Virchows Arch. Abt. A Path. Anat. 358, 113—136 (1973)

Nervous Elements in the Human Colon of Hirschsprung's Disease

(With Comparative Remarks on Neuronal Profiles in the Normal Human and Monkey Colon and Sphincter Ani Internus)

H. G. Baumgarten *, A. F. Holstein, and F. Stelzner

Department of Neuroanatomy and Department of Functional Anatomy, University of Hamburg
Department of Surgery, University of Frankfurt

Received September 27, 1972

Summary. Neuronal profiles in the human and monkey colon and sphincter ani internus were analyzed microscopically and compared to those in the constricted aganglionic transitional and dilated, hypertrophic sections of colon from 3 cases of Hirschsprung's disease. Light microscopy revealed two different types of nerve bundles in the normal colon: 1. aniline-blue-positive nerve trunks (conventional peripheral type) containing endoneural collagen and layers of perineural sheath cells, in all probability of extrinsic origin and autonomic in nature, and 2. azocarmine-positive, large multiaxonal Schwann units in Auerbach's plexus, devoid of collagen and hardly ensheathed by perineurium (bundles of intrinsic type), resembling central nervous neurophil formations (with or without ganglion cells interspersed). Preterminal and terminal varicose axons in Auerbach's plexus—not distinguishable by light microscopy—were classified according to their vesicle populations and suspected transmitter type into adrenergic, cholinergic, p-type and sensory fibres.

The internal anal sphincter receives predominantly large bundles of the conventional peripheral type containing a conspicuous amount of myelinated axons, as does the aganglionic portion of the Hirschsprung colon. Vesicle-filled profiles among the smooth muscle cells of the sphincter are considered adrenergic and cholinergic, but p-type fibres are present in the junctional area of the lower rectum and upper sphincter. In the aganglionic, constricted section of the colon from two cases of Hirschsprung's disease, adrenergic and cholinergic axons establish frequent synaptic contacts with smooth muscle cells by exposed varicosities.

Throughout the aganglionic colon section, processes of intrinsic neurons are absent; but close to the cone-shaped transitional section abnormal nerve fascicles are present, bound by hypertrophic perineurium, and contain masses of collagen in distended endoneurial interspaces. Schwann cell units inside these bundles are mono- or oligoaxonal. The ultramorphology of supporting glial cells reveals features of immature, undifferentiated Schwann cells. These findings are interpreted as indicative of abortive regeneration and axonal sprouting from intact intrinsic pericarya in the transitional section of the Hirschsprung colon.

Noradrenaline concentrations in both the normal internal anal sphincter and the aganglionic colon section from one case of Hirschsprung's disease are similar and about twice as high as in all colon sections from healthy individuals, indicating a functionally important, direct adrenergic innervation of smooth musculature in both situations.

The lack of inhibitory extrinsic and intrinsic innervation in the constricted gut section in Hirschsprung's disease is considered to be the cause of its spastic contraction in vivo. Similarities and differences in innervation and pharmacological behavior of the internal anal sphincter in man and monkey and in the aganglionic colon section in Hirschsprung's disease are pointed out.

Zusammenfassung. Im Colon und Sphincter ani internus des Menschen und des Affen wurden Nervenfasertypen licht- und elektronenmikroskopisch analysiert und mit den Nervenelementen in den verschiedenen Abschnitten des Colon von drei Hirschsprungfällen verglichen.

* Supported by grants from the Deutsche Forschungsgemeinschaft.

Lichtmikroskopisch ließen sich zwei Arten von Nervenbündeln im normalen Colon beschreiben: 1. anilinblaupositive Nervenkabel (vom gewöhnlichen peripheren Typ), die endoneurales Kollagen und Lagen von perineuralen Hüllzellen besitzen und vermutlich extramuralen Ursprung haben und autonomer Natur sind; 2. azokarminpositive, große multiaxonale Schwannzelleinheiten im Auerbachschen Plexus, die praktisch kein Kollagen enthalten und von einer diskontinuierlichen Lage von Perineuralzellen umgeben sind (Bündel vom intramuralen Typ). Das ultrastrukturelle Bild dieser Nervenfasern ähnelt zentralnervösem Neuropil. Präterminale und terminale, varicöse Axone des Auerbachschen Plexus werden aufgrund ihrer Vesikelpopulationen und des vermuteten Transmittertyps in adrenerge, cholinerge, p-Fasern und sensorische Fasern eingeteilt.

Der Sphincter ani internus empfängt vorwiegend große Nervenkabel vom gewöhnlichen peripheren Typ. Ebenso wie im aganglionären Abschnitt des Hirschsprungdarmes fallen sie durch ihren Reichtum an markhaltigen Fasern auf. Vesikelhaltige Axone zwischen glatten Muskelzellen des Sphincter internus sind entweder adrenerg oder cholinerg; in der Übergangsregion von distalem Rectum und proximalem Sphincter kommen außerdem p-Fasern vor. Nackte Axone adrenerger und cholinerger Neurone nehmen zu Muskelzellen des aganglionären Abschnitts im Hirschsprungdarm synaptische Beziehungen auf. Fortsätze intramuraler Neurone fehlen im aganglionären Colonabschnitt. In der Übergangszone zum hypertrophierten Abschnitt finden sich abnorme Nervenbündel mit hypertrophiertem Perineurium und Massen von Kollagenfibrillen. Die in diesen Bündeln vorkommenden Schwannzelleinheiten sind mono- oder oligoaxonal. Die Ultrastruktur des Gliagewebes erinnert an unreife, undifferenzierte Schwannzellen. Diese Befunde werden als Anzeichen für eine vergebliche Regeneration intramuraler Perikaryen im Übergangsabschnitt des Hirschsprungdarmes gewertet.

Sowohl im normalen Sphincter ani internus als auch im aganglionären Abschnitt eines Hirschsprungfalles ist der Noradrenalingehalt etwa gleich hoch, im Vergleich zu allen Colonabschnitten eines gesunden Individuum jedoch mehr als zweimal so hoch.

Das Fehlen einer extra- und intramuralen Hemminnervation im aganglionären Teil des Hirschsprungdarmes ist die Ursache seiner spastischen Dauerkontraktion. Gemeinsamkeiten und Unterschiede im Innervationsmuster und im pharmakologischen Verhalten des Sphincter ani internus und des aganglionären Colonabschnittes bei der Hirschsprungschen Krankheit werden hervorgehoben und diskutiert.

Introduction

In an attempt to establish a morphological background for the unique physiological behaviour of the smooth muscle of the internal sphincter of man—i.e. its permanent constriction under resting conditions, providing the terminal colon with continence function—an ultrastructural analysis of neuronal elements in the human, monkey and guinea-pig colon was carried out (Baumgarten, Holstein and Owman, 1970) and the results compared to those obtained on nerve fibre profiles in the cat, monkey and human sphincter internus (Baumgarten, Holstein and Stelzner, 1972). According to the studies mentioned, three main morphological findings may account for the differences in motility behaviour of colonic and sphincteric smooth muscle: 1. an absence of intrinsic nerve cell pericarya in Auerbach's and Meissner's plexus of the most distal portion of the anal canal (cf. Holstein, 1959; Fleischhauer, Holstein and Stelzner, 1966; Stelzner, Fleischhauer and Holstein, 1966), 2. a gradual decrease in the number of processes of intrinsic neuronal pericarya in the proximal and their virtual absence in the terminal part of the internal sphincter muscle (designed as p-type fibres: Baumgarten, Holstein and Owman, 1970), and 3. an increased frequency of nerve fibre profiles innervating the smooth muscle cells of the internal sphincter compared to the colon and rectum, tentatively classified as processes of extrinsic cholinergic and adrenergic neurons (Baumgarten, Holstein and Stelzner, 1972).

The idea emanating from these findings is that the tonic contraction of the sphincter segment is—apart from differences in the distribution of receptor types on smooth muscle cells of sphincteric and non-sphincteric sections of the colon—mainly due to the absence in the sphincter of inhibitory influences of specialized intrinsic neurons of the mammalian intestine (Baumgarten, Holstein and Stelzner, 1972). This implies that intestinal smooth muscle cells deprived of inhibitory neuronal control tend to contract spontaneously, a behaviour which seems to be reinforced by a tonic excitatory influence of the many adrenergic (and cholinergic) terminals under resting conditions.

The clinical observation that constricted narrow sections of the human colon affected by Hirschsprung's disease behave strikingly similar to the continence segment of the human anal canal (except for a "relaxation failure" since the aganglionic colon section in Hirschsprung cases is certainly not connected to the spinal centres mediating the defecation reflex as is the internal sphincter) was considered to be an ideal test object for our hypothesis on the organization of neuronal control of sphincter function in man. The light and electron microscopical analysis of neuronal elements in the human gut of Hirschsprung's disease presented here is based on resection material of three cases.

Material and Methods

Electron microscopy of normal human colon (caecum, appendix vermiformis, colon transversum, sigmoideum and rectum) was performed on biopsy specimens (glutaraldehyde immersion fixation) obtained at operations on patients (aged 15–68), not suffering from any obvious disease of the colon (for details, see Baumgarten, Holstein and Owman, 1970). Small tissue pieces of the internal anal sphincter and lowermost rectum, dissected from amputation preparations, removed from six male patients (aged 41, 49, 55, 56, 58 and 62) because of cancer of the rectum, were processed identically. Corresponding parts of the alimentary canal from rhesus monkeys fixed by intravascular perfusion with 6% buffered glutaraldehyde before postfixation in 1% phosphate buffered OsO_4 solution were available from earlier studies (see Baumgarten, Holstein and Owman, 1970; Baumgarten, Holstein and Stelzner, 1972). Finally, small specimens from all parts of resection material from three Hirschsprung cases (males, aged 12, 15 and 18 years) were fixed by immersion in glutaraldehyde (1 hr) and OsO_4 ($2^1/_2$ hrs) and embedded in epon 812, all according to Luft (1961). Semi thin and ultrathin section were prepared on a Reichert OmU2, using glass and diamond knives respectively, and counterstained with toluidine blue pyronine for light and lead citrate for electron microscopy. Electron microscopy was performed in a Philips EM-300.

Larger specimens from resection material of human sigmoideum, rectum and sphincter internus, and from all parts of the Hirschsprung material mentioned above were either directly frozen in liquid nitrogen for analysis of tissue noradrenaline content[1] (using Häggendal's modification of the trihydroxyindole method, 1963) or immersed in 4% formaldehyde for 3 days, dehydrated in alcohol and embedded in paraffin. 10 μ sections were stained either with azocarmine-anilinblue (Azan) or according to Goldner (see Romeis, 1948). Corresponding material from 5 rhesus monkeys was treated as described above for the human material.

Observations

Nervous Elements and Their Supporting Structures in the Normal Colon of Man

a) Light Microscopy. Conventional paraffin sections taken from different regions of the human colon stained with Azan reveal two distinct types of nerve

1 For laboratory facilities we are deeply indebted to Prof. E. Rosengren, Lund, supported by a grant from Ford Foundation (No. 68-383), New York.

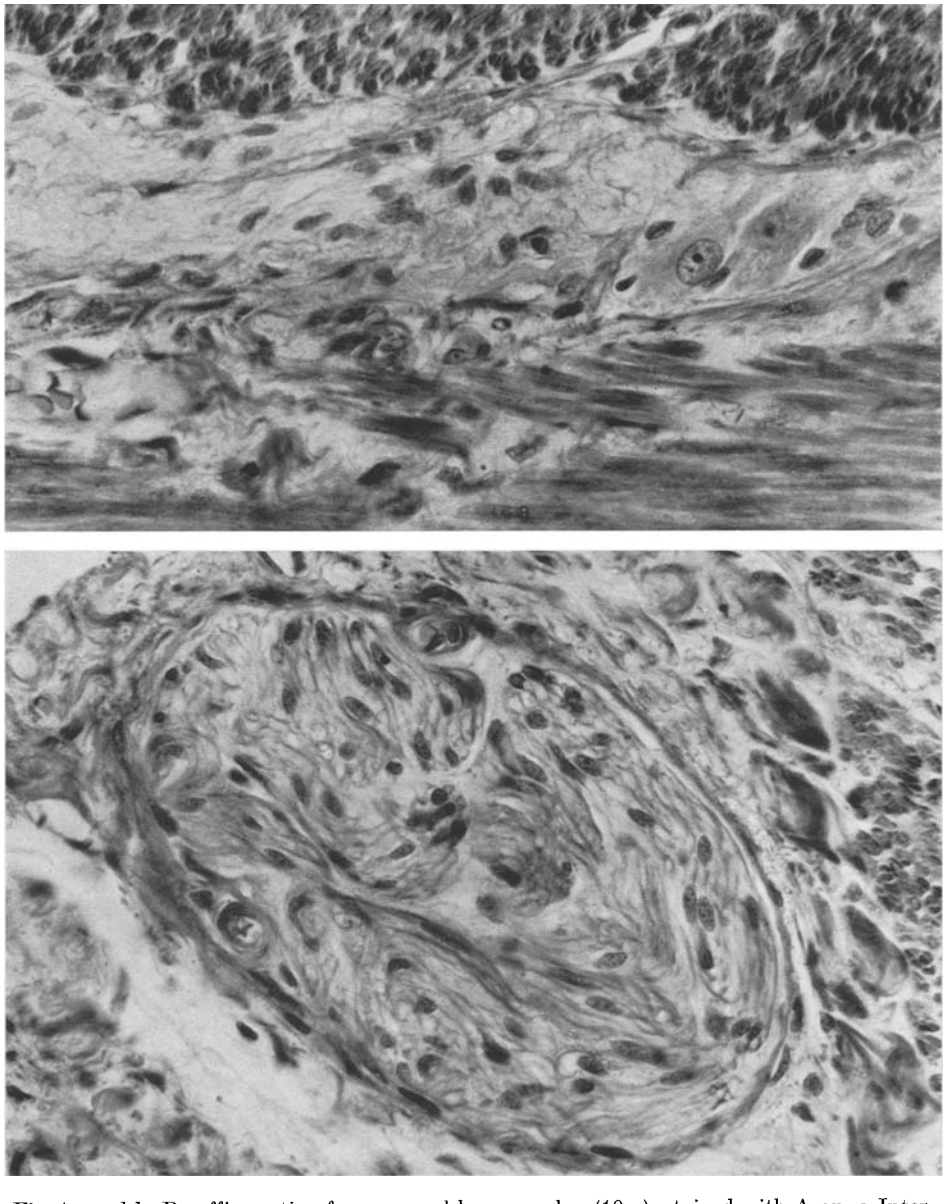

Fig. 1a and b. Paraffin section from normal human colon (10 μ), stained with Azan. a Interconnecting (left) and intraganglionic (right) portion from Auerbach's plexus appearing as delicate meshwork of pink stained cytoplasmic elements (Schwann cell processes), small nuclei of glial cells and large ganglion cells. b Nerve trunk of the conventional peripheral bundle type in between smooth muscle layers showing deeply blue stained endo- and perineural collagen and capsular cells. ×400

trunks (Fig. 1a, b): among bundles of the outer longitudinal smooth muscle layer nerve fascicles of varying size are traced which contain appreciable amounts of deeply blue stained collagen fibres in between losely arranged Schwann cell units carrying groups of unmyelinated axons (Fig. 1b). Nerve fascicles of this type are well separated from surrounding tissue elements by a perineurium and thus resemble typical peripheral nerve bundles. When joining strands of Auerbach's plexus located in septa between longitudinal and circular smooth muscle layers they in part lose their prominent perineurium and connective tissue elements. This is reflected by a change in staining behaviour. Blue stained perineural components and collagen fibres are replaced by interweaving networks of lightly pink stained cytoplasmic elements, most probably Schwann cell processes supporting the dense neuropil of Auerbach's plexus (Fig. 1a). Nodes of Auerbach's plexus comprise ganglion cells of different size and a complex embracing neuropil that are devoid of connective tissue components and blood vessels. Semi thin section from Auerbach's plexus stained with toluidine blue pyronine (Fig. 2a, b) furthermore show that nerves and glial structures are only incompletely separated from neighbouring connective tissue and smooth muscle by a discontinuous layer of small spindle shaped capsular cells with thin elongated processes (Fig. 2a). Ganglion cells are easily distinguished from glial pericarya by a large transparent spherical nucleus with one, sometimes two, prominent nucleoli (Fig. 2a). The lack of intraganglionic connective tissue elements and close mutual aggregation and packing of neural elements in neuropil formations of Auerbach's plexus does not permit with the light microscope to safely distinguish individual Schwann cell units and their distribution and relationship in the myenteric plexus (Fig. 2a, b). Thus, due to the architecture and staining properties of neural and glial components, Auerbach's plexus strikingly resembles central nervous tissue rather than constituents of peripheral nervous system in mammals.

b) Electron Microscopy. Strands of Auerbach's plexus and their extensions into both smooth muscle layers differ in their architecture from occasional nerve trunks seen between bundles of longitudinal smooth muscle of the gut. These latter trunks are composed of many moderately multi-axonal Schwann cell units, well separated from each other by wide collagen filled interspaces; the whole fascicle is delineated from surrounding tissue elements by one to five layers of flattened perineural sheath cell processes, each bordered at its inner and outer surface by a basal lamina. Axons inside these fascicles are generally unmyelinated and mostly small sized, preterminal fibre sections, containing scattered neurotubules, filaments or mitochondria, or ocasionally vesicle filled, expanded sections representing varicosities. This cable type will be called conventional peripheral bundle type (for a typical example, see Fig. 3 from monkey sphincter internus) in contrast to the intrinsic, non-peripheral type of nerve bundle lacking connective tissue fibres between the extremely multi-axonal Schwann cell units, not showing notable interspaces between the tightly packed carrier units and being incompletely separated from surrounding tissue by single, discontinuous fibrocytes (see e.g. Fig. 9).

Nerve bundles conforming to this description are intraganglionic and interconnecting strands of Auerbach's plexus and their ramifications entering the smooth muscle layers. They bear features of central nervous neuropil formations

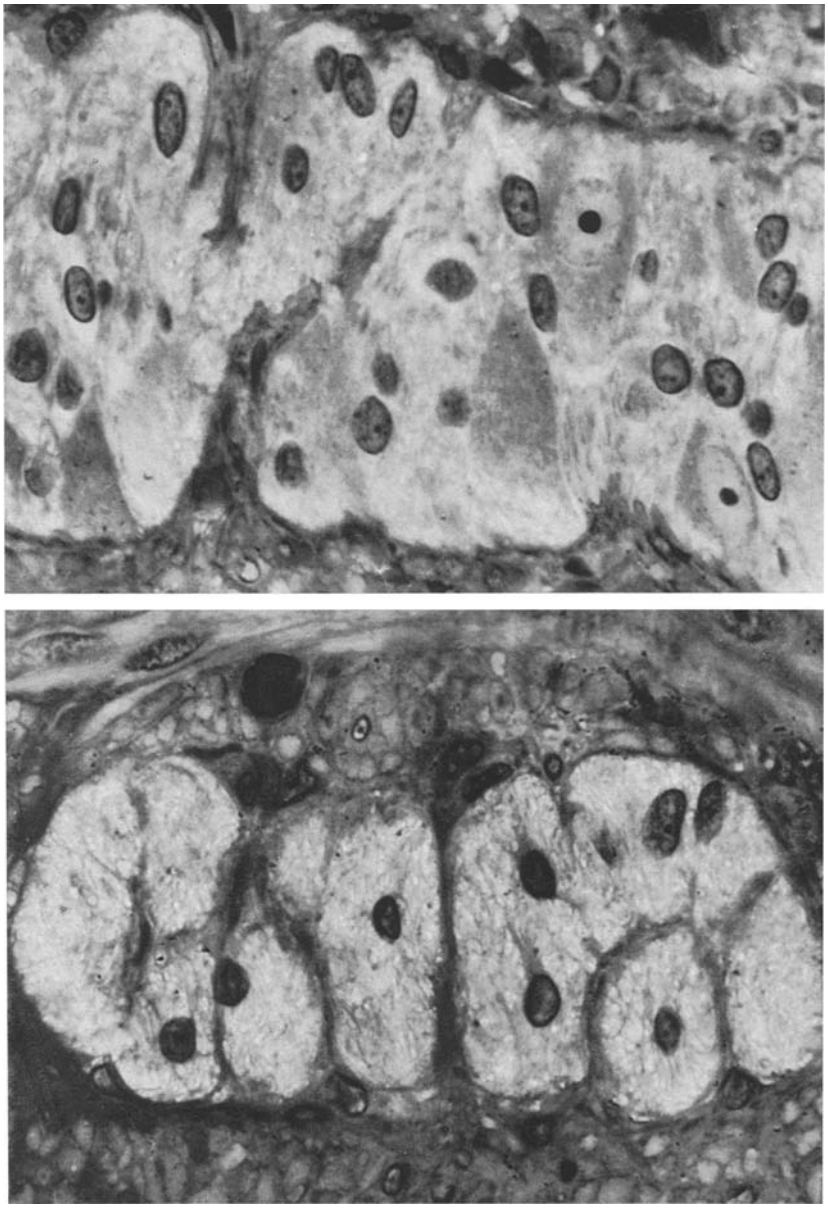

Fig. 2a and b. Semi thin sections from normal human colon (1 μ), stained with toluidine blue pyronine. a Intraganglionic portion of myenteric plexus consisting of ganglion cells, nuclei of glial pericarya and densely packed neuropil formations. Note absence of collagen and capillaries. b Interconnecting portion of myenteric plexus, consisting of multi-axonal Schwann units which are difficult to distinguish from each other. ×650

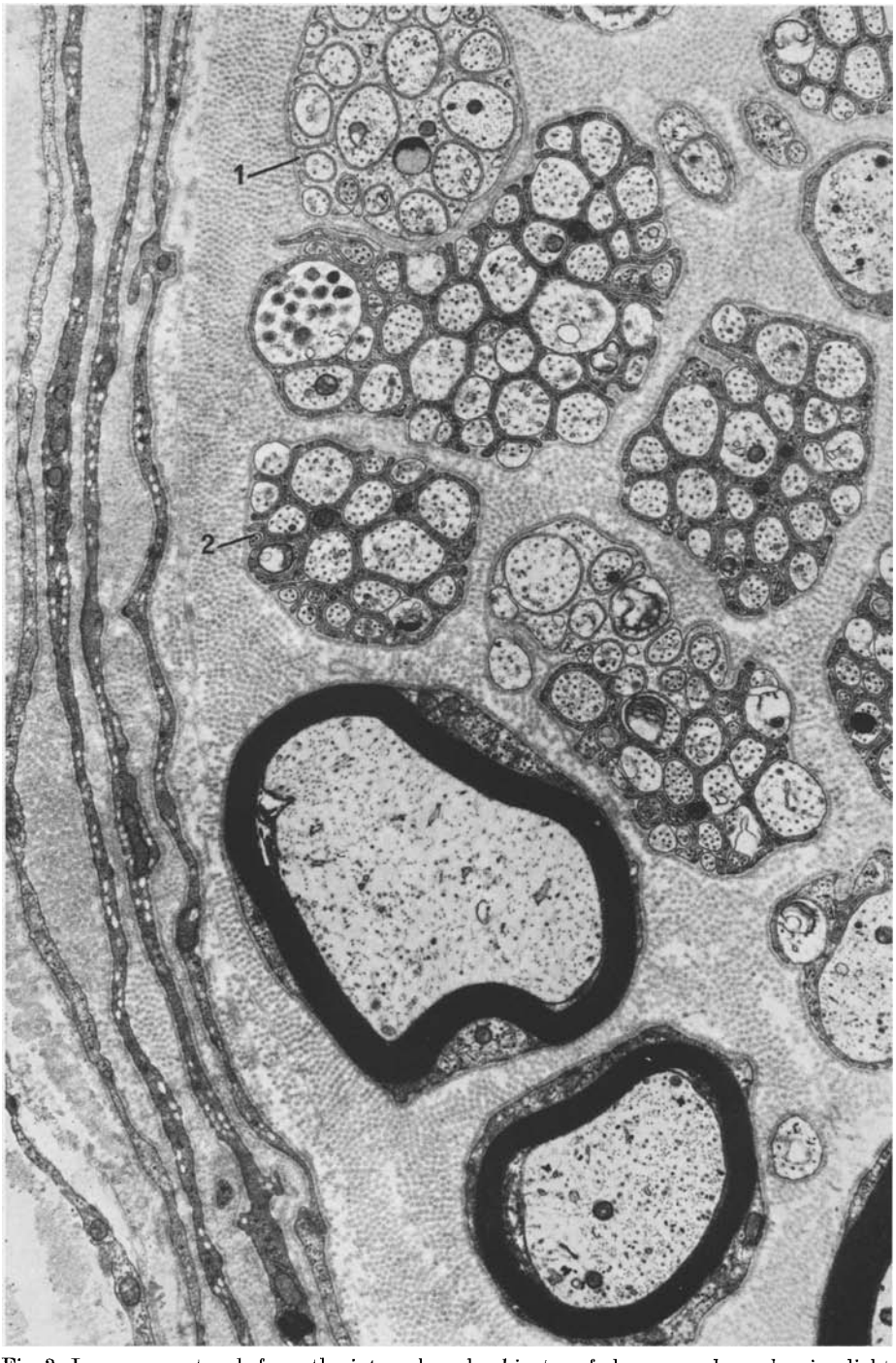

Fig. 3. Large nerve trunk from the internal anal sphincter of rhesus monkey, showing light (*1*) and dark (*2*) Schwann cell processes. Note separation of individual units by collagen filled endoneural spaces. The fascicle is ensheathed by multiple layers of perineurium. ×14000

that are devoid of diffusion-barrier constituting perineurium and lack any definite cable architecture and topographical order of axons carried by individual glial cells.

Intermediate bundle varieties are occasionally seen besides the elaborate extreme fascicle types characterized above indicating that both bundle types join to aggregate units of different origin or to exchange and redistribute axons of different units. Axons inside such extremely multiaxonal Schwann units of Auerbach's neuropil either correspond to non-terminal thin axon sections or they represent varicose swelling showing features of synapses. Groups of vesicle filled axon swellings and strands of undulating, non terminal small sized axons alternate in unpredictable fashion inside the same units of plexus neuropil (cf. Fig. 9) but varicose expansions are more numerous in intraganglionic portions of the myenteric plexus and its ramifications penetrating septula between smooth muscle cells.

According to the composition and feature of vesicles stored in such swellings, the axons of extrinsic and intrinsic neurons in the colon wall may be—for the sake of comparison only and not for completeness—classified into five categories:

1. cholinergic profiles; 2. p-type fibres; 3. adrenergic profiles; 4. large, distended axons comprising clusters of mitochondria, neurotubules and multilamellar and lipofuscine-like bodies, most possibly presynaptic dilatations of sensory axons; 5. some, apparently non-terminal large axons are myelinated, but it has been impossible to establish their relationship to any type of axonal varicosities described above (for details, see Baumgarten, Holstein and Owman, 1970).

Besides axons of the categories characterized above, intraganglionic portions of normal myenteric neuropil contain ganglion cells and Schwann cells. The ultrastructure of nerve and glial cells and their processes and synaptic relationship in the normal myenteric neuropil of the human, monkey and guinea-pig colon and guinea-pig small intestine have been described by Baumgarten, Holstein and Owman (1970) and Gabella (1972).

Nervous Elements in the Sphincter Ani Internus of Man and Monkey

Large nerve trunks of the conventional peripheral bundle type are prominent inside the concentrically arranged wide interstitial spaces separating bundles of circular smooth muscle from the sphincter internus in man and monkey (cf. Fig. 3). Oligo- and moderately multiaxonal units are embraced by continuous basal laminae and loosely distributed in the collagen filled endo- and perineural space bound by prominent perineural sheath cells. Individual units are supported by either transparent or electron dense Schwann cell processes (Fig. 3) suggesting that different types of glia (or different functional states of glial cells) may exist in the peripheral nervous system of primates (see also Baumgarten, Holstein and Rosengren, 1971). Myelinated fibres are regular constituents of axon populations in these large nerve trunks.

The nerve fascicles of the sphincter are distributed in wide muscle separating interspaces that differ with respect of their structural components from the narrow interstitial spaces separating muscle bundles in the colon. Large connective tissue interspaces in the sphincter reveal concentrically arranged layers of flattened fibrocyte processes dividing the spaces into compartments filled with

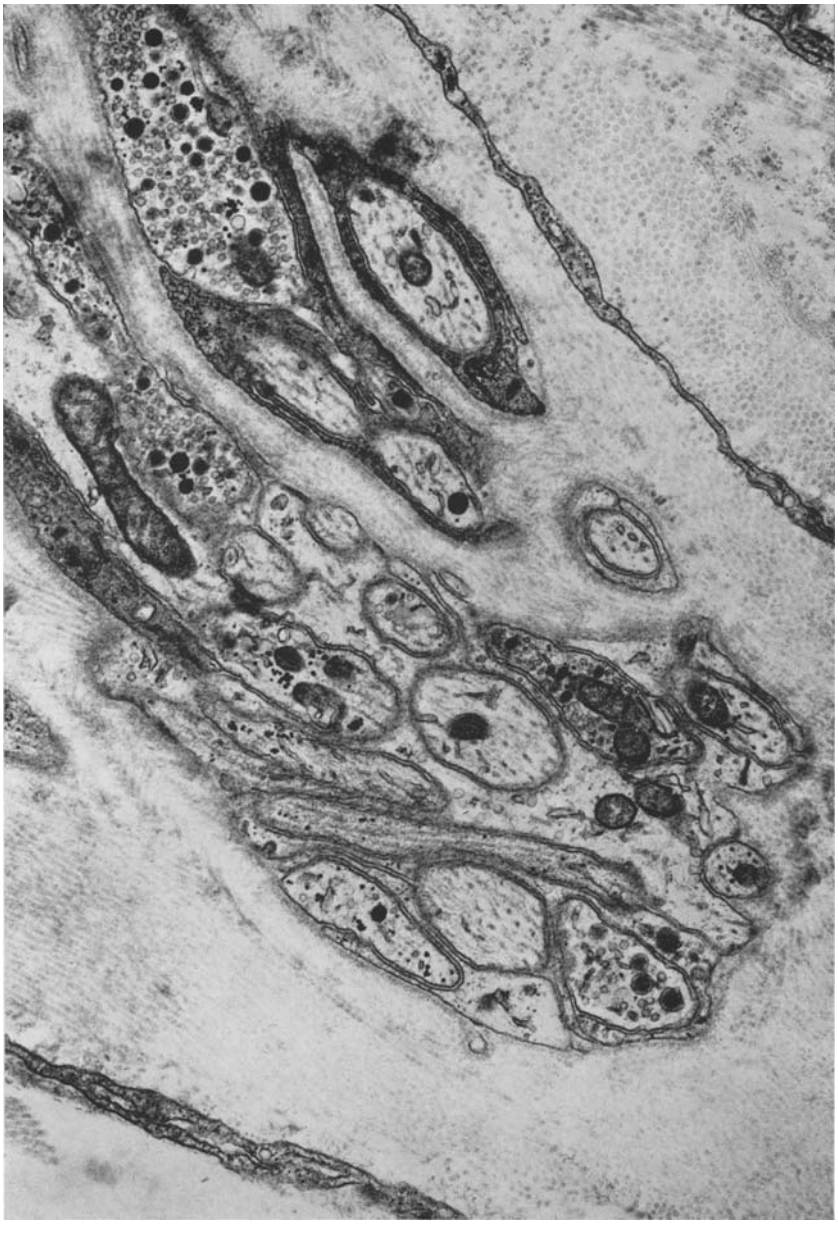

Fig. 4. Small nerve fascicle inside connective tissue compartment of the human internal ana sphincter. Note absence of perineurium and frequency of vesicle filled axons, partly exposed from Schwann cell covering. Dark and light Schwann cell processes as in Fig. 3. ×20000

large bundles of collagen fibrils of alternating orientation (Fig. 4). Among the collagen bundles patches and complexes of elastic tissue are interspersed—in part embraced by fibrocyte processes.

Aggregated or individual Schwann cell units leave the large nerve trunks and approach smooth muscle cells while developing many exposed varicose axonal swellings filled with heterogeneous vesicle populations (Fig. 4). Careful analysis of the fibre types indicates that adrenergic and cholinergic profiles are present in about equal proportions throughout the internal sphincter muscle in both species (Baumgarten, Holstein and Stelzner, 1972) whereas p-type fibres are rare and mainly confined to cranial portions of the sphincter, near to its junction with the circular muscle of the rectum.

Noradrenaline Content of the Human Colon and Sphincter Internus

The concentration of noradrenaline (NA) in different sections of the gut from Hirschsprung case 3 (unaffected sigmoid; hypertrophic portion of the colon sigmoideum and narrow, constricted section of the proximal rectum) was determined spectrofluorimetrically and compared to that of different colon parts (biopsy specimens) from patients not suffering from any obvious diseases of the large intestine. In the normal gut, the lower portion of the rectum comprising the internal sphincter (external sphincter removed) show higher concentrations of noradrenaline than the remaining colon sections, except the appendix vermiformis which was, however, removed from cases of supposed appendicitis. Comparable portions from the colon of the Hirschsprung case reveal concentrations of NA similar to those of apparently unaffected normal colon, but those in the constricted, sphincter-like section of aganglionic rectum are notably higher and resemble the NA values found in the sphincter portion of the rectum from healthy individuals (see Table 1).

Nervous Elements and Their Supporting Structures in the Hirschsprung Gut

a) Light Microscopy. Toluidine blue pyronine stained semi thin sections taken from the constricted portion of the Hirschsprung gut reveal important differences in morphology of nervous structures located in between the two smooth muscle layers when compared to those of colon portions from apparently healthy individuals. Nerve trunks observed in locations normally occupied by Auerbach's plexus are unusually large and well separated from the surrounding tissue by an outstanding, multilayered perineural sheath (Fig. 5b) that is virtually absent and discontinuous on nerve bundles in the unaffected gut. The compactness of dense neuropil supported by relatively few Schwann cells, typical for the myenteric plexus of normal gut (Figs. 2b, 9), is replaced by interweaving though separated strands of individual small fibre bundles showing relatively increased numbers of Schwann cells (Fig. 5b). Individual subfascicles in big nerve trunks of narrow, aganglionic sections are delimitated from neighbouring fascicles by distinct patches of connective tissue, containing masses of fine collagen fibrils. Another important feature which distinguishes these large nerve fascicles from typical, ordinary neuropil of myenteric ganglia is the occurrence of capillaries among the undulating nerve bundles. Furthermore, at higher magnification, it is immediately evident that there exists a striking difference in fibre morphology and composition and microtopography of fibres in nerve trunks of constricted gut portions in Hirschsprung cases: Individual axons, belonging to single Schwann cell units, show widely varying diameters (Fig. 5a, b), from 0.1 up to 10 and even 15 μ, and

Table 1. Noradrenaline content of the human colon from controls and Hirschsprung case 3 (in μg/g fresh tissue weight)

Region analyzed	Sex and age	NA content
Sphincter internus	♂, 62	0.44
	♂, 58	0.51
Colon rectum	♂, 62	0.27
	♂, 58	0.30
	♀, 61	0.25
	♀, 61	0.27
Sigmoideum	♀, 64	0.18
	♀, 64	0.19
Colon transversum	♀, 48	0.25
Appendix vermiform.	♀, 16	0.44
	♂, 15	0.43
Aganglionic portion, rectum	♂, 15	0.46
Cone shaped, transitional portion	♂, 15	0.19
		0.17
		0.14
		0.14
Hypertrophic portion, sigmoid	♂, 15	0.15
Normal portion, proximal sigmoid	♂, 15	0.17

the number of axons supported by individual lemnocytes is considerably lower than in bundles of normal myenteric neuropil. In addition, varying numbers of medium to strongly myelinated axons (Fig. 5b) occur among a majority of non-myelinated fibres whereas myelinated axons are very rare in or absent from bundles of healthy myenteric plexus. Finally, nerve cell pericarya are lacking in nerve trunks from constricted sections of Hirschsprung gut.

Big hypertrophic nerve trunks that resemble those in the constricted gut section are frequently seen in between the smooth muscle layers of the transitional, cone shaped segment, but with the difference of occasional ganglion cells interspersed in seemingly normal myenteric neuropil (Fig. 5a) some of which show a conspicuous vacuolization of their cytoplasm. Nerve trunks of pathologic feature are gradually replaced by strands of myenteric neuropil in the true hypertrophic gut section, that resemble those of unaffected gut portions. Concomitantly, the relationship of neuropil to pericarya is restored to normal in the ganglionic nodes of the myenteric plexus.

b) Electron Microscopy. 1. Large nerve trunks in connective tissue spaces between smooth muscle layers. Nerve trunks in the narrow constricted portion of Hirschsprung's megacolon as identified in the light microscope are far from uniform in morphology when viewed with the electron microscope. One type which is commonly seen in the constricted section of the Hirschsprung gut resembles in

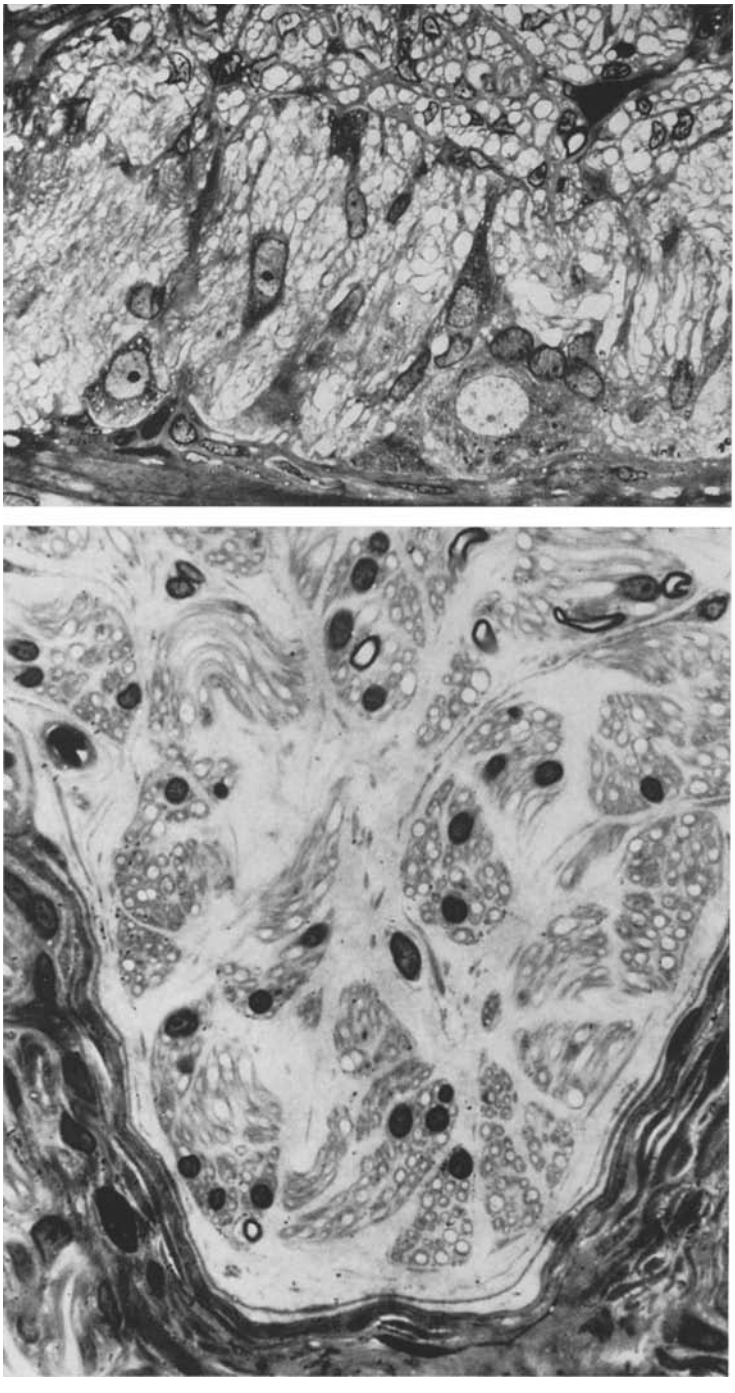

Fig. 5. a Semi thin section from cone-shaped, transitional portion of Hirschsprung gut, stained with toluidine blue pyronine. Intraganglionic part of myenteric plexus with ganglion cells and neuropil formations showing many unusually distended axons. Compare to Fig. 2a.

most details typical conventional peripheral nerve bundles; another type bears features of intrinsic myenteric plexus neuropil and peripheral bundles at the same time. Accordingly, such nerve cables are made up few multiaxonal Schwann units engulfing varicose and non-varicose portions of axons. Similar to extensions of plexus neuropil into colonic circular smooth muscle of healthy individuals, the trunks of narrow gut sections possess a loose discontinuous layer of thin slender processes of perineural sheath cells; in both instances the peripherally located axon swellings are partly exposed from the lemnocyte cytoplasm covered only by a basal lamina. The basal lamina is, however, more elaborate and deeper in staining at nerve bundles of constricted gut sections. Furthermore, while collagen fibrils are rather loose and dispersed in and outside the perineural covering of peripheral nerve bundles in healthy colon (and virtually absent from plexus neuropil) and the ground substance among them appears indistinct, collagen fibrils are densely packed in the endo- and perineural space of nerve trunks in the constricted gut section, and the interstitial ground substance is prominent, sometimes floccular in texture. Nerve trunks from normal gut inside connective tissue septula of circular smooth muscle contain axonal swellings equipped with heterogeneous types of granular and electron lucent vesicles whereas those in narrow gut sections from Hirschsprung cases show a more uniform composition of mainly clear small vesicles or medium sized to large granular among small empty vesicles (most probably representing cholinergic or adrenergic fibre profiles).

Another type of nerve trunk never observed in normal colon comprises many dispersed mono- or oligo-axonal Schwann cell units of characteristic ultramorphology. Mono-axonal units (Fig. 6) exhibit a Schwann cell cytoplasm of poor substructure and electron contrast hardly containing gliofilaments or tubules typical for mature lemnocytes. Processes of the rarefied lemnoplasm either form an incomplete cover on the large axons or embrace it completely giving access to the interstitial space by a small mesaxon. Occasionally small distended sheaths of Schwann cell processes engulf the axon from two opposing sides partly overlapping each other. At portions of its circumference the axon may even be covered by staggered layers of thin undulating lemnoplasmic membranes. The nucleus of the presumptive Schwann cell reminds of the nucleus from an undifferentiated endoneurial fibroblast rather than of a mature lemnocyte since it lacks the prominent condensations of chromatin at the nucleolemmal envelope (Fig. 6). Each unit has a distinct continuous basal lamina and is embedded in a vast intercellular space filled with patches of floccular, moderately osmiophilic ground substance and interweaving strands of small and medium sized collagenous fibrils.

Oligo-axonal units (Fig. 7) carry thin and extremely distended intervaricose smooth axon sections besides few vesicle containing axonal enlargements. Large

$\times 530$. b Semi thin section from constricted, aganglionic portion of Hirschsprung gut. Large nerve trunk in connective tissue space between the longitudinal and circular smooth muscle layer, normally occupied by Auerbach's plexus. The trunk is embraced by multilayered dark stained perineurium and composed of multiaxonal Schwann units. Note small sized and deeply blue stained glial cell nuclei and many unusually distended axons in individual units from each other. Increased frequency of myelinated fibres. Compare to Fig. 2. $\times 650$

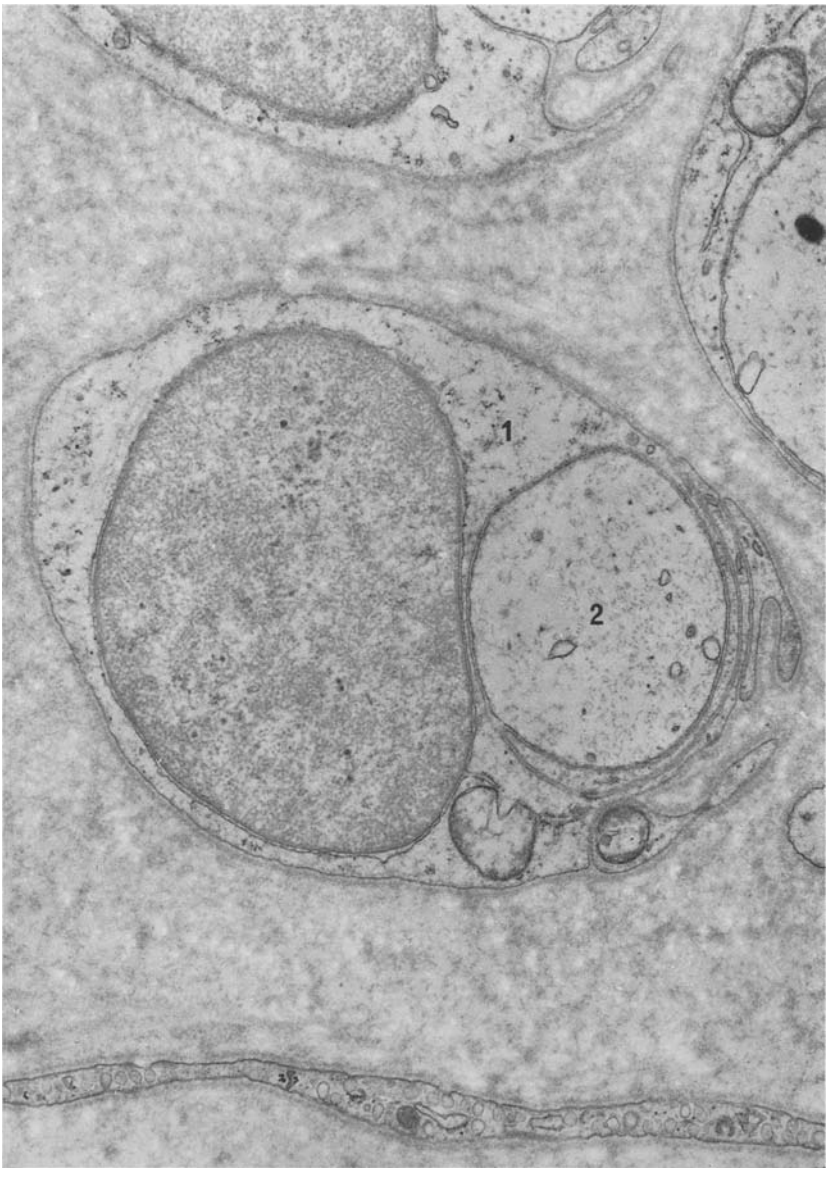

Fig. 6. Monoaxonal Schwann unit from constricted, aganglionic portion of Hirschsprung gut. The undifferentiated Schwann cell (*1*) carries a large sized, apparently non-terminal axon (*2*) containing scattered filaments in transparent axoplasm. Note extensive endoneural space filled with small sized collagen fibrils. ×20000

sized axons in mono- and oligo-axonal units are monotoneous in feature (Figs. 6, 7) and mainly filled with few microfilaments and scattered tubules in a translucent axoplasm, poor in organelles. Longitudinal sections from such axons clearly reveal that the large axons are distended over distances and that they do not conform to

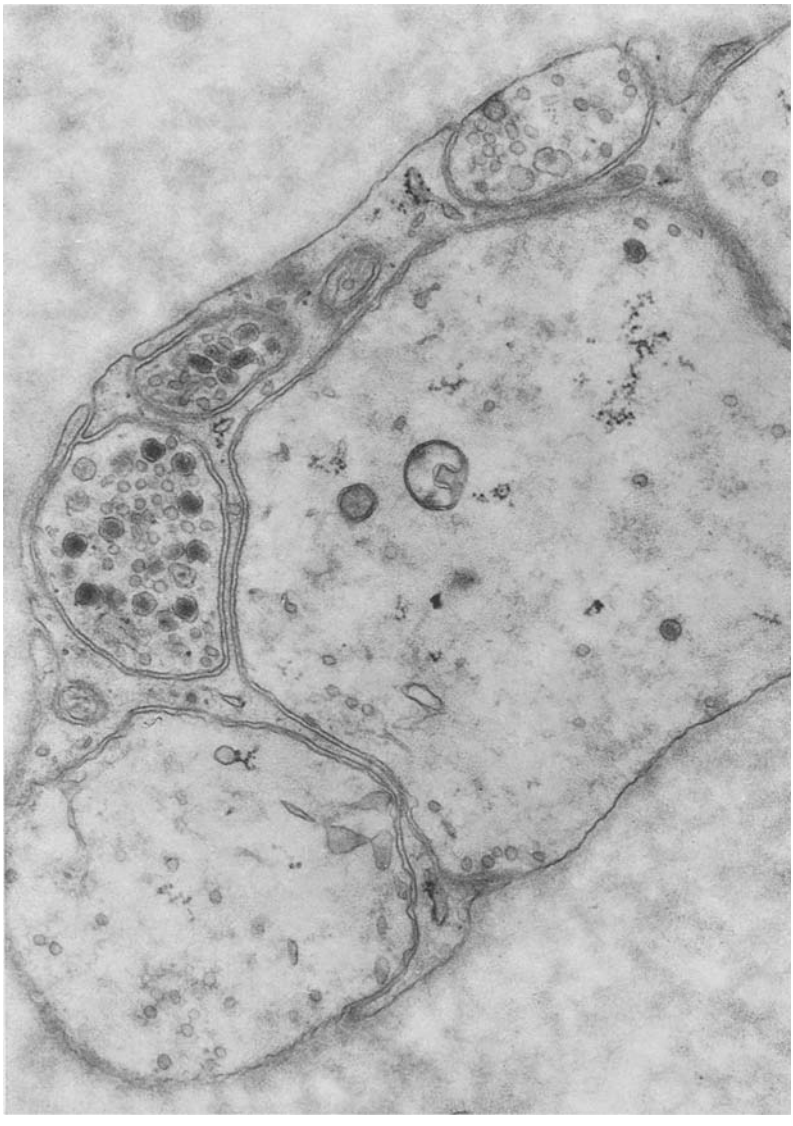

Fig. 7. Similar situation as in Fig. 9, depicting oligo-axonal unit carrying extremely distended, seemingly non-terminal axons and vesicle filled preterminal fibre swellings, either covered by Schwann cell cytoplasm or exposed. ×30000

varicosities. Groups of mono- and/or oligo-axonal units described above are ensheathed by multiple, coherent layers of perineural cells.

Besides, large nerve bundles are seen to contain both conventional multi-axonal and pathologic oligo- or mono-axonal Schwann units. In these bundles endoneurial fibrocyte processes tend to incompletely subdivide the different units of the large nerve trunk into compartments. Thus, a wide spectrum of different nerve bundle types is found in the sphincter-like section of the Hirschsprung gut.

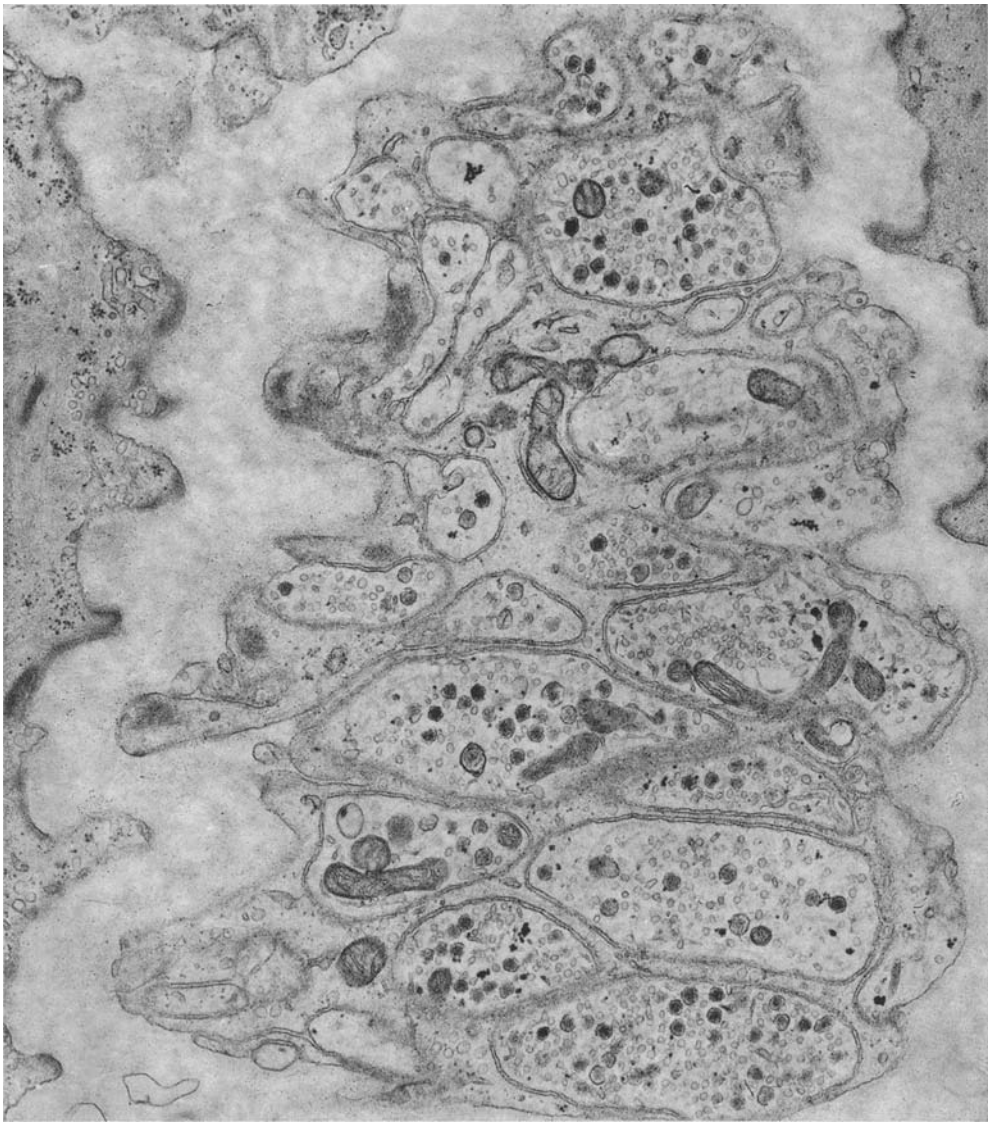

Fig. 8. Individual Schwann cell unit inside circular smooth muscle layer from aganglionic, constricted portion of Hirschsprung gut. Abundant near-terminal swellings of axons filled with small empty and medium sized to large granular vesicles (most probably adrenergic varicosities) some of which are partly uncovered from glial cell processes. ×20000

2. Small nerve fascicles in connective tissue septula between smooth muscle bundles of circular and longitudinal layer. In the constricted colon segment, ramifications from the large nerve trunks frequently enter small interstitial spaces separating bundles of smooth muscle cells from either layer. When invading the septula, they gradually lose their perineural sheath cells. Simultaneously most axons develop prominent vesicle filled expansions (Fig. 8) that are more con-

spicuous and more numerous (per Schwann cell) than in bundles from comparable locations of the normal colon. At random along the Schwann cell unit surface, axons are exposed to face the interstitial space eventually approaching smooth muscle cells at a minimum distance of about 2500 Å. In contrast to the complex equipment of varicose terminal axons with heterogeneous vesicle and organelle populations in circular smooth muscle of normal colon, those from narrow segments of aganglionic Hirschsprung gut in part show a preponderance of either small empty or varying proportions of small electron lucent and medium sized and large granular vesicles (Fig. 8). Occasional fibre swellings are characterized by accumulations of mitochondria, lysosomes and few large electron opaque vesicles (processes of sensory axons?). Those terminal axon dilatations that resemble adrenergic profiles are most frequent, those poor in vesicles but rich in axoplasmic organelles are very rare. All fascicles traced between smooth muscle bundles inside the layers are composed of multi-axonal, conventional Schwann cell units. The number of axons per Schwann cell is higher than in corresponding units of normal colon, as is the number of exposed varicosities.

3. Nerve bundles in the hypertrophic gut section. Except for an increased frequency of occurrence in between the muscle layers and except for their greatly increased cable size (and number of multi-axonal Schwann units), the nerve trunks in the interlayer connective tissue spaces (Fig. 9) and in between the circular muscle bundles of transitional and hypertrophic gut sections resemble those in unaffected portions of the colon from the same individuals.

Discussion

One of the most intriguing findings that seems to have diagnostic value for investigations on nervous elements in peripheral organs is the light microscopical observation of two easily distinguishable types of nerve bundles in the normal colon and sphincter internus: the differentiation of anilinblue-positive from anilinblue-negative, but azocarmine-positive nerve trunks. The deeply blue stained bundles contain endoneurial collagen and perineural sheath cells and thus reveal features of ordinary bundles of peripheral nervous tissue; the pink stained bundles lack significant amounts of collagen or perineurium and thus conform to bundles of myenteric neuropil resembling central nervous tissue. Electron microscopy confirmed this light microscopical distinction of two extreme types of nerve bundle organization. The virtual absence of nerve trunks of the intrinsic bundle type in the aganglionic parts of Hirschsprung gut and of the lowermost sphincter internus and their richness in bundles of the peripheral type suggests that both colon sections receive mainly extrinsic autonomic connections. It should be kept in mind, however, that the distinction of bundle types as outlined above holds for large nerve trunks only and not for individual Schwann units that finally establish synaptic or synaptoid contact to the effector tissue.

The identification of different types of neuronal processes in the human and monkey colon and sphincter internus is based on a classification of characteristic vesicle populations in varicose enlargements of axons. The identification of adrenergic and so called p-type fibres is reliable and safe, since adrenergic profiles in the colon can be easily recognized by a mixed population of medium sized and large vesicles with cores of medium to high electron density in glutaraldehyde/

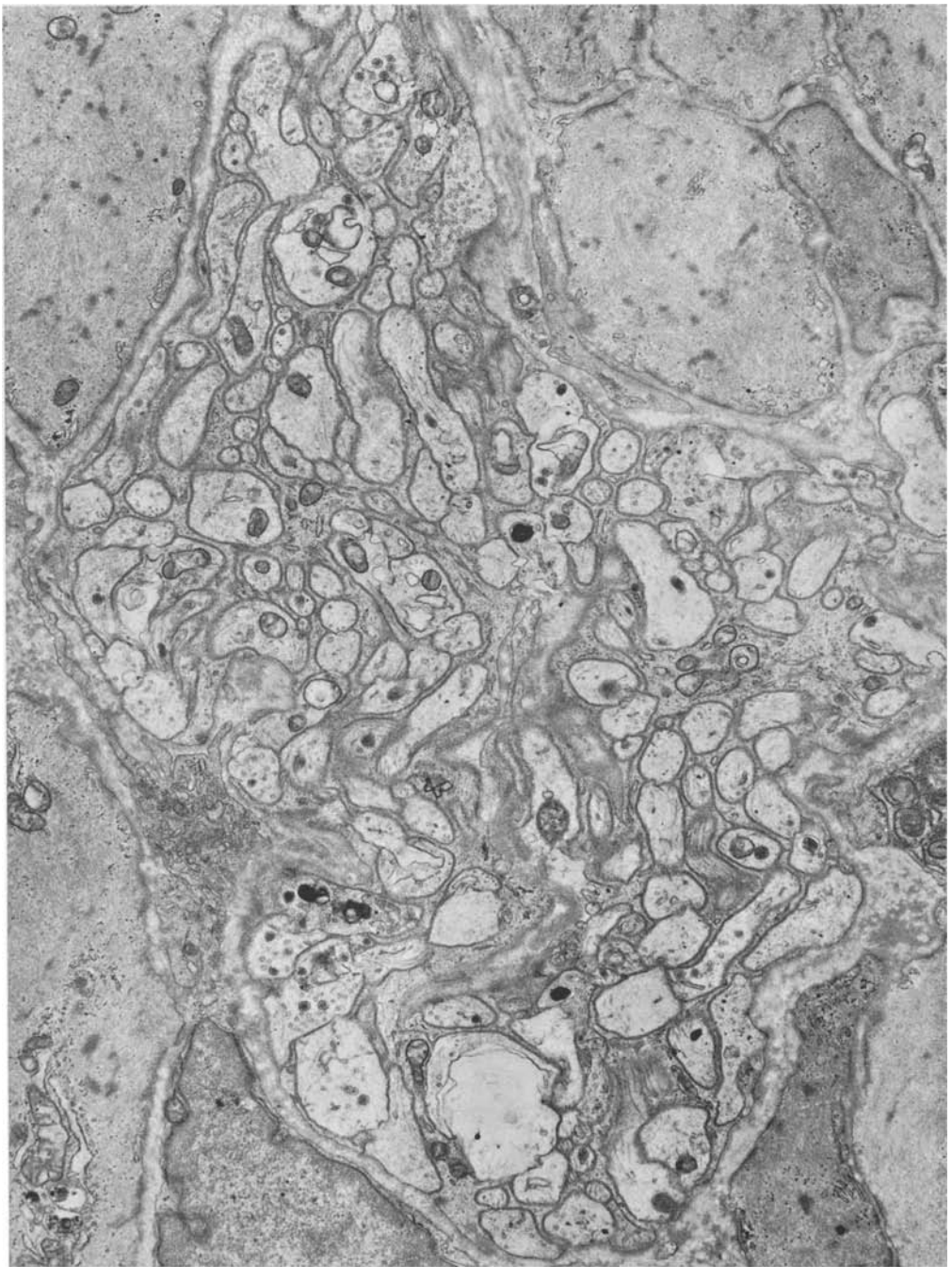

Fig. 9. Typical aspect of interconnecting strand of myenteric neuropil from proximal, apparently healthy sigmoid of Hirschsprung case 3. ×12000

osmium fixed specimens; p-type axonal varicosities on the other hand contain large granular vesicles of varying ultramorphology and electron density among clusters of small faintly electron opaque, synaptic vesicles. The electron opacity and amount of granular material in vesicles of adrenergic fibres can be enhanced by short term pretreatment of animals with the false neurotransmitters 5- or 6-hydroxydopamine (Baumgarten, Holstein and Owman, 1970; Baumgarten, Holstein and Stelzner, 1972) and the axons can be made to completely degenerate by long term treatment with high doses of 6-hydroxydopamine. This treatment schedule leaves all other types of axons unaffected. The classification of axonal dilatations with small empty vesicles as cholinergic is based on previous electron microscopy of peripheral nervous tissue known by pharmacological evidence to contain abundant acetylcholine releasing nerve fibres. No attempt has been made to divide these axons any further into subclasses according to the shape of vesicles (spherical or flattened) since this distinction cannot at present be positively related to differences in transmitter type or origin of the nerves involved (see Gabella, 1972). The description of organelle-rich, vesicle-poor axonal enlargements as part of near-terminal sensory neurons is arbitrary and not related to sufficient experimental evidence.

This differentiation of axons according to more or less distinct vesicle populations enabled us to recognize differences in the nervous supply of the normal and aganglionic colon as well as of the internal anal sphincter. If the classification of axons according to transmitters synthesized and stored by them is valid, a coincidence with established knowledge on the pharmacological behaviour of nerve-smooth muscle interaction in colon and sphincter should be obtained.

In currently available literature there exists but one communication on the pharmacological behaviour of the primate sphincter taking into account that a true lower internal sphincteric region should be distinguished from a transitional upper segment of distal colonic circular smooth muscle. This is the study by Rayner (1970) on the functional internal anal sphincter of the vervet monkey. He investigated a sequence of consecutive strips of circular smooth muscle, two strips comprising the anal sphincter and two strips the transitional lower rectum. Interestingly, the two lower strips responded to noradrenaline, adrenaline and phenylephrine by a contraction indicating a true sphincter area, whereas the third strip showed an ambiguous reaction towards noradrenaline and the upper strip was relaxed (like colon in general by sympathetic stimulation). These effects could be blocked by phentolamine and phenoxybenzamine suggesting the presence of alpha-adrenoreceptors. The two lower strips were found to receive a high number of adrenergic nerve terminals. This finding in the vervet monkey sphincter is paralled by our observations on an exceptionally dense adrenergic innervation of the human internal sphincter (Baumgarten, 1967) and of the corresponding region in the rhesus monkey. Both the human and monkey sphincter internus contain amounts of noradrenaline significantly higher than in remaining sections of the human or monkey colon (about twice as much in the sphincter). Therefore, the sphincteric region of the circular smooth muscle of the anal canal in primates is delimitated from neighbouring circular smooth muscle of the terminal rectum by quantitative differences in the adrenergic innervation. A similar observation has been made in fluorescence microscopical (Bennett, Garrett and Howard,

1968; Gannon, Noblett and Burnstock, 1969) and chemical investigations on noradrenergic nerve density and transmitter content (this study) of the constricted gut section of Hirschsprung cases, pointing to a similar extrinsic innervation pattern. These random observations should be controlled in larger series of Hirschsprung cases, especially since Ehrenpreis, Norberg and Wirsen (1968) did not notice any compensatory hypertrophy of adrenergic nerve fibres in their Hirschsprung cases (in contrast to the other investigators).

It therefore would seem, that rectal (and colonic) circular smooth muscle differs from that of the internal anal sphincter by quantitative differences in the adrenergic nerve supply and by qualitative differences in adrenoreceptor types (alpha-inhibitory in the colon; alpha-excitatory in the internal sphincter). The alpha-inhibitory receptors are most likely confined to intrinsic ganglion cells and their dendritic processes, where most of the inflowing varicosities of extrinsic adrenergic fibres establish their synaptic contacts along the entire human, monkey and mammalian colon. Compared to these functionally important adrenergic synapses in the colon, the few adrenergic terminals distributed in between circular smooth muscle may be physiologically insignificant. But at present it cannot be excluded that these adrenergic fibres in the smooth muscle layers activate alpha-excitatory adrenoreceptors the function of which is only masked by the overwhelming number of inhibitory receptors on ganglion cells. Thus, the differences between rectal and sphincteric smooth muscle are quantitative rather than qualitative what concerns the adrenoreceptors in the smooth musculature.

If excitatory alpha-adrenoreceptors are present already in the normal colon (Gagnon, 1970), they supposedly will also persist in the aganglionic part of Hirschsprung gut and become functionally important, since alpha-inhibitory influences, normally confined to nervous tissue (Kosterlitz, 1968; Daniel, 1968), are completely lacking due to the absence of ganglion cells. Thus a situation similar to that in the normal internal sphincter exists in the constricted aganglionic gut section. Consequently, adrenergic constrictory influences in an abnormally densely innervated, permanently contracted section of Hirschsprung gut would reinforce the tendency of the smooth muscle towards spontaneous contraction. The tendency towards spontaneous contraction is once more increased by the removal of the inhibitory influences of the ATP-releasing intrinsic neurons (our p-neurons and their processes) which thus mediate relaxation of gut smooth muscle under healthy conditions (Bennett, Burnstock and Holman, 1966; Burnstock, Campbell, Satchell and Smythe, 1970).

It has been claimed by Ehrenpreis (1970) that the hypertrophic adrenergic (and cholinergic) nerves resemble blindly ending amputation neuromas being devoid of functionally meaningful synaptic relationships. This cannot be decided by light microscopy. Our observations on significantly increased numbers of exposed, vesicle filled varicosities facing smooth muscle cells at distances compatible with effective neurotransmission (as close as 2500 Å) rather point to an enhanced influence of extrinsic nerves on the constricted, intrinsically denervated gut section. A similar view has been expressed by Howard and Garrett (1970).

For a substantiation of our concept, that the tonic contraction of the sphincter internus as well as that of the smooth muscle in the constricted section of the aganglionic Hirschsprung colon, is dependent on an absence of the intrinsic in-

hibitory neurons, it was essential to demonstrate that processes of p-neurons are either absent or rare in both situations. Electron microscopical analysis of the frequency distribution of p-type varicosities has indeed shown that they are virtually absent in the lowermost parts of the internal anal sphincter in man and monkey as well as in the constricted aganglionic section of the Hirschsprung colon, but sparsely present in the uppermost portions of the anal internal sphincter as well as close to the cone shaped transitional section in the still aganglionic proximal colon portion of our Hirschsprung cases. These observations suggest that intrinsic pericarya of Auerbach's plexus have relatively short projections in the colon of mammals and are thus unable to bridge larger, ganglion-cell devoid territories by means of their processes.

It has been claimed that the increased acetylcholinesterase activity confined to hypertrophic nerve bundles of the aganglionic section of Hirschsprung gut reflects an increased cholinergic inflow to the intrinsically denervated gut (see Kamijo, Hiatt and Koelle, 1953; Niemi, Kouvalainen and Hjelt, 1963; Meier-Ruge and Morger, 1968). This is based on insufficient evidence, since recent investigations on acetylcholinesterase staining of truely adrenergic sympathetic nerves of the mammalian pineal clearly indicate that this enzyme activity can be localized to adrenergic nerves (Eränkö and Eränkö, 1971). On the other hand, our electron microscopical investigation revealed besides many adrenergic also exposed cholinergic axonal varicosities implying that acetylcholine may also influence smooth muscle activity of the aganglionic gut section. So far, nothing is known about the effect of acetylcholine on gut smooth muscle in this special situation and thus any consideration on its role in the maintenance of spastic contraction of ganglion cell deprived gut sections in Hirschsprung's disease remains speculative. The established, classical concept of parasympathetic influence suggests that cholinergic stimulation mediates relaxation, or at least inhibition of contraction, in the internal sphincter and would thus be important in decreasing sphincter tone at the onset of defecation. Contrary to this, recent experiments on nembutal anaesthetized intact cats indicate that acetylcholine induces contraction of the sphincter that can be blocked by atropine, and, that atropine by itself reduces resting sphincter tone (Garrett and Howard, 1971). Supposed a nicotinic action of high amounts of acetylcholine in the intact cat can be excluded as can be direct effects of acetylcholine on the release of neuronal noradrenaline, adrenergic and cholinergic nerves thus may act synergistically upon the sphincter internus. The morphological aspect of some of the nerve bundles of the upper aganglionic gut section in our Hirschsprung cases was abnormal in many respects. These bundles had a hypertrophied, multilayered perineurium, wide collagen filled endoneural spaces and, sometimes, abnormal mono- or oligoaxonal Schwann cell units. Furthermore, many Schwann cell pericarya inside such bundles looked undifferentiated, similar to what is seen in peripheral nervous system during embryonic development (Kubozoe, Daikoku and Takita, 1969). This raises the question as to the nature and significance of these nerve bundles and as to the origin of their abnormal Schwann units and axons. Evidence available from investigations on developing peripheral nerves (Tennyson, 1962, 1963, 1965), on de- and regeneration processes in peripheral nervous system (see Morris *et al.*, 1971 a, b, c, d) suggests that the abnormally looking Schwann units signify regenerative,

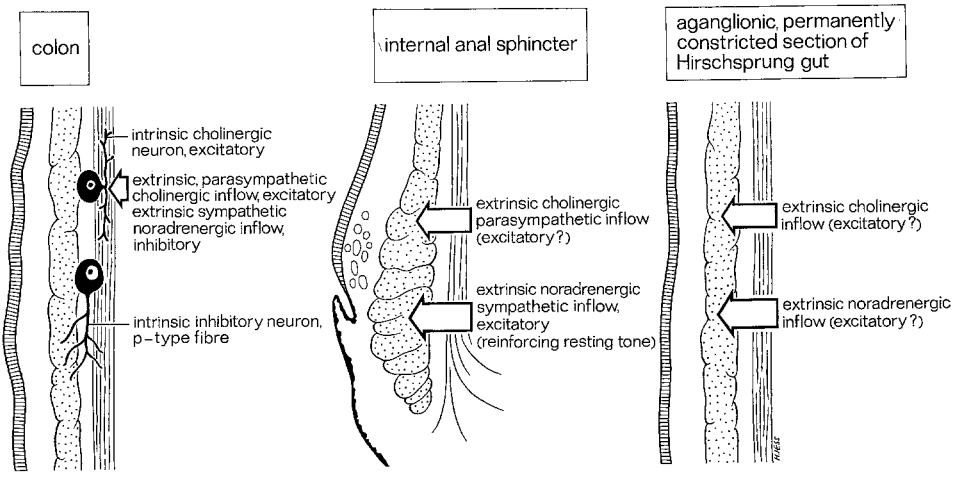

Fig. 10. Simplified, strongly schematic diagram of main nervous control of smooth muscle motility in the mammalian colon, sphincter internus and constricted section of Hirschsprung gut. For explanation, see "Discussion"

possibly frustrating efforts of intrinsic pericarya located in the junctional region of the aganglionic and the dilated, coneshaped section of Hirschsprung gut. The monotoneous feature of translucent axoplasm, poor in organelles, inside the distended preterminal axons does not seem compatible with the idea that they are involved in degeneration processes. Their supporting cells bear resemblance to newly formed young Schwann cells rather than reactive, fully developed lemnocytes as seen in degeneration events. Finally, the greatly disturbed numerical relationship between Schwann cells and axons supported by them (mainly monoaxonal units) would not seem compatible with degeneration but rather with regeneration events. The unusual multilayered perineurium either indicates that the sprouting units penetrate an unfavourable environment and need a special protective covering, or that regeneration processes induce a hypertrophy of perineurium-forming fibrocytes, subdividing groups of sprouting axons and their Schwann cells into separate fascicles.

Based on these considerations, the following hypothetical scheme (Fig. 10) on basic organization of neural influences upon the smooth muscle motility in the normal colon, the normal internal anal sphincter and the constricted, aganglionic section of Hirschsprung colon is presented. The striking similarity in nerve supply of the true internal anal and the abnormal sphincter-like contracted colon section is immediately evident. Differences may exist: a) in receptor types and their quantitative distribution in both tissues, and b) in the involvement of the internal anal sphincter in highly organized recto-anal, spinal reflexes that enhance resting tone but mediate relaxation at rectal distension, transmitted by extrinsic autonomic nerves. These pathways mediating reflex relaxation are not included in the scheme presented.

Our concept of nervous influences in the Hirschsprung colon is supported by recent electrophysiological and pharmacological investigations (Frigo, Torsoli, Lecchini, Falaschi and Crema, 1972).

References

Baumgarten, H. G.: Über die Verteilung von Catecholaminen im Darm des Menschen. Z. Zellforsch. **83**, 133–146 (1967).

Baumgarten, H. G., Holstein, A. F., Owman, Ch.: Auerbach's plexus of mammals an man: electron microscopical identification of three different types of neuronal processes in myenteric ganglia of the large intestine from rhesus monkeys, guinea-pigs and man. Z. Zellforsch. **106**, 376–397 (1970).

Baumgarten, H. G., Holstein, A. F., Rosengren, E.: Arrangement, ultrastructure, and adrenergic innervation of smooth musculature of the ductuli efferentes, ductus epididymidis and ductus deferens of man. Z. Zellforsch. **120**, 37–79 (1971).

Baumgarten, H. G., Holstein, A. F., Stelzner, F.: Unterschiede in der Innervation des Dickdarms und des Sphincter ani internus bei Säugern und beim Menschen. Erg.-Bd. Anat. Anz. **130**, 43–47 (1972).

Bennett, M. R., Burnstock, G., Holman, M. E.: Transmission from intramural inhibitory nerves to the smooth muscle of the guinea-pig taenia coli. J. Physiol. (Lond.) **182**, 541–558 (1966).

Bennett, A., Garrett, J. R., Howard, E. R.: Adrenergic myenteric nerves in Hirschsprung's disease. Brit. med. J. **1968 I**, 187.

Burnstock, G., Campbell, C., Satchell, D., Smythe, A.: Evidence that adenosine triphosphate or a related nucleotide is the transmitter substance released by non-adrenergic inhibitory nerves in the gut. Brit. J. Pharmacol. **40**, 668 (1970).

Daniel, E. E.: Pharmacology of the gastrointestinal tract. In: Handbook of physiology, vol. IV, p. 2267–2324, ed. by Ch. F. Code. Washington D.C.: Amer. Physiol. Soc. 1968.

Ehrenpreis, Th.: Hirschsprung's disease. Chicago/Ill.: Yearbook Medical Publishers Inc. 1970.

Ehrenpreis, Th., Norberg, K. A., Wirsen, C.: Sympathetic innervation of the colon in Hirschsprung's disease: A histochemical study. J. pediat. Surg. **3**, 43 (1968).

Eränkö, O., Eränkö, L.: Loss of histochemically demonstrable catecholamines and acetylcholinesterase from sympathetic nerve fibres of the pineal body of the rat after chemical sympathectomy with 6-hydroxydopamine. Histochem. J. **3**, 357–363 (1971).

Fleischhauer, K., Holstein, A. F., Stelzner, F.: Über das Fehlen von Ganglienzellen im Bereich des Musculus sphincter ani internus des Menschen. Z. Zellforsch. **70**, 515–518 (1966).

Frigo, G. M., Torsoli, A., Lecchini, S., Falaschi, C. F., Crema, A.: Recent advances in the pharmacology of peristalsis. Arch. int. Pharmacodyn., Suppl. **196**, 9–24 (1972).

Gabella, G.: Fine structure of the myenteric plexus in the guinea-pig ileum. J. Anat. (Lond.) **111**, 69–97 (1972).

Gagnon, D. J.: Intestinal smooth muscle: Demonstration of catecholamines-induced contraction mediated through alpha-adrenergic receptors. Europ. J. Pharmacol. **10**, 297–300 (1970).

Gannon, B. J., Noblett, H. R., Burnstock, G.: Adrenergic innervation of bowel in Hirschsprung's disease. Brit. med. J. **1969**, III, 338–340.

Garrett, J. R., Howard, E. R.: Effects of rectal distension on the internal anal sphincter of cats. J. Physiol. (Lond.) **222**, 86–86 P (1971).

Häggendal, J.: An improved method for fluorimetric determination of small amounts of adrenaline and noradrenaline in plasma and tissues. Acta physiol. scand. **59**, 242–254 (1963).

Holstein, A. F.: Morbus Hirschsprung in Vergangenheit und Gegenwart mit einer histologisch-anatomischen Untersuchung am Rectum normaler Menschen. Diss. Hamburg 1959.

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

Kamijo, K., Hiatt, R. B., Koelle, G. B.: Congenital megacolon, a comparison of the spastic and hypertrophied segments with respect to cholinesterase activities, and sensitivities to acetylcholine, D.F.P. and the barium ion. Gastroenterology **24**, 173–185 (1953).

Kosterlitz, H. W.: Intrinsic and extrinsic nervous control of motility of the stomach and the intestines. In: Handbook of physiology, vol. IV, p. 2147–2171, ed. by Ch. F. Code. Washington D.C.: Amer. Physiol. Soc. 1968.

Kubozoe, T., Daikoku, S., Takita, S.: Electronmicroscopic observations on Auerbach's plexus in a 12 mm human embryo. J. Neuro-visceral Rel. **31**, 291–307 (1969).

Luft, J. H.: Improvements in epoxy resin embedding methods. J. biophys. biochem. Cytol. **9**, 409–414 (1961).

Meier-Ruge, W., Morger, R.: Neue Gesichtspunkte zur Pathogenese und Klinik des Morbus Hirschsprung. Schweiz. med. Wschr. **98**, 209 (1968).

Morris, J. H., Hudson, A. F., Weddell, G.: A study of degeneration and regeneration in the divided rat sciatic nerve based on electron microscopy. I. The traumatic degeneration of myelin in the proximal stump of the divided nerve. Z. Zellforsch. **124**, 76–102 (1972). II. The development of the "regenerating unit". Z. Zellforsch. **124**, 103–130 (1972). III. Changes in the axons of the proximal stump. Z. Zellforsch. **124**, 131–164 (1972). IV. Changes in fascicular microtopography, perineurium and endoneurial fibroblasts. Z. Zellforsch. **124**, 165–203 (1972).

Niemi, M., Kouvalainen, K., Hjelt, L.: Cholinesterase and monoamine oxidase in congenital megacolon. J. Path. Bact. **82**, 363 (1961).

Rayner, V.: Observations on the functional internal anal sphincter of the vervet monkey. J. Physiol. (Lond.) **213**, 27–28P (1970).

Romeis, B.: Mikroskopische Technik. München: R. Oldenbourg 1948.

Stelzner, F., Fleischhauer, K., Holstein, A. F.: Die Bedeutung des Sphincter internus für die Analkontinenz. Langenbecks Arch. klin. Chir. **314**, 132–136 (1966).

Tennyson, V. M.: Electron microscopic observations of the dorsal root ganglion in the rabbit embryo. Proc. 2nd Ann. Meeting Amer. Soc. Cell Biol., 187 (1962).

Tennyson, V. M.: Fine structure of the embryonic dorsal root ganglion of the rabbit. Anat. Rec. **145**, 292 (1963).

Tennyson, V. M.: Electron microscopic study of the developing neuroblast of the dorsal root ganglion of the rabbit embryo. J. comp. Neurol. **124**, 267–318 (1965).

Priv.-Doz. Dr. H. G. Baumgarten
Neuroanatomisches Institut
der Universität
Prof. Dr. A. F. Holstein
Anatomisches Institut
der Universität
D-2000 Hamburg 20
Martinistraße 52
Federal Republic of Germany

Prof. Dr. F. Stelzner
Chirurgische Klinik
der Universität
D-6000 Frankfurt a. Main
Ludwig-Rehn-Straße 14
Federal Republic of Germany