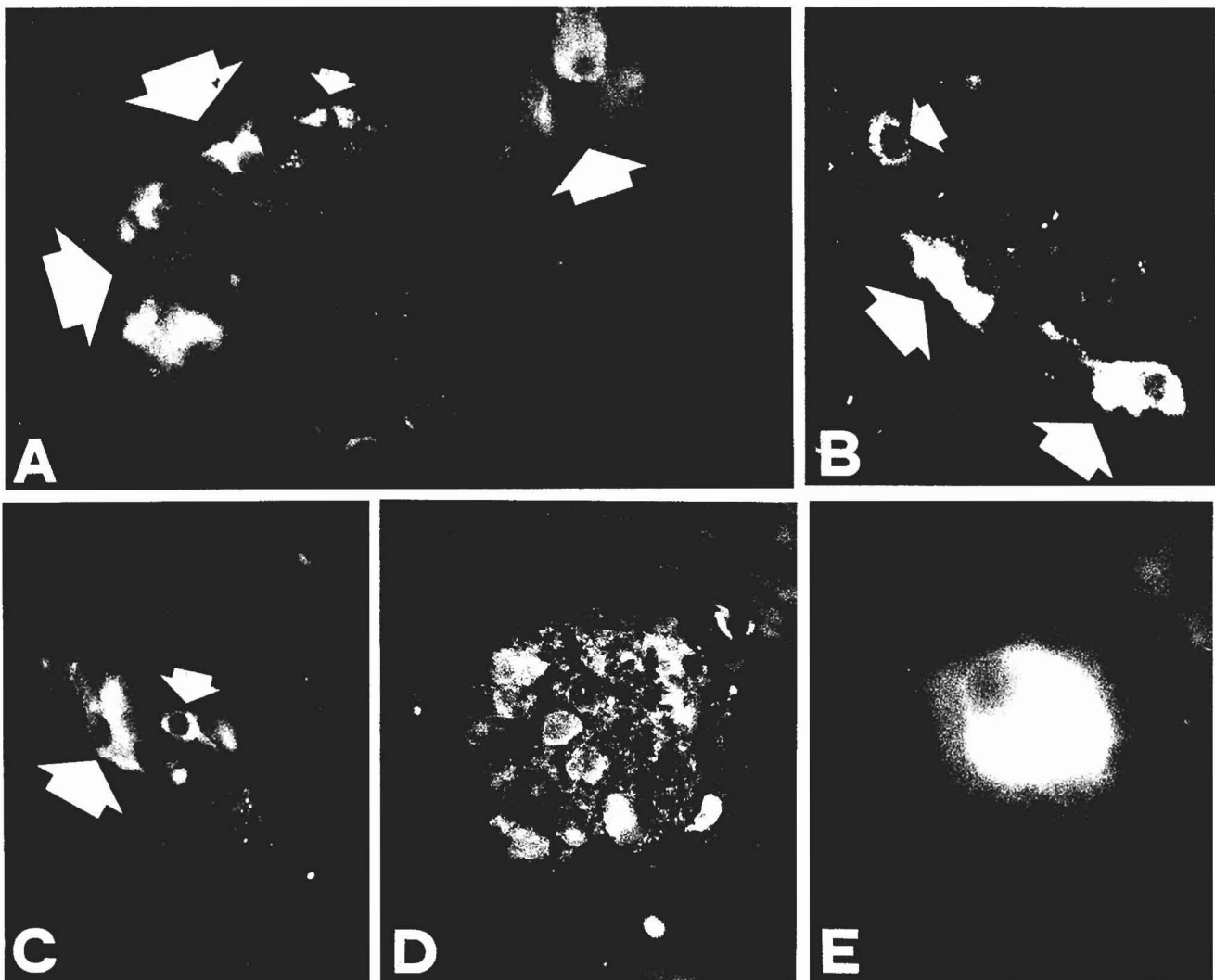

Fig. 9. Ileocecal and cecocolonic junctions. NOS-immunoreactive (IR) neurons. **A and B.** Myenteric plexus. NOS-IR neurons (arrows) are numerous in A (control subjects), while they are few in B (constipated subjects). A, x 400; B, x 400. **C-E.** Submucous plexus. NOS-IR neurons (arrows) are fewer in C (control subjects), than in D (constipated subjects). This ganglion is the same as that shown in Fig. 1C. E. One large-sized NOS-IR neuron (the same as in Fig. 1D) in the 31-year-old patient. C and D, x 400; E, x 1,000

possible clinical relevance. Inhibitory motor neurons of the SMP have been proposed as regulating the activity of the colonic pacemaker area (the submucosal interstitial

cells of Cajal plus the smooth muscle cells of the inner circular muscle layer (Faussone-Pellegrini and Cortesini, 1984; Faussone-Pellegrini et al., 1990a, 1993b;
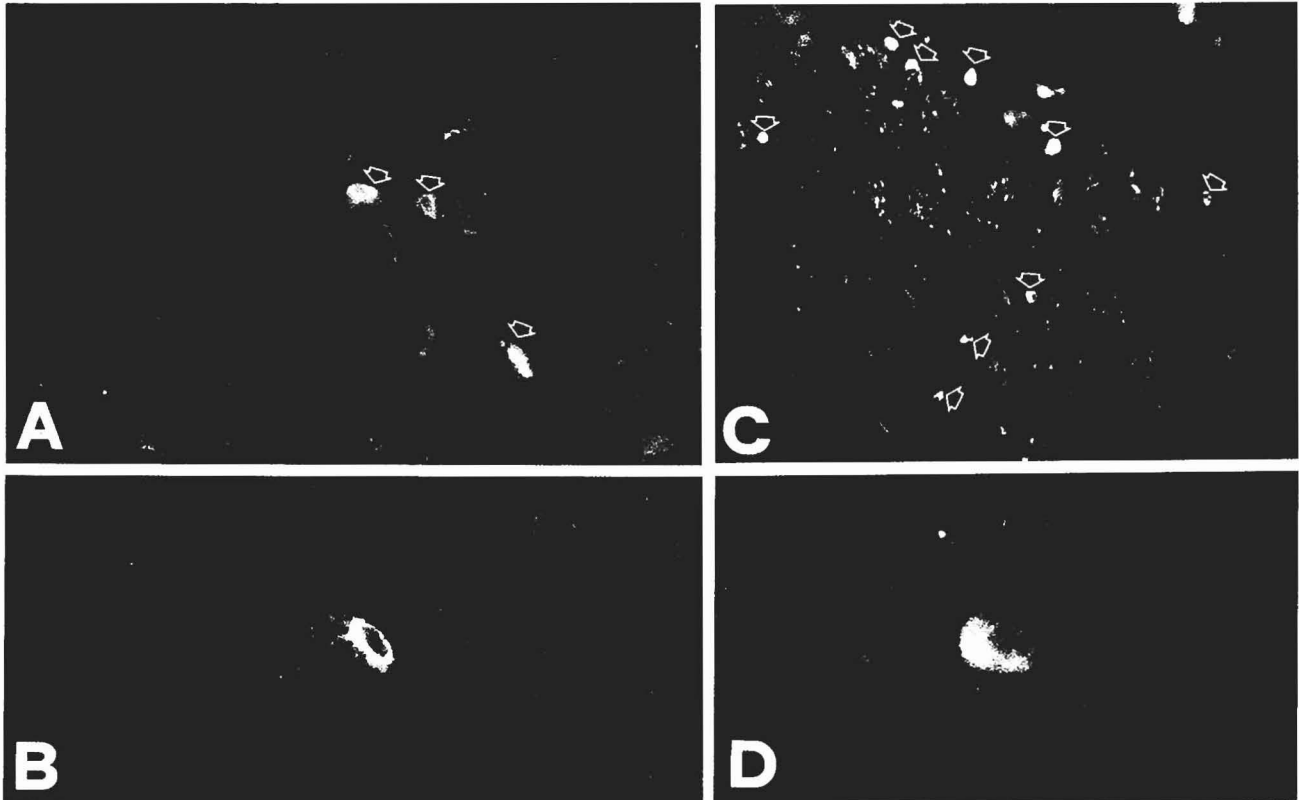

Fig. 10. Ileocecal and cecocolonic junctions in idiopathic chronic constipation. NOS- and VIP-immunoreactive (IR) mast cells. **A and B.** NOS-IR mast cells (arrows) at the submucosal border of the inner circular layer and within it. A, x 400; B, x 1,000. **C and D.** VIP-IR mast cells (arrows) at the submucosal border of the inner circular layer and within it. C, x 200; D, x 1,000
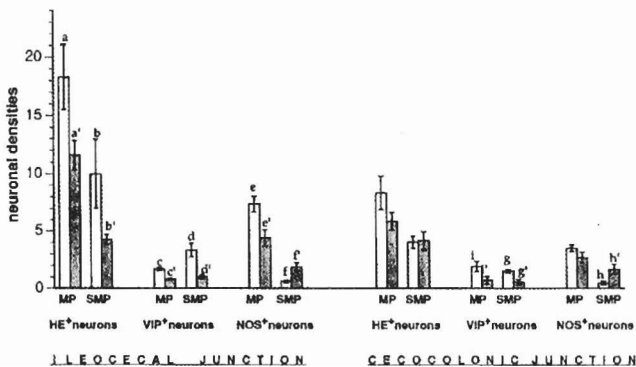

Fig. 11. Neuronal densities (HE-, VIP-, NOS-stained neurons per mm) in the myenteric (MP) and submucous (SMP) plexuses at the ileocecal and cecocolonic junctions in normal and constipated subjects. Differences between control subjects (empty columns) and constipated subjects (stippled columns) were statistically evaluated by means of the two tail Student t-test. Significance levels were as follow: a vs. a', b vs. b', e vs. e', f vs. f', g vs. g': p<0.01; c vs. c', d vs. d': p<0.0001; h vs. h', i vs. i': p<0.05. Error bars, mean±S.E.
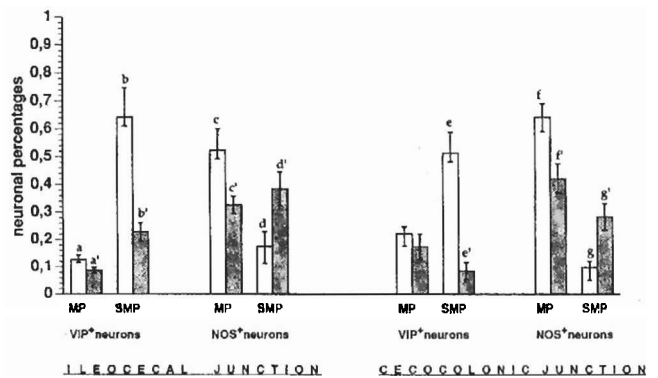
Fig. 12. Percentages of VIP- and NOS-stained neurons in the myenteric (MP) and submucous (SMP) plexuses at the ileocecal and cecocolonic junctions in normal and constipated subjects. Differences between control subjects (empty columns) and constipated subjects (stippled columns) were statistically evaluated by means of the two tail Student t-test. Significance levels were as follow: a vs. a': p<0.05; b vs. b', e vs. e': p<0.0001; c vs. c', d vs. d', f vs. f', g vs. g': p<0.01. Error bars, mean±S.E.

Rumessen et al., 1993). It would be of high interest to ascertain whether the pacemaker activity of this region is impaired in such patients in the presence of an increased number of NO-producing neurons at the SMP and of an apparently normal pacemaker tissue.

Interestingly, mast cells were numerous at the ICJ and CCJ of idiopathic chronic constipated patients and shared intense VIP- and NOS-positivity. Speculatively, since the VIP-positive mast cells were particularly numerous at the inner portion of the circular muscle layer and at its submucosal border, the VIP these cells release may provide this layer with large amounts of a myorelaxant molecule and contribute to its hypomotility.

In conclusion, data we obtained in idiopathic chronic constipated patients i) show that the motility behaviour of the ileocecal region is impaired; ii) demonstrate the existence of an important neural impairment at the ICJ and CCJ, and, since this impairment is similar to that already described at the right and left colon, greatly support that this disease is a neuropathy extended along the entire colonic length; and iii) exclude the presence along the entire colonic length of a myopathy and/or an involvement of the colonic pacemaker tissue (interstitial cells of Cajal plus smooth muscle cells of the inner circular muscle layer). From a pathophysiological point of view, other possible regulatory molecules other than VIP and NO might be involved, although it remains that there is an important deficiency of VIP-producing neurons and a considerable variation in NO-producing neurons. A different release in both these molecules with known relaxant effects points towards a possible mechanism. We have, however, very little knowledge of the motor patterns or tone of the colon in health, and even less in intractable constipation, but possible other mechanisms might also be involved. According to the present data, it can be hypothesized that the colonic slow transit characteristic of this pathology might be dependent at a proximal level on a compromised relaxation of the ileocecocolonic junctions and on an impaired onset of the propagating contractions.

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