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An Electron Microscope Study of the Auerbach's Plexus and Determination of Substance P of the Colon in Hirschsprung's Disease

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Summary. The AA present a study of 3 cases of Hirschsprung's disease performed under electron microscopy and whose data seen to suggest that substance P, which is always found in larger amounts in the ganglionic portion of the colon (than in the aganglionic one), may be related with the granular vesicles of Auerbach's plexus.

Hirschsprung's disease (congenital megacolon, hypoganglionosis or aganglionosis of the colon), although clinically and morphologically well known has not yet been thoroughly understood as its etiology, pathogenesis and physiopathology. Ehrenpreis and Pernow (1953) demonstrated that, in the disease, there can be observed small concetrations of substance P in the aganglionic segment of the colon, whereas large ones may be seen in the ganglionic portion. Gadun (1959) reported that such substance must be related to Meissner's plexus. Tafuri (1964) suggested its probable relation to the vesicular component of the intestinal plexuses. In human trypanosomiasis cruzi, where intense hypoganglionosis is present, especially in Auerbach's plexus, quantitative and volumetric alterations of the granular vesicles can be observed in the megaesophagus and megacolon, which could partially account for the peristaltic disturbances occuring in that disease. Hial and Cols (1973) conducted a quantitative study of substance P in the megaesophagus and megacolon and, based on Tafuri and Cols' work (1971) suggested that such substance might be related with the neurons still at work in the organs.

This paper presents, from 3 surgical cases of Hirschsprung's disease, a quantitative determination of substance P in the stratum of the muscle layer of both narrowed and dilated zones of the colon, as well as a study of the alterations developed in the ultrastructures of Auerbach's plexus, in the dilated segment of the colon and in the inner nerve fibers of the narrowed zones.

Material and Methods

The material for this study consisted of snips from 3 surgical cases of congenital megacolon, collected in the surgery room. The procedure was as follows:

a) for Electron-Microscopy, muscle fragments from both dilated (proximal) and narrowed (distal) portions of the colon were placed in a 3% solution of glutaraldehyde, with phosphate buffer (pH 7.4), for 5 hours; afterwards, they were washed in saccharose at 0.3 M, re-fixed in 1.5% osmium for 1.5 hours and, then, dehydrated and embedded in epon. Auerbach's ganglia in the proximal portion, as well as bundles of nerve fibers in the narrowed segment, could be detected, through tick cuts, with a dissecting microscope. All sections were performed on a Porter-bloom microtome, fished with copper nets, stained in uranila and lead acetate and, finally, microphotographed on Zeiss EM9A.

b) Substance-P Determination. substance P was extracted and determined according to Pernow (1951, 1953) modified as follows: 1) about 2.1 g of defatted material was chopped in smaller fragments and boiled for 15 min. in twice its volume of distilled water acidified to pH 4.0 with HCl; 2) the extracted material was filtered and the filtrate was tested directly, after neutralization with NaOH, on isolated guinea-pig's ileum, suspended in an intestinal bath with 4 ml Tyrode's solution containing glucose and areated with air; 3) Atropine sulphate (1:2.5 millions) and Fenergan, Rhodia (1:1 million) were added to the Tyrode's solution; 4) isolated guinea-pig ileum and rat duodenum were used to test the pharmacological effect of substance P. A bradykinin standard (3 Units/ml) was used for comparison purposes.

Results

A. Auerbach's Plexus—Proximal Portion

Most times, in the 3 cases studied, isolated Auerbach's ganglia showed, under optical microscopy, to be normal and displayed voluminous neurons and nerve fibers with enlarged caliber. Nevertheless, under electron microscopy, some of these very same neurons were seen to be (sometimes extensively) altered and lying by the side of normal or, even, hypertrophic ones (Figs. 1, 2 and 4).

1. Neuron Body

Its perikarion presents rather diverse morphology. The lesions most frequently observed were: a) partial, focal or, sometimes, diffuse tigrolysis; b) tumefaction, partial or total cristolysis, and lysis of the mitochondria matrix; c) enlargement of the cisternae of the endoplasmic reticulum; d) presence of numerous liposomes and lysosomes and eventual loss of the whole architecture, a totally vacuolated perikarion being then present.

The neuron nuclei most deeply effected were electrodense, wrinkled and pyknotic. The hypertrophic neurons were more electrodense and displayed numerous ribosomes, especially free polysomes randomly distributed or agglomerated close to the membranes of the endoplasmic reticulum. In the zones where ribosomes were more scarce, well-developed, neurofilamentous and neurotubular, structure could be observed (Fig. 3). Similarly hypertrophic was the Golgi complex, which was surrounded by several dense vesicles. Granular vesicles were scattered all over the perikarion (Fig. 3).

2. Nerve Fibers—Proximal Portion

All nerve fibers were unmyelinated, sectioned in various directions, and displayed different calibers, some of them being hypertrophic, even strikingly so, as compared with the others. They were always filled with vesicular components (dense granular vesicles, as well as intermediary and clear ones (Fig. 4). Such

Fig. 1. Dilated colon Auerbach's ganglion. Slightly altered neurons (*N*); Schwann cells (*SC*) and numerous unmyelinated nerve fibers (*UF*), some of them (arrows) full of dense granular vesicles. Capsule with fibroblasts (*Fu*), collagenic (*C*) and capillar (*Cp*). ×7200

vesicles called the investigator's attention by their wide variety in caliber and shape. Actually, Fig. 6 shows that they varied from 250 Å to 3000 Å. All vesicles presented a 75 Å—tick membrane with finely-granulated osmiophilic contents or were instead, dense or clear. Besides their variability in shape and caliber, they were also increased in number. There could be counted 48 clear, 1459 dense and 213 intermediary vesicles in an axonal area of 100 μ^2.

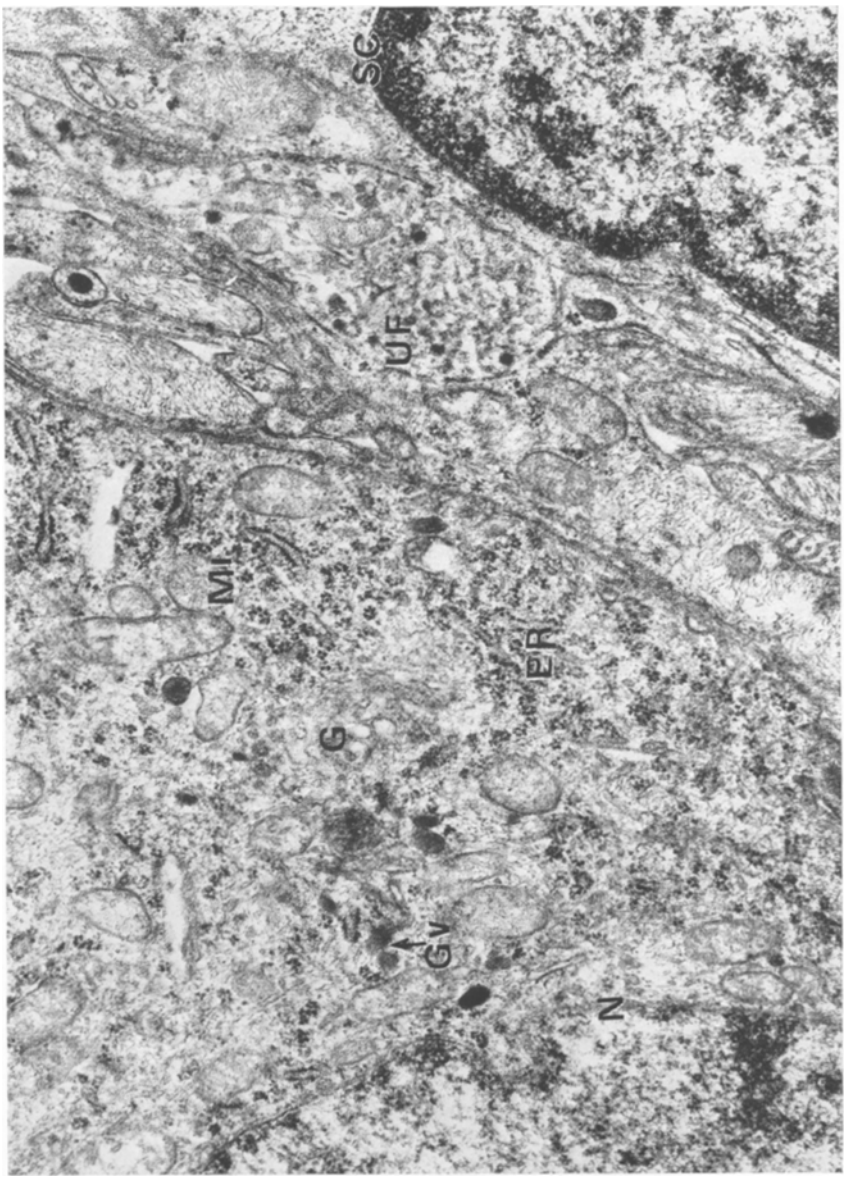

Fig. 2. Dilated colon—Auerbach's ganglion. Normal neuron (*N*) with its nucleus, mitochondria (*Mi*), Golgi complex (*G*) granular vesicles (*Gv*). Well developed endoplasmic reticulum (*ER*), Schwann cell (*SC*) and unmyelinated nerve fibers (*UF*). ×28000

Not all nerve fibers were normal. A large number of them presented vacuolated mitochondria and lysis of the neurofilaments and neurotubules.

3. Schwann Cells

Not great alterations could be observed. Their cytoplasm looked more developed and presented well-individualized gliofilaments. It was also observed the presence of lysosomes and dense granular vesicles, morphologically identical to those of the nervous fibers.

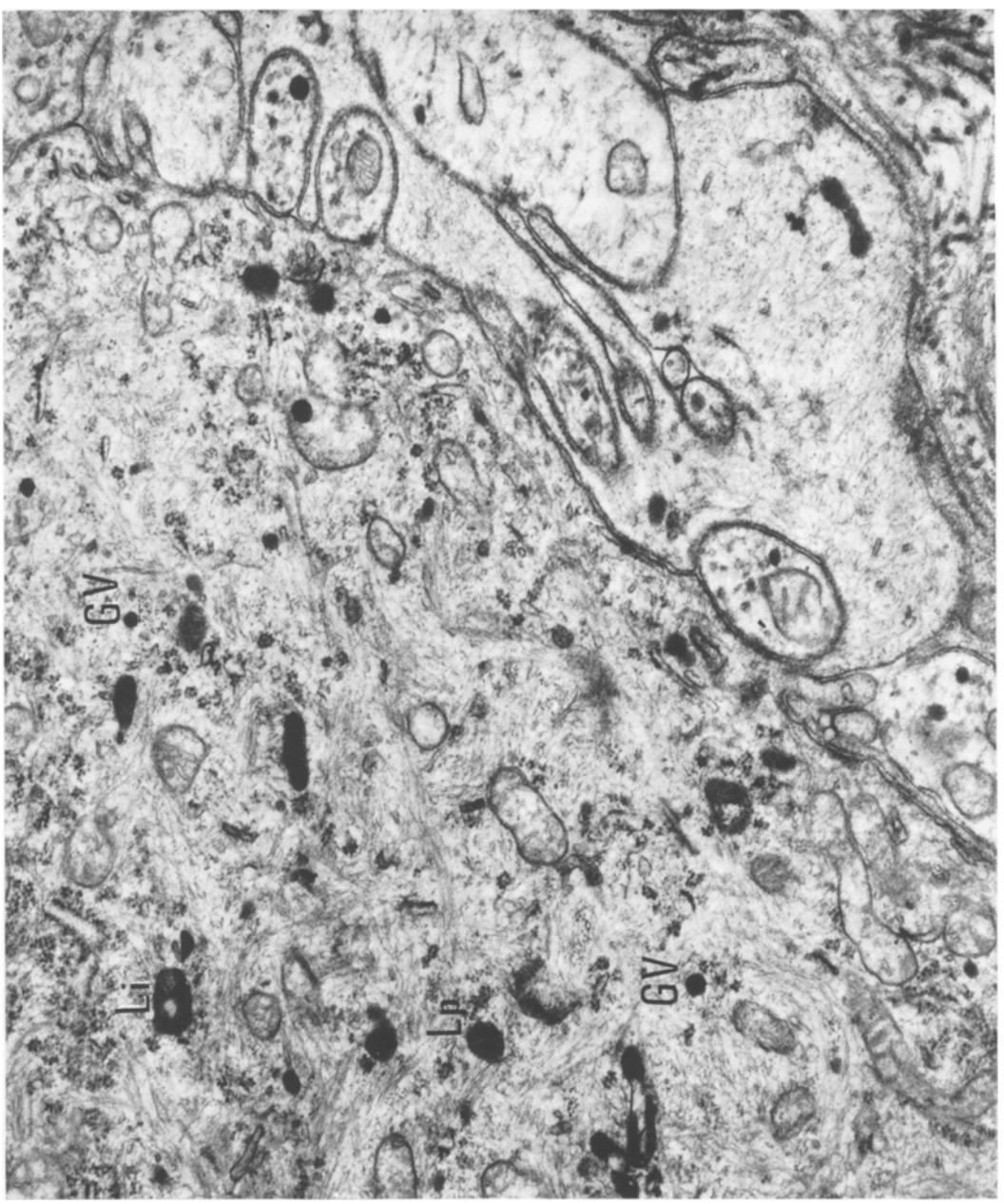

Fig. 3. Dilated colon—Auerbach's ganglion. Perikarion of one neuron with few ribosomes and evident neurofilamentous architecture. Dense granular vesicles (*GV*). Liposomes (*Lp*) and lysosomes (*Li*). ×28000

Fig. 4. Dilated colon—Auerbach's ganglion. Unmyelinated nerve fibers (*UF*) with widely variable caliber, the largest one being full of vesicular component, also very variable in shape and caliber. ×28000

Extrinsic Nerve Fibers of the Distal Portion. Such fibers were easily identifiable in a thick section. In general, they were seen in bundles, the fibers disorderly following various directions and showing to be increased in tickness and enveloped in a sheath of connective tissue. Neurons were scarcely detected and just one of them, presenting narrow perikarion and normal morphology, could be microphoto-

Fig. 5. Distal colon. Normal (*UF*) and altered (arrow) unmyelinated nerve fibers with dense granular vesicles enveloped in collagenic tissue. ×28000

graphed. All nerve fibers were unmyelinated, most of them, which showed to be unipaired, were seen to be lying by the side of others displaying normal morphology, number and diameter (Fig. 5).

Fig. 6 presents the data showing the mean diameters of the dense granular vesicles. Most diameters ranged from 600 to 1000 Å. In 100 μ^2 of axonal area, 250 clear, 273 dense and 131 intermediary vesicles could be observed.

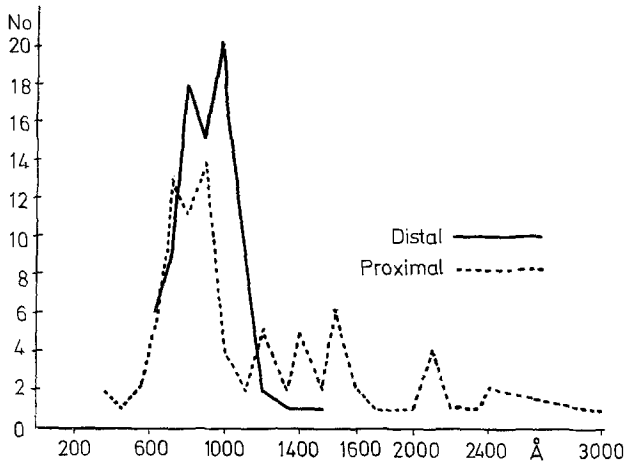

Fig. 6. Histogram showing the mean diameters of the dense granular vesicles of the distal and proximal colons

B. Determination of Substance P

Fig. 7 shows the figures representing the amounts of substance P found in the proximal and the distal portions of the colon in the 3 investigated cases of Hirschsprung's disease. Substance P could be determined by comparing its action over the ileum of the guinea pig with that of bradykinin. As can be noticed, larger amounts of such substance were always found in the proximal portion of the colon. The action of the extract over the rat's duodenum proved to be quite contrary to that of bradykinin, since whereas the former induced contractions, the latter produced relaxation. In further test with trypsine and bradykinin potentiating factor (BPF), the extract was seen to behave as substance P.

Comments

It was observed, in the 3 cases of Hirschsprung's disease, significant increase of substance P in the dilated part of the colon as compared with that in the narrowed segment. This finding confirms the reports of Ehrenpreis and Pernow (1953).

Despite the extensive literature on Hirschsprung's disease already available, very little is known about the alterations occurring in the ultrastructures of the intramural nervous plexuses. Farrell (1970), conducting a histochemical and ultrastructural study of the distal portion of the aganglionic megacolon, could not detect nerve fibers making up plexuses. The formations are positive acetylcholinesterases, which end blindly, with no basket-like architecture. Under electron microscopy the nerve fibers appear to be all of glycogen and myelin figures. Farrell believes these fibers to be cholinergic and, possibily, preganglionic. Howards *et al.* (1970), wishing to know, in detail, the inervation of the aganglionic portion of the colon in Hirschsprung's disease, conducted an electron-microscopical study of 10 such cases. The largest number of nerve fibers was found in

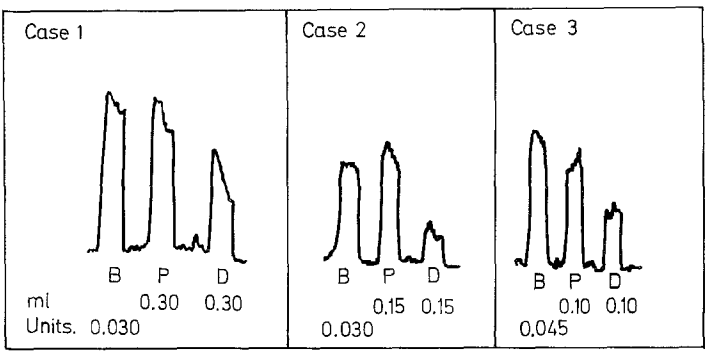

Fig. 7. Figures representing the amounts of substance P found in the proximal (*P*) and the distal (*D*) portions of the colon in the 3 investigated cases of Hirschsprung's disease. *B* bradykinin

the circular layer of the distal part of the rectum. They were seen to be connected with the Schwann cells in the same way as in the proximal portion. Aspects similar to those of the neuro-effector junctions could be observed (space of 500 Å, nerve fibers with no Schwann sheath, vesicles and mitochondria). Concerning the dilated portion, the AA reported variations in the morphology of Auerbach's plexus and, in 3 cases, myelinated fibers from controverted origin. Baumgarten *et al.* (1973) studied the nervous elements in the colon of Hirschsprung's disease and compared their findings with those of the normal human and monkey colon. They classified the axon of Auerbach's plexus according to their vesicle populations in 4 types, that is, adrenergic, cholinergic, p-type and sensory fibers. Based in our material it was not possible to distinguish these 4 types.

Our material allowed a more detailed study of the changes in the ultra-structures of Auerbach's plexus and of the extrinsic nerve fibers of the narrowed segment. In our experience, the most striking features were the quantitative and qualitative changes in the vesicular components, especially those of the granular vesicles. In fact, the hypertrophic axons of Auerbach's plexus were completely full of those vesicles displaying widely variable calibers (Figs. 1, 5) and density. On the other hand, normal neurons could be detected (Fig. 2) by the side of others showing great alterations. The abnormal neurons presented a larger number of dense granular vesicles diffusely scattered over their perikarion. Comparing the number of dense vesicles in each 100 μ^2 of axonal area, in both dilated and narrowed portions of the colon, as well as their histograms (Fig. 6), we can state that, in the first case investigated, the vesicles demonstrated to have increased in volume and in number. This fact seems to suggest that, in the dilated segment, the integral neurons of Auerbach's plexus developed a hypersecretion mechanism. Nozelof *et al.* (1970) postulated that in Hirschsprung's disease, the neurons of Auerbach's plexus undergo hypertrophy and hyperplasia. Such phenomena is probably due to the obstacle to be overcome in the colon or in the distal rectum (spasm). It could also be supposed that hypersecretory neurons display a large number of

vesicles because the effector cells are not to use up the mediator substances. In our investigation, no neuro-muscular junctions could be detected, as reported by Howards (1970).

Larger granular vesicles predominated in both proximal and distal segments of the colon. They presented a wide variety of morphological aspects, some of them resembling those described by Pellegrino de Iraldi (1969) in the regenerating sciatic nerve. The A admit that such vesicles can be originated from neurotubules in the axon. There is some indication that large granular vesicles contain Na, 5HT, ATP and substance P. As, in the 3 cases described in the paper, a larger increase of substance P was observed in the ganglionic portion of the colon, where the number of dense vesicles was likewise, much larger than that of the aganglionic segment, we assume that such vesicles may be related with substance P.

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