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Histochemical and ultrastructural features of the developing enteric nervous system of the human foetal small intestine

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Summary. The developing enteric nervous system of the human foetus has been analyzed at the 10th and 18th week of gestation with a special reference to the development of nerve-muscle contacts. The myenteric plexus formation was analyzed by means of electron microscopy and on whole-mounts after NADH diaphorase histochemistry. The development of noradrenergic innervation as an extrinsic inhibitory supply was followed by means of a glyoxylic acid-induced fluorescence method. Differentiated neurons and neuroblasts both occurred in myenteric ganglia of the 10- and 18-week-old foetus although the ganglionic neuropil was almost unidentifiable ultrastructurally at the 10th week of gestation but was mature looking at the 18th week. The nerve plexuses connecting the ganglia frequently formed distant and close myoneural contacts. NADH-diaphorase histochemistry revealed that in the 18-week-old human foetus most of the neural perikarya was within the ganglia. Also, at the 18th week of gestation a well-defined fluorescent network was demonstrated within the ganglia, as well as in the internodal segments. On the basis of these observations we concluded that the time between the 10th and 18th week of gestation has paramount importance for both the morphological and functional maturation of the enteric nervous system.

Key words: Human foetal development, Enteric nervous system, Ultrastructure, Histochemistry

Introduction

Clinical studies have revealed that congenital malformations of the enteric nervous system (ENS) seriously affect gut motility, gastric acid secretion, water and electrolyte transport (Okamoto and Ueda, 1967). Consequently, the clinical aspects of the studies that concentrate on the development of the human ENS are evident. Since the innervation of the mammalian gut is relatively mature at birth (Gershon et al., 1981; Furness and Costa, 1987) to study the development of the ENS requires the use of foetal tissue. Some ultrastructural studies have already been performed on the developing myenteric plexus (Gershon et al., 1981) though only on small laboratory animals. Because of the differences in the organization and function of the enteric plexuses between large mammals, including man, and small laboratory animals (Brookes et al., 1991) the rodent cannot be used as a valid model to study the development of human ENS. To establish the basic rules of the human ENS development and to find the time relation between the morphological and functional maturation in order to understand the reason for congenital mal formation leading to the misfunction of the gastrointestinal system, investigation of human foetal material is necessary. Because of the unavailability of tissue specimens, only sporadic data have hitherto been published on the morphological and functional development of the human foetus (Read and Burnstock, 1970; Daikoku et al., 1975). Neither the fine-structural characteristics of the neuromuscular junction nor the nature of the chemical substances transmitting the impulses from the ENS to the target cells in the gut wall are known. This study describes the fine structural organization of the ENS in the 10- and 18-week old human foetus and attempts to correlate the morphological maturation and the development of aminergic innervation, using glyoxylic acid-induced histofluorescence (De la Torre and Surgeon, 1976). Using the nitroblue tetrazolium/nicotinamide adenine dinucleotide (NBT/NADH) method (Gabella, 1969) the formation of enteric ganglia was followed. The present investigation, together with immunocytochemical studies in progress, may lead to a clarification of the period important for the normal development of human ENS.

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Materials and methods

Tissue sources

Human foetuses were obtained immediately after

legally approved or spontaneous abortions. The crown-heel length was used to assign gestation age. Three foetuses of both ages were used for each examination.

NBT/NADH method

For the NADH-diaphorase method (Gabella, 1969), pieces of 10- and 18-week-old foetal small intestines were distended with Krebs solution, then incubated in a solution containing 5 mg nitroblue tetrazolium (NBT) and 10 mg nicotinamide adenine dinucleotide (NADH) in 100 ml 0.1M phosphate buffer. After incubation for 60 min at room temperature, the tissue pieces were fixed in 10% formalin for 24 h, and whole-mounts were made.

Histofluorescence studies

For the histochemical detection of monoamines, the sucrose-phosphate glyoxylic acid method (De La Torre and Surgeon, 1976) was applied to whole-mount stretch preparations of 10- and 18-week-old human foetal intestine. Immediately after abortion, the small intestine was removed and exposed to the ice-cold incubation medium (6.8 g sucrose, 3.2 g KH_2PO_4 and 1g glyoxylic acid in 100 ml H_2O). After incubation for 30 min, the muscle layers were separated and stretched on microscope slides, dried and exposed to a temperature of 95 °C for 4 min. After mounting in liquid paraffin, the preparations were viewed in a Leitz Orthoplan microscope equipped with an HBO 50 W high-pressure mercury lamp.

Electron microscopy

The foetal small intestine was immersed in an aldehyde-containing fixative: 2% paraformaldehyde, 2% glutaraldehyde, 0.5% acrolein, and 0.5% dimethyl-sulphoxide. The pH was adjusted to 7.4 with 0.1M cacodylate buffer. Prefixation was performed overnight. After a short rinse in 0.1M cacodylate, postfixation was performed in 2% OsO_4 at 4 °C for 2 h. Samples were dehydrated in an ascending alcohol series. Block

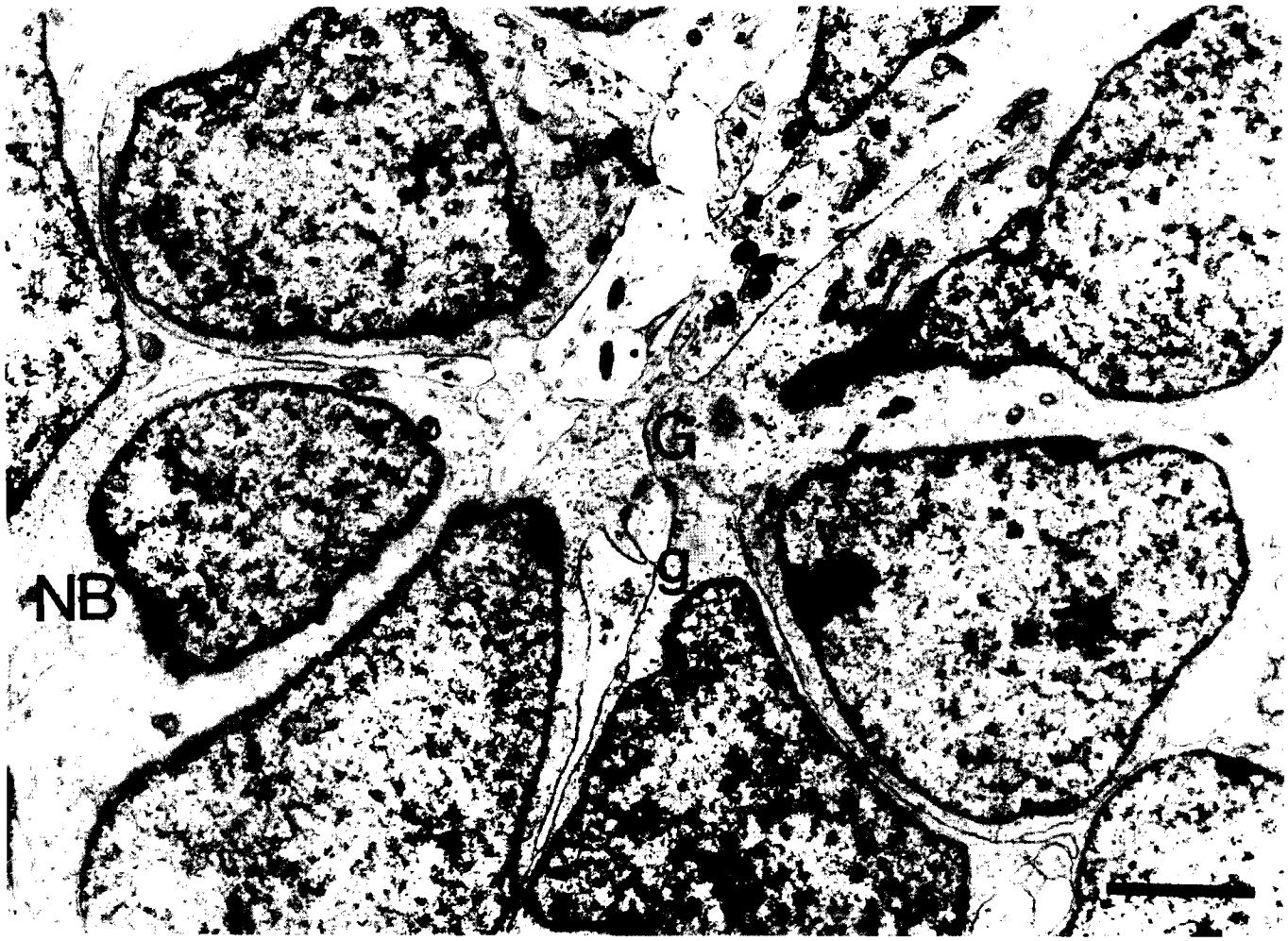

Fig. 1. Neuroblasts (NB) and ganglion cells (G) compose a loose myenteric ganglion (g) in the gut wall of a 10-week-old human foetus. Bar=1 μm.

contrasting was performed for 30 min with saturated uranyl acetate in 75% alcohol. Durcupan ACM was used for embedding. The ultrathin sections were post-contrasted with lead according to Reynolds (1963). Electron micrographs were taken with a TESLA BS 500 and JEM 100B electron microscope.

Results

10-week-old human foetus

Most of the neural cells seemed to be neuroblasts, which formed compact intramural ganglia (Fig. 1). The nuclei were rich in both hetero- and euchromatin. Two nuclei could often be found in one neural cell (Fig. 1). The perikaryon was usually narrow along the nucleus and contained a large number of free ribosomes, fewer mitochondria and a few tubules of rough-surfaced endoplasmic reticulum. Among the neuronal cells, only primordial neuropil could be found at this stage (Fig. 2). Most of the neural processes contained abundant microtubules, spherical clear, and a few dense-core vesicles. Synaptic contacts between neuronal elements were rare, although a few axosomatic synapses were observed (Fig. 2, insert). The number of synaptic vesicles in the synapsing axon terminals was rather low. Morphological specialisation typical of a neuromuscular junction was not distinguishable at this age (Fig. 3). Free axon terminals among intestinal smooth muscle cells were rarely observed in the 10-week-old human foetus. Neither amine-specific fluorescence nor NADH activities could be detected at this time of gestation.

18-week-old human foetus

Neuroblasts and a large number of mature ganglion cells could be seen in the myenteric plexus at this stage of the human foetus (Fig. 4). The perikaryon of the ganglion cells was wide around the nucleus and it contained a large number of cell organelles (Fig. 4). The

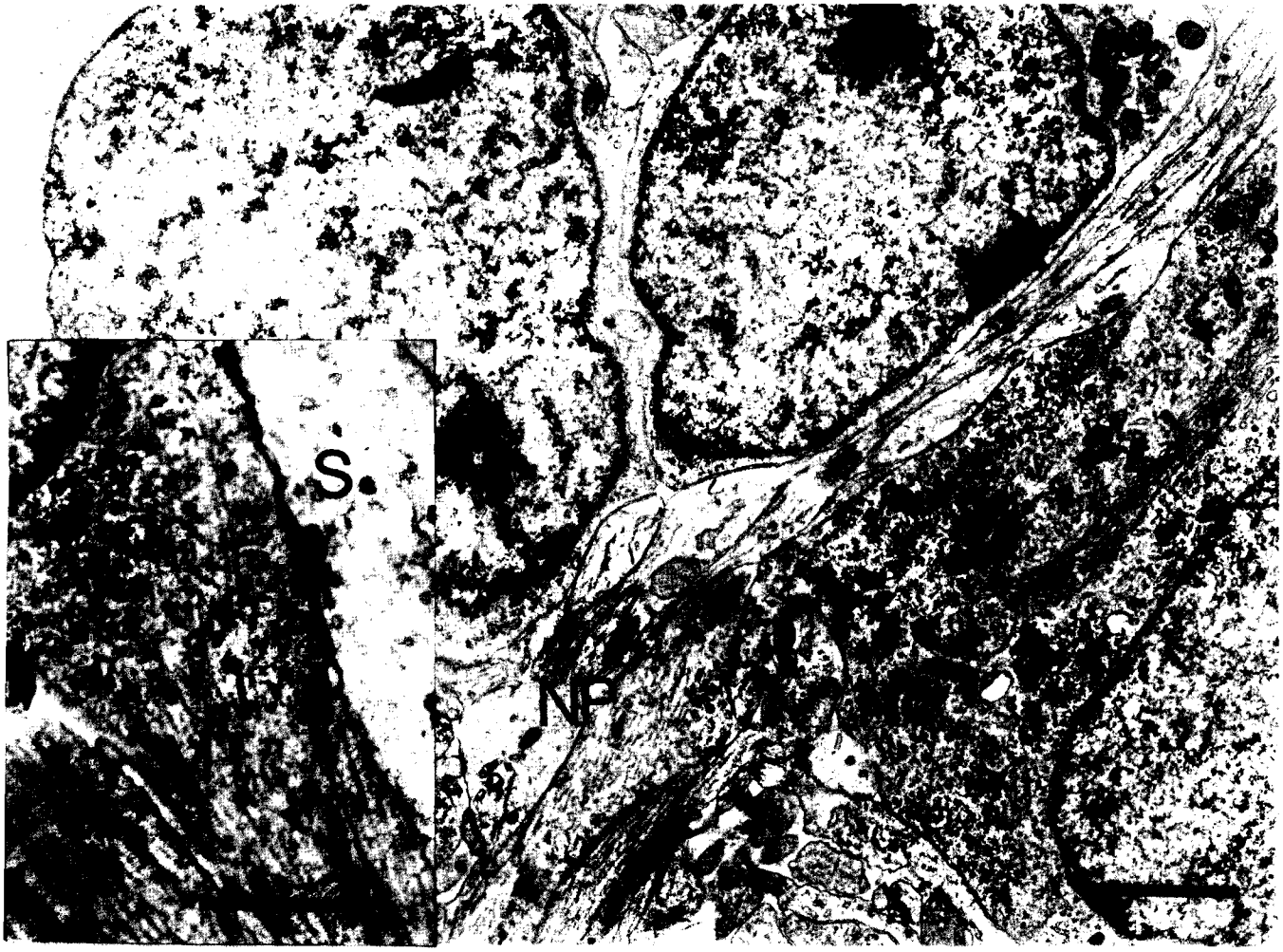

Fig. 2. Simple neuropil (NP) exists among intestinal neurons in the 10-week-old foetus. nc: nerve cell. Bar=1 μm. **Insert.** S: axosomatic synapse; dc: dense core vesicle in the soma; MT: microtubules. Bar=0.5 μm.

mitochondria, the tubules of rough-surfaced endoplasmic reticulum and the Golgi apparatus were evenly distributed in the cytoplasm. Microtubules were common in every neuron. Lysosomes and also dense-core vesicles were numerous in the perikarya. Neuropil occurred among the ganglion, cells and also in the internodal segments (Figs. 4, 5). The axon profiles contained agranular small vesicles (Fig. 4), large semiopaque neurosecretory, and dense-core granules (Figs. 4, 5). Axosomatic synapses were often detected (Fig. 4). Plexuses were frequently found in the proximity of smooth muscle cells (Fig. 5), forming «distant» and «close» contacts with each other. In the 18-week-old foetal intestine an intensive amine-specific fluorescence was detected in the myenteric plexus (Fig. 6). The fluorescent fibres frequently formed pericellular baskets around the non-fluorescent cell bodies (Fig. 6).

NADH-diaphorase staining revealed that most of the neurons were already ganglionated in the 18-week-old foetal intestine (Fig. 7), although small groups of solitary neuroblast-like cells were frequently found close to the mesenteric border.

Discussion

Our knowledge of the general structure of the enteric plexuses in adults is based on silver impregnation studies (Stach, 1989), methylene blue staining (Schabadash, 1930), glyoxylic acid-induced fluorescence (Scheuermann and Stach, 1984) and neuron-specific enolase histochemistry (Scheurmann et al., 1989). The ontogenic sequence of the formation of the myenteric plexus in chicken was followed on whole-mounts after NADH diaphorase histochemistry (Fekete et al., 1991). The present investigation follows the ultrastructural changes between the 10th and 18th week of gestation. During this period the primordial ganglia formed at the 10th week undergo a very pronounced structural maturation.

Peristalsis of the small intestine has been recorded from week 12 of gestation (Stach, 1989), which means that intestinal transit takes places in the foetus at this

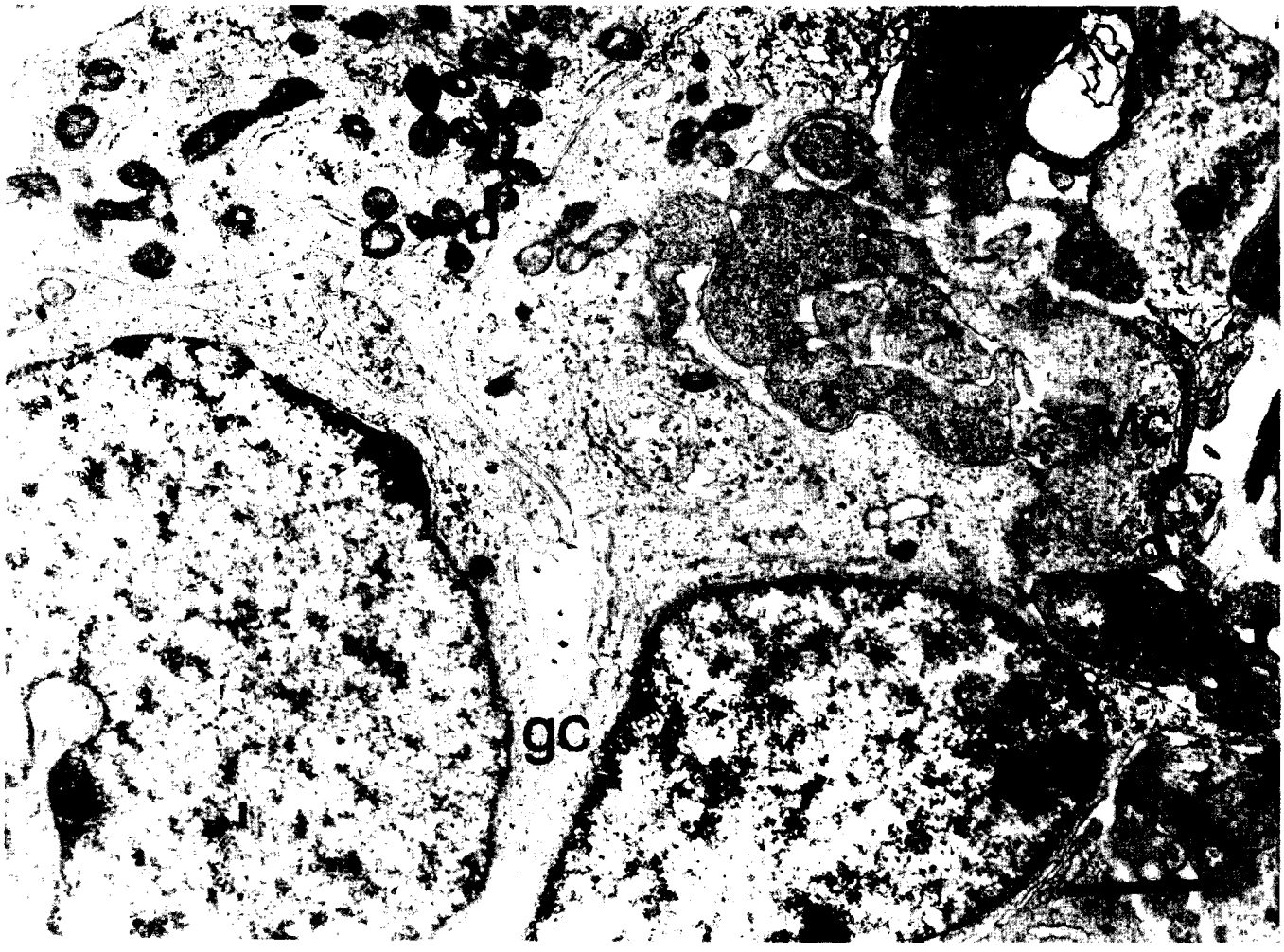

Fig. 3. Smooth muscle cells (Mc) are in close contact with nerve cell bodies (gc) in the gut wall of a 10-week-old human foetus. Bar=1 μm.

age. Migration of neuroblasts in the vagal trunk begins in about week 5, and neuroblasts reach the rectum in week 12 (Okamoto and Ueda, 1967). All these facts suggest that to gain information about the structural organization of the human ENS, morphological studies must be performed as early as possible. Satisfactory ultrastructural preservation has been achieved in the 10-week-old foetal intestine and it has been proven that most of the neuronal cells are poorly developed neuroblasts and that the neuropil is also far from mature. So the neuronal circuits required for integrated peristalsis are lacking. This conclusion is according to the observation of Daikoku et al. (1975), who reported that the longitudinal muscle layer develops only in weeks 10-12, and the monodirectional (oroanal) peristalsis begins only in weeks 27-30. At the same time, the close proximity of the neuroblasts and myoblasts is common. These contacts without any morphological differentiation might be the sites of direct trophic effects between smooth muscle cells and nerves. Reports suggesting a trophic influence of sympathetic nerve on smooth muscle in vitro (Chamley and Campbell, 1975) allow us to suppose similar links between the elements of the ENS and the smooth muscle in the gut wall. The morphological changes revealed by electron microscopy are prominent by the 18th week of gestation. Nerve terminals appear among muscle cells and probably modify the contraction of the muscle cells as occurs in adults. Electron microscopic studies revealed a large number of mature ganglia in the myenteric plexus. The existence of these ganglia was confirmed with the NADH-diaphorase method. The number of structurally mature neurons was clearly increased, and transmitter-containing vesicles also appeared in the perikarya. Histofluorescence observations revealed a well-developed aminergic fibre system in the myenteric plexus of the 18-week-old foetal intestine. The lack of fluorescent cell bodies suggests that the aminerg plexuses are extrinsic in origin.

Although more than twenty neurotransmitters may occur in the adult ENS (Schultzberg et al., 1980), little is known about the appearance of the different transmitters

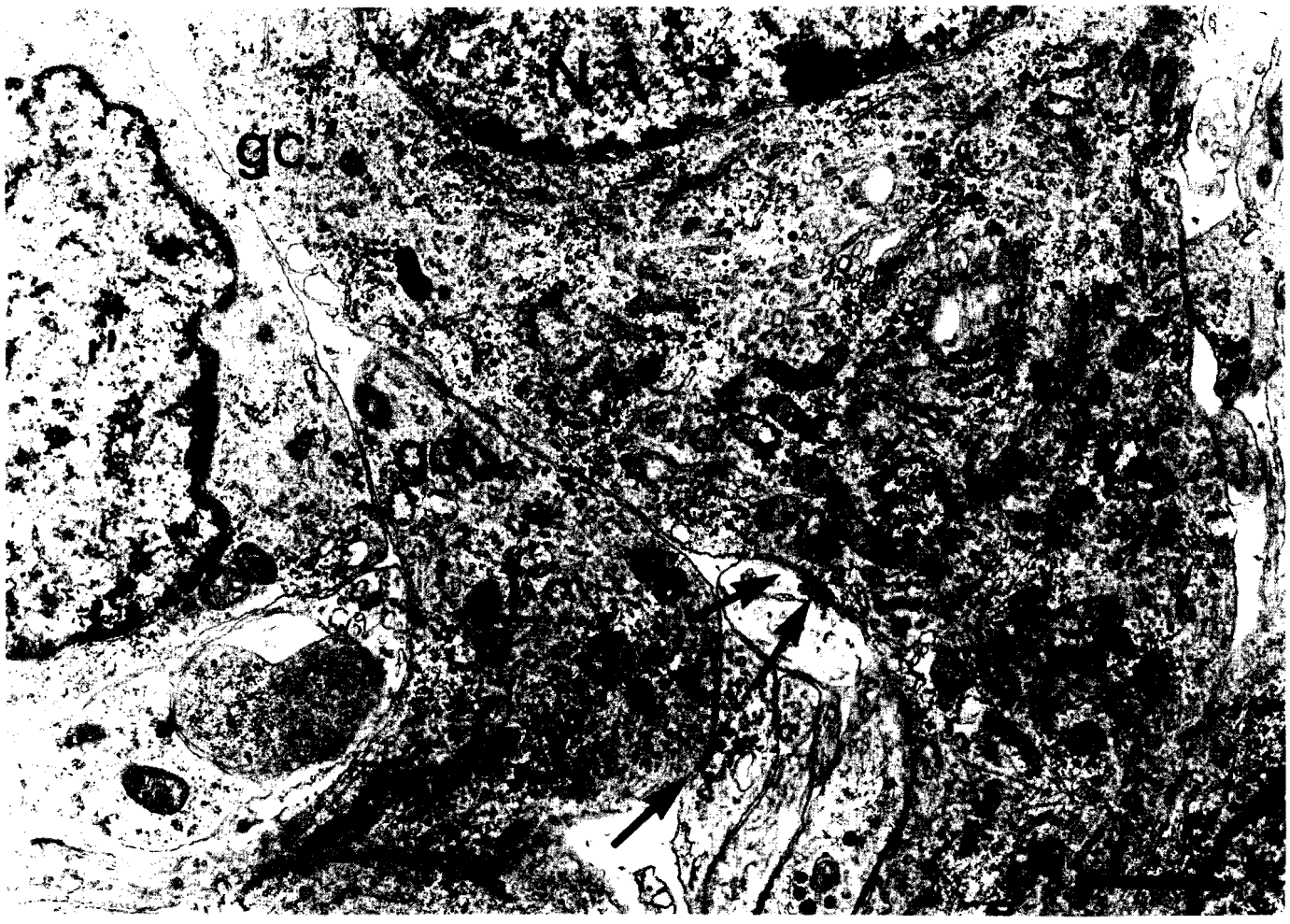

Fig. 4. Ganglion cells (gc) and axosomatic synapses (arrows) in the myenteric plexus of an 18-week-old human foetus. L: lysosome. Bar=0.5 µm.

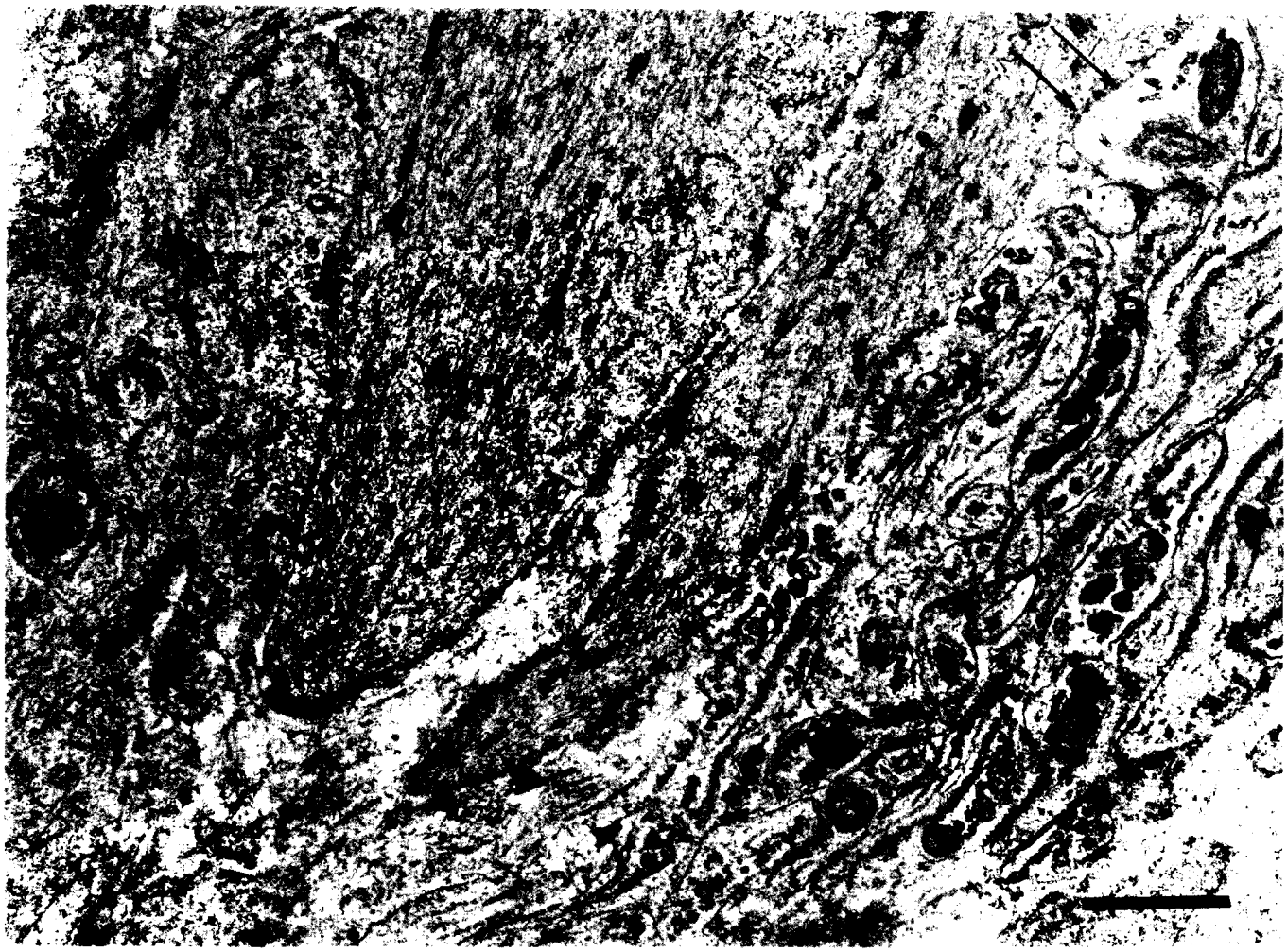

Fig. 5. Close (arrow) and distant (arrowhead) neuromuscular contacts in the jejenum of an 18-week-old human foetus. a: axon; m: muscle cell. Bar=0.5 μm.

Fig. 6. Whole-mount preparation from the intestine of an 18-week-old human foetus after glyoxylic acid-induced fluorescence. A dense fluorescent network is present within the myenteric ganglia (arrowhead) and in the internodal segments (arrow). Bar=50 μm.

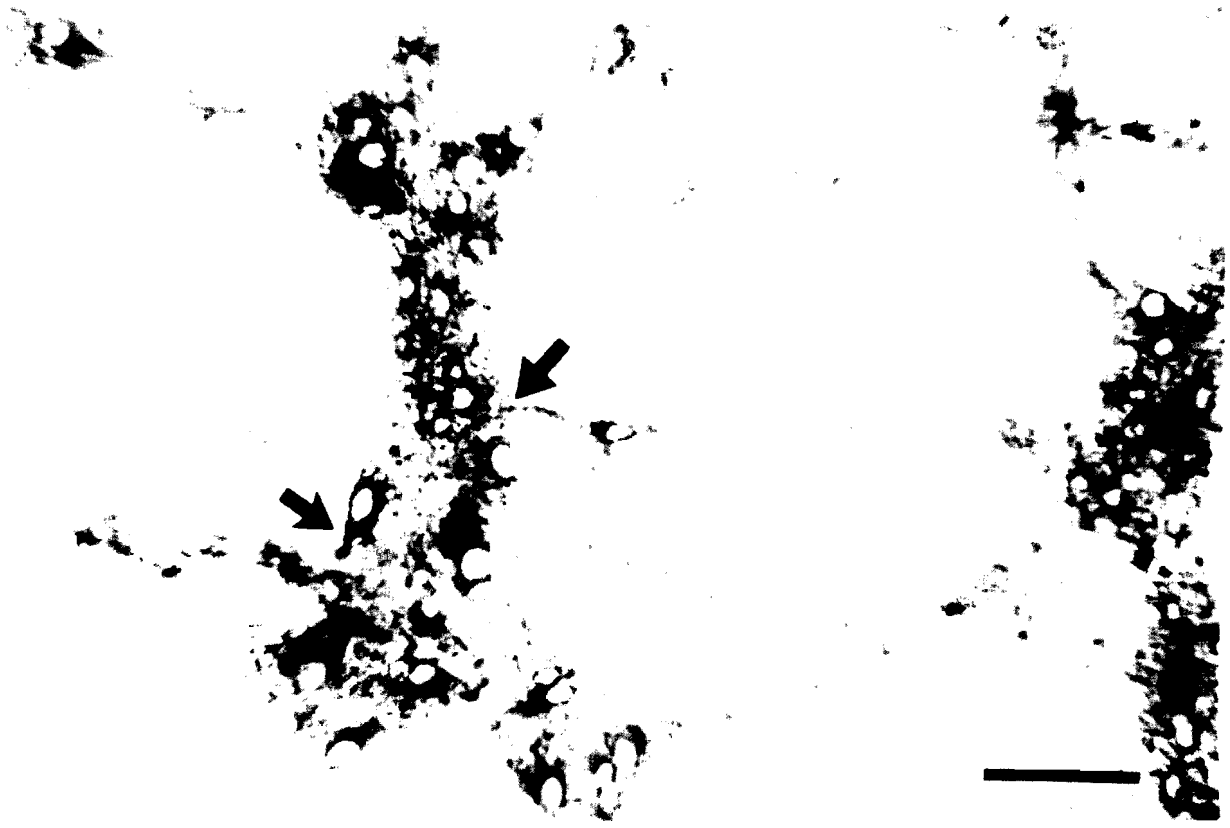

Fig. 7. Whole-mount preparations from the intestine of an 18-week-old human foetus after the NBT/NADH reaction. Arrows point to neural cell bodies within the ganglia. Bar=50 μm.

during human foetal development. Present ultrastructural studies strongly suggest that besides the aminergic profiles cholinergic and peptidergic fibres are also already present in the neuropil, and this highly organized neuronal network is able to effectively modulate the motor activity of the 18-week-old foetal gastrointestinal system. Based on these results systematic immunohistochemical studies are in progress in our laboratory to determine the chronological sequence which may exist in the appearance of the different neuronal transmitters.

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