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REVIEW ARTICLE

Prenatal development of the myenteric plexus in the human fetal small intestine

Éva Fekete[1]*, Mária Bagyánszki[1], Béla A. Resch[2]

[1]Department of Zoology and Cell Biology, [2]Department of Obstetrics and Gynaecology, University of Szeged, Szeged, Hungary

ABSTRACT The enteric nervous system is large, complex and independent of the central nervous system. Its neural-crest-derived precursors migrate along defined pathways to colonize the bowel. It has been established that signalling molecules produced by the developing neurons and the mesenchyma of the gut wall play a critical role in the development of the mammalian enteric nervous system. Recent studies have further characterized the roles of the different cellular and molecular elements that are critical for enteric ganglia formation. The application of modern neuroanatomical techniques revealed that the enteric nervous system contains a considerable number of neuronal subpopulations. Most of our knowledge concerning the functional features of the enteric neurons, e.g. chemical coding, neuronal connectivity and electrophysiological behaviour, was derived from studies of the guinea-pig small intestine. In light of the interspecies differences, comparison of the findings on different species is mandatory. Consequently, the investigation of human fetal material is necessary in order to establish the basic rules of the development of the human enteric nervous system and to find the time relation between the morphological and functional maturation, thereby permitting an understanding of the causes of congenital malformation leading to misfunction of the gastrointestinal system.

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KEY WORDS

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Since the first description of the myenteric plexus (Auerbach 1864) and Meissner's plexus (Meissner 1857), many reports have been published on the morphological and functional organization of the enteric nervous system (ENS). The ENS is composed of a collection of autonomic ganglia and associated neural connectives in the wall of the intestines (Taxi 1965; Furness and Costa 1987; Gershon et al. 1994). Even though the ENS is a component of the peripheral nervous system (PNS), it is unlike any other. In contrast with extra-enteric peripheral ganglia, myenteric ganglia lack collagen and receive their support not from Schwann cells, but from astrocyte-like enteric glia. In fact, the ultrastructure of the ENS resembles that of the central nervous system (CNS) more than that of the rest of the PNS (Boros and Fekete 1993; Fekete et al. 1996). The most striking peculiarity of the ENS which distinguishes it from the other two autonomic divisions (the sympathetic and the parasympathetic) is that the majority of enteric neurons are not directly innervated by the brain or spinal cord. This unique independence of the ENS enables the intestine to manifest reflex activity in the absence of CNS input (Costa and Furness 1976; Furness et al. 1992). The ENS can also influence other organs. Neurons within the gut project out of the bowel to innervate pre-vertebral sympathetic ganglia, as well as ganglia in the gall bladder and pancreas. The ENS is thus an independent nervous system that structurally resembles the brain. The phenotypic diversity of its components, neurons and non-neuronal (NN) cells transcends that found in other ganglia and includes every class of neurotransmitters found in the CNS. A correlation between the structural and functional features of the enteric neurons was first suggested by Dogiel (Dogiel 1896). In the late 1970s, the development of new techniques, and particularly immunohistochemistry, led to a dramatic increase in our knowledge of the diversity of the enteric neurons (Furness et al. 1991). The diversity of chemically defined and functionally differing subtypes of enteric neurons was established in the guinea-pig, rat and pig (Furness and Costa 1987; Costa et al. 1991; Sundler et al. 1993; Timmermans et al. 1997). It is difficult to transfer concepts from these species to the human gut. Recently, human enteric neurons were morphologically described and classified through the use of different methods (Timmermans et al. 1994; Wattchow et al. 1995, 1997; Porter et al. 1996; Fekete et al. 1997). These results reinforced the view that results on small laboratory animals cannot simply be extrapolated to humans (Hoyle and Burnstock 1989; Scheuermann et al. 1989). The same conclusions were drawn after developmental studies (Gershon et al. 1981; Brookes et al. 1991). At the same time, clinical studies revealed that congenital

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*Correspondening author. Phone: 36(62)544-123, Fax: 36(62)544-049, E-mail: efekete@bio.u-szeged.hu

malformations of the ENS seriously affect the gut motility, gastric acid secretion, and water and electrolyte transport (Okamoto and Ueda 1967). Consequently, the clinical aspects of studies that concentrate on the development of the human ENS are evident.

This review will focus on the morphological and neurochemical changes in the cellular elements of the myenteric plexus (MP) and the muscle coat in the human fetal small intestine from the 10th to the 26th week of gestation. Special attention will be paid to the histochemical and ultrastructural features of the developing human ENS, the ultrastructural changes in the nerve-muscle contacts and also the interstitial cells - neuronal contacts during this developmental period. The review will also focus on the development of the nitrergic neurons. These neurons are involved in inhibitory non-adrenergic, non-cholinergic (NANC) neurotransmission in the ENS of various mammalian species (Stark and Szuszewski 1993).

Ultrastructural features of the myenteric plexus of the human fetal small intestine

In the electron microscope the ganglia of the MP of the human fetal small intestine appear as very compact structures, completely surrounded by a basal lamina and isolated from the connective tissue and blood vessels (Fig. 1). All spaces are occupied by nervous and glial elements, constituting a dense neuropil, with a gap of 20 nm between adjacent membranes. The compactness of the structure, the isolation from blood vessels and connective tissue, the virtual absence of extracellular space, and the occurrence of a dense neuropil are reminiscent of the CNS rather than of other autonomic ganglia. The nerve cells display a high variability in shape, structure and size, they have a large, round or oval nucleus. The nucleoplasm is finely granular with a few condensations of chromatin attached to the nuclear envelope. The morphological characteristics of the nucleus afford a consistent criterion whereby nerve cells can be distinguished from glial cells. Glial cells are numerous in the ganglia and the connecting strands of the MP (Gabella 1972; Cook et al. 1992).

Glial cells, ultrastructurally similar to central astrocytes and called enteric glial cells, are found in the myenteric ganglia (Gabella 1981). Throughout their course in the gut wall, the nerve bundles are accompanied by Schwann cells, which partially ensheathe single fibres or fibre bundles. In the ganglia, the nuclei of the glial cells appear to outnumber the nuclei of the neurons by 2 or 3 to 1. The cell bodies of the glial cells are generally smaller than those of the neurons. The nucleus is oval with large patches of dense material attached to the nuclear envelope. The neurons, the glial cells and their processes are closely packed together within the ganglia. Outside the ganglia and the large interconnecting strands lie interstitial cells which are flattened and have long laminar processes.

Types of neurons in myenteric ganglia

In terms of size, the enteric neurons are distributed over an extremely wide range (Gabella 1971). The histograms of neuron sizes vary in the different parts of the alimentary tract. Elaborate classifications of the enteric neurons have been produced on the basis of silver impregnation and methylene blue studies. The best-known of them is that of Dogiel (1896), who described three types of methylene-blue-stained neurons according to the number, extent and branching characteristics of the neuronal processes. More recently, tracers such as horseradish peroxidase, Procion Yellow and Lucifer Yellow have been injected into the myenteric neurons, and it has become possible to study the morphology of enteric ganglion cells in a highly selective way (Hodgkiss and Lees 1983). The development of immunohistochemistry led to a substantial increase in our knowledge of the diversity of enteric neurons (Furness et al. 1991). It was realized that the ENS contains a considerable number of neuronal subpopulations (Gershon and Erde 1981; Furness and Costa 1982).

Conventional ultrastructural studies revealed different types of synaptic vesicles in the enteric neurons (Baumgartner et al. 1970; Gabella 1972; Wilson et al. 1981; Fekete et al. 1995). Several kinds of vesiculated nerve processes form synapses with intramural neurons. In the MP of humans, rhesus monkeys and guinea pigs, three types of nerve profiles have been described (Baumgarten et al. 1970). One type is characterized by numerous agranular vesicles. These varicosities are interpreted as cholinergic. They form typical synaptic junctions, the majority of which are on perikarya or somatic spines. A second type of endings contains vesicles 50 to 90 nm in diameter, with an intensely osmiophilic granule. These endings are never found to form synaptic contacts and are interpreted as adrenergic. Endings of the third and most common type contain, in addition to a few agranular vesicles, vesicles 85 to 160 nm in diameter, with a large granule of medium electron density. These nerve profiles are labelled p-type or peptide-containing varicosities.

An extremely puzzling observation is the occurrence of morphological specializations in axons contacting glial cells (Murphy et al. 1995; Fekete et al. 1997). The number of contacts is rather high and it is very probable that each glial cell has one or more.

The MP is situated between the layers of the muscle coat. The muscle cells lie approximately parallel to each other, usually forming sheet-like layers or coats. The MP can be visualized as mesh-like laminar structures, i.e. wide and thin ganglia spread over a surface and joined to each other by connecting strands (Figs. 2 and 3). The mesh formed by the ganglia and the connecting strands has a regular pattern, which is characteristic of each segment of the alimentary tract and to some extent also of the animal species. Whether these patterns have any significance and whether they bear any relation to the functional properties of the organ is

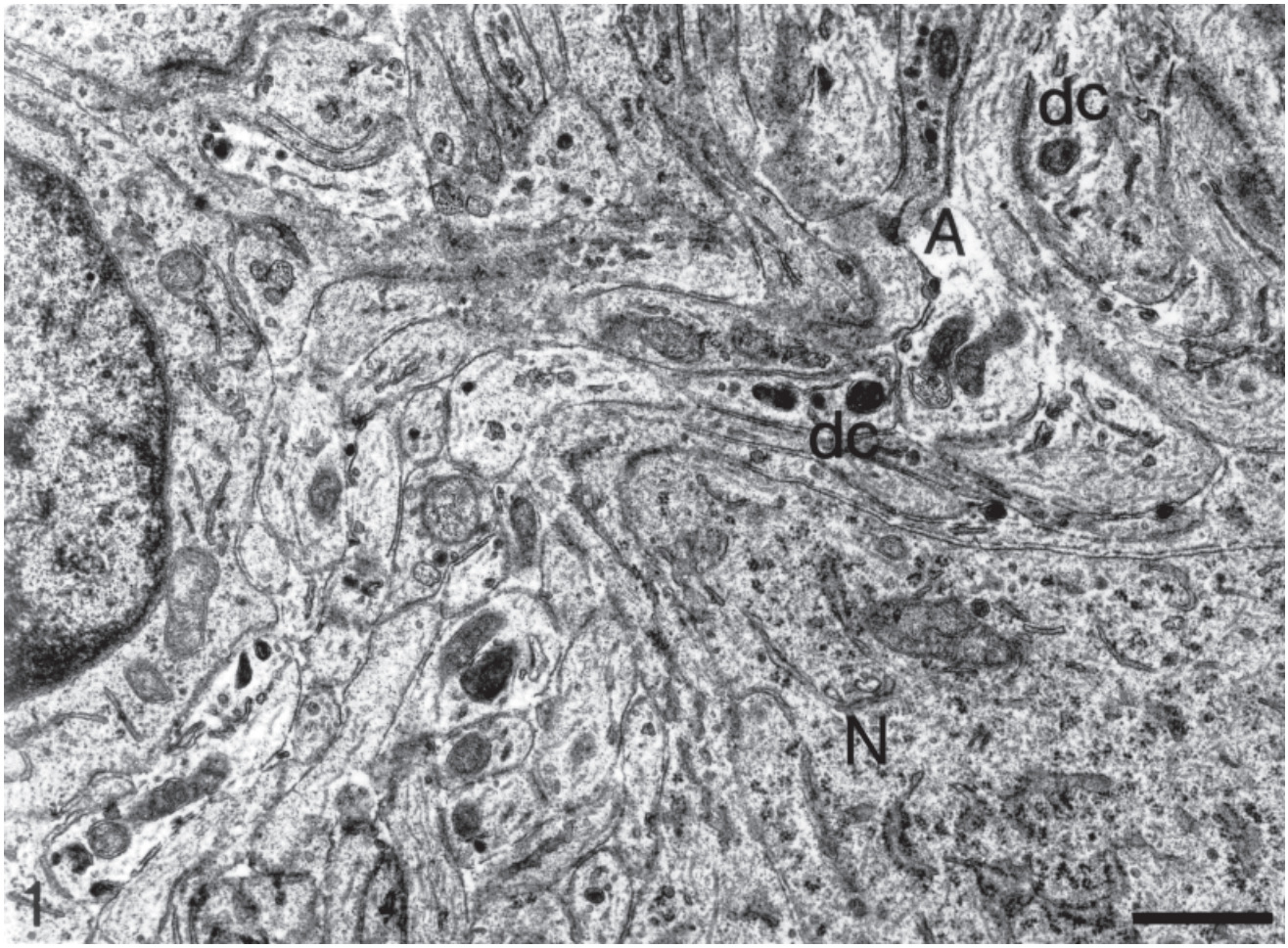


Figure 1. General view of the myenteric plexus in the small intestine of a 26-week-old human fetus. One nerve cell body (N) is partially visible. A dense neuropil is apparent. dc: dense core vesicle, A: axon profile. Bar: 0.5 μm.

unclear. As a manifestation of order, they pose a challenging problem of morphogenesis and intercellular organization.

Prenatal development of the myenteric plexus in the human fetal small intestine

Despite the high degree of complexity and its similarities to the CNS, the ENS is derived from the neural crest (NC), the source of all other branches of the PNS (Le Douarin 1982; Gershon 1998). Prospective enteric NC cells emigrate from two main regions of the neural tube: the vagal region, which corresponds to somites 1-7, and the sacral region posterior to somite 28. Vagal-derived ENS progenitors, which give rise to the majority of neurons and glia of the enteric ganglia, enter the foregut mesenchyma and migrate in an anteroposterior direction, colonizing the entire length of the gut (Le Douarin and Teilett 1971). Despite the detailed characterization of the origin and the migratory pattern of the preenteric NC (Pomeranz and Gershon 1990; Burns and Le Douarin 1998), a number of critical question remain unanswered. One of the most interesting questions, concerning the development of the human fetal ENS concerns the mechanisms which control the formation of enteric ganglia in the appropriate locations within the gut wall. Since the innervation of the mammalian gut is relatively mature at birth (Gershon et al. 1981; Furness and Costa 1987), study of the development of the ENS requires the use of fetal tissue. Because of the differences in the organization and function of the enteric MP between large mammals, including man, and small laboratory animals (Brookes et al. 1991), the rodent cannot be used as a valid model for study of the development of the human ENS. To establish the basic rules of the development of the human ENS the investigation of human fetal material is necessary. Peristalsis of the human fetal small intestine has been recorded from week 12 of gestation (Stach 1989), which means that intestinal transit takes place in the fetus at this age. Migration of neuroblasts in the vagal trunk begins in about week 5, and neuroblasts reach the rectum in week 12 (Okomato and Ueda 1967). All these facts suggest

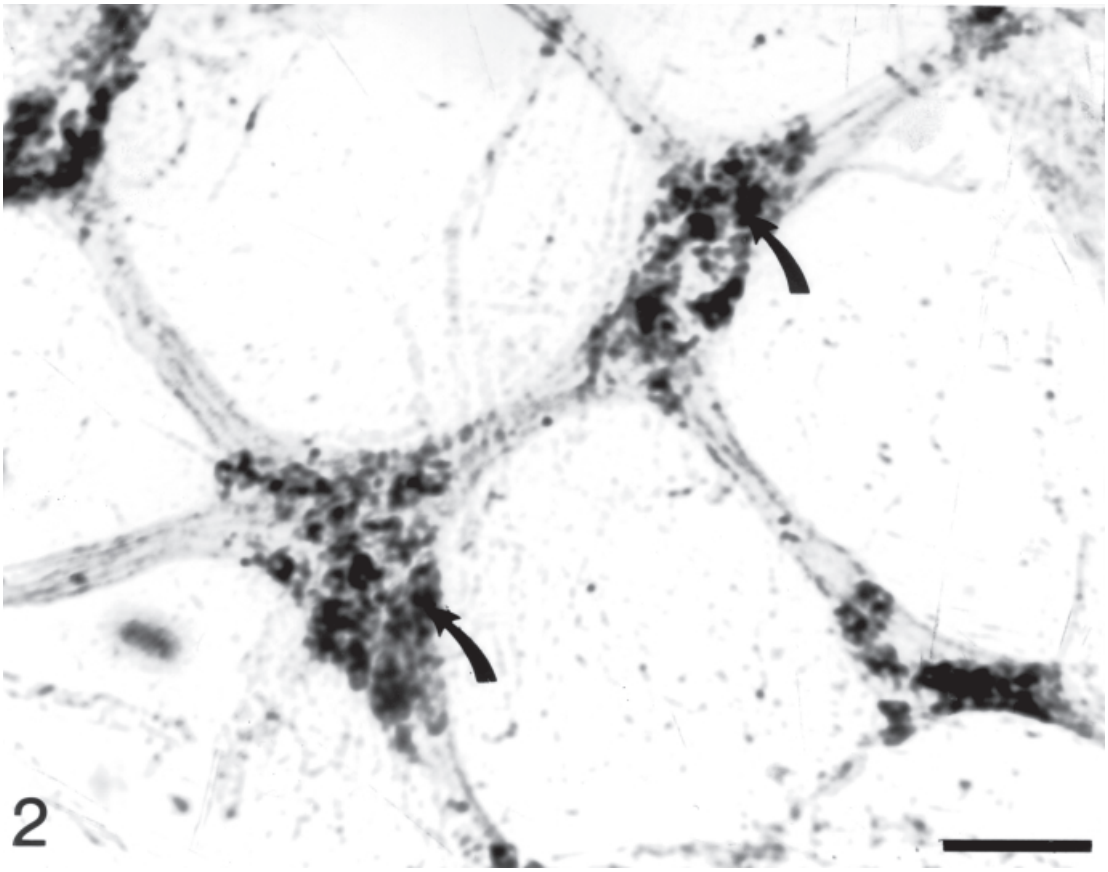


Figure 2. Whole-mount preparation from the small intestine of a 12-week-old human fetus after NADPHd histochemical reaction. Arrows point to neural cell bodies within the ganglia. Bar: 50 μm.

that morphological and histochemical studies must be performed as early as possible in order to gain information on the structural organization of the human ENS. MP formation has been analyzed by means of electron microscopy and on whole-mounts after NADH-diaphorase histochemistry (Fekete et al. 1995). Satisfactory ultrastructural preservation has been achieved in the 10-week-old fetal intestine. It has been proved that most of the neuronal cells and the neuropil are then far from mature. Most of the neural cells seem to be neuroblasts, which form compact intramural ganglia (Fig. 4). The nuclei are rich in both hetero- and euchromatin, and two nuclei can often be found in one neural cell. Among the neuronal cells, only primordial neuropil can be found at this age (Fig. 5). Synaptic contacts between neuronal elements are rare, although a few axosomatic synapses are observed (Fig. 5, insert). Morphological specialization typical of a neuromuscular junction is not distinguishable at this age (Fig. 6). Free axon terminals among intestinal smooth muscle cells are rarely observed in the 10-week-old human fetus.

The histochemical investigations revealed neither amine-specific fluorescence nor NADH activities at this stage of gestation. These observations led to the conclusion that the neuronal circuits required for integrated peristalsis were lacking. This conclusion was in accordance with former observations (Daikoku et al. 1975). It has been reported that the longitudinal muscle (LM) layer develops only in weeks 10-12, and that 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 nerves on smooth muscle *in vitro* (Chamley and Campbell 1975) allow the supposition of 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 week 18 of gestation (Fekete et al. 1995). Some neuroblasts and a large number of mature ganglion cells can be seen in the MP at this stage of the human fetus (Fig. 7). Neuropil occurs among the ganglion cells and also in the internodal segments. The axon profiles contain agranular small vesicles, and large

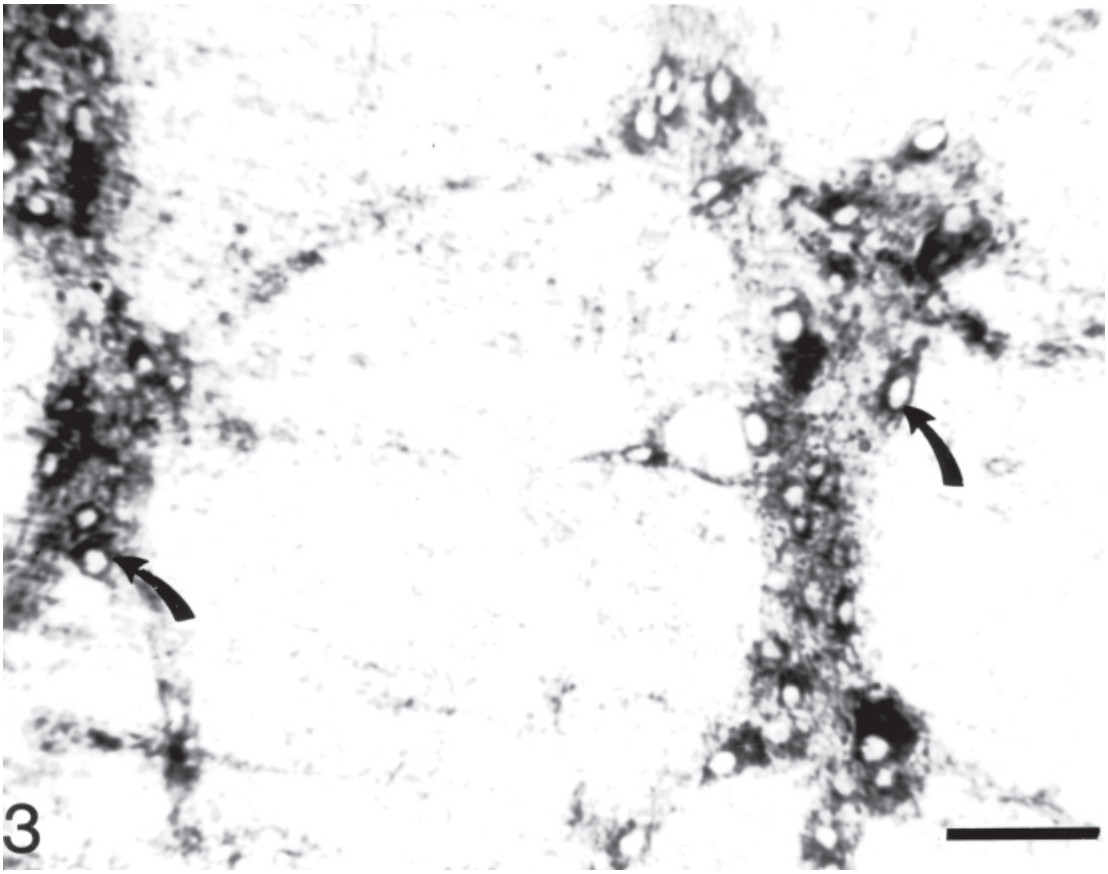


Figure 3. Whole-mount preparation from the small intestine of an 18-week-old human fetus after NADPHd histochemical reaction. Arrows point to neural cell bodies within the ganglia. Bar: 50 μm.

semiopaque neuro-secretory and dense-core granules. Axosomatic synapses are often detected. MPs are frequently found in the proximity of smooth muscle cells, forming distant and close contacts with each other (Figs. 8 and 9). The nerve terminals appearing among the muscle cells probable modify the contraction of the muscle cells. The existence of a large number of mature ganglia, revealed by electron microscopy, has been confirmed with the NADPH-diaphorase method (Figs. 2 and 3). Histofluorescence observations reveal a well-developed aminergic fibre system in the MP of the 18-week-old fetal intestine. The lack of fluorescent cell bodies at the same time suggests the extrinsic origin of the fetal aminergic plexuses. Although more than 20 neurotransmitters may occur in the adult human intestine (Schultzberg et al. 1980), little is known about the appearance of the different transmitters during human fetal development. Recent ultrastructural and immunohistochemical studies (Fekete et al. 1997) strongly suggest that, besides the aminergic profiles, cholinergic and peptidergic fibres are also present in the neuropil of the 18-week-old fetal gastrointestinal system, and these transmitters are effectively able to modulate the motor activity of the fetal intestine. By means of neuron-specific enolase (NSE) immunocytochemistry and electron microscopy, a changing topographic relation between the elements of the MP and the muscle coat has been revealed in the human fetal small intestine between weeks 10 and 26 of gestation (Fekete et al. 1996). In sections of the 10-week-old human fetal small intestine, NSE-immunopositive aggregates of enteric neurons can be distinguished on the outer surface of the newly-formed circular muscle (CM) layer (Fig. 10). Throughout week 18 of gestation, the CM provides the mechanical surface for the developing MP, which is attached firmly to this muscle layer. Around week 18 of gestation, the mechanical points of attachment shift from the CM to the LM layer. Concomitantly, the MP adheres to the LM layer, while strips of CM can be easily removed. This relocation may be accompanied by the appearance of specific surface molecules recognized by developing neurons, as shown in *in vitro* systems (Domoto et al. 1990). The changing pattern of nerve-muscle contact is also reflected at the ultrastructural level. Although both the CM and the MP appear by week 10 of gestation, they cannot be recognized

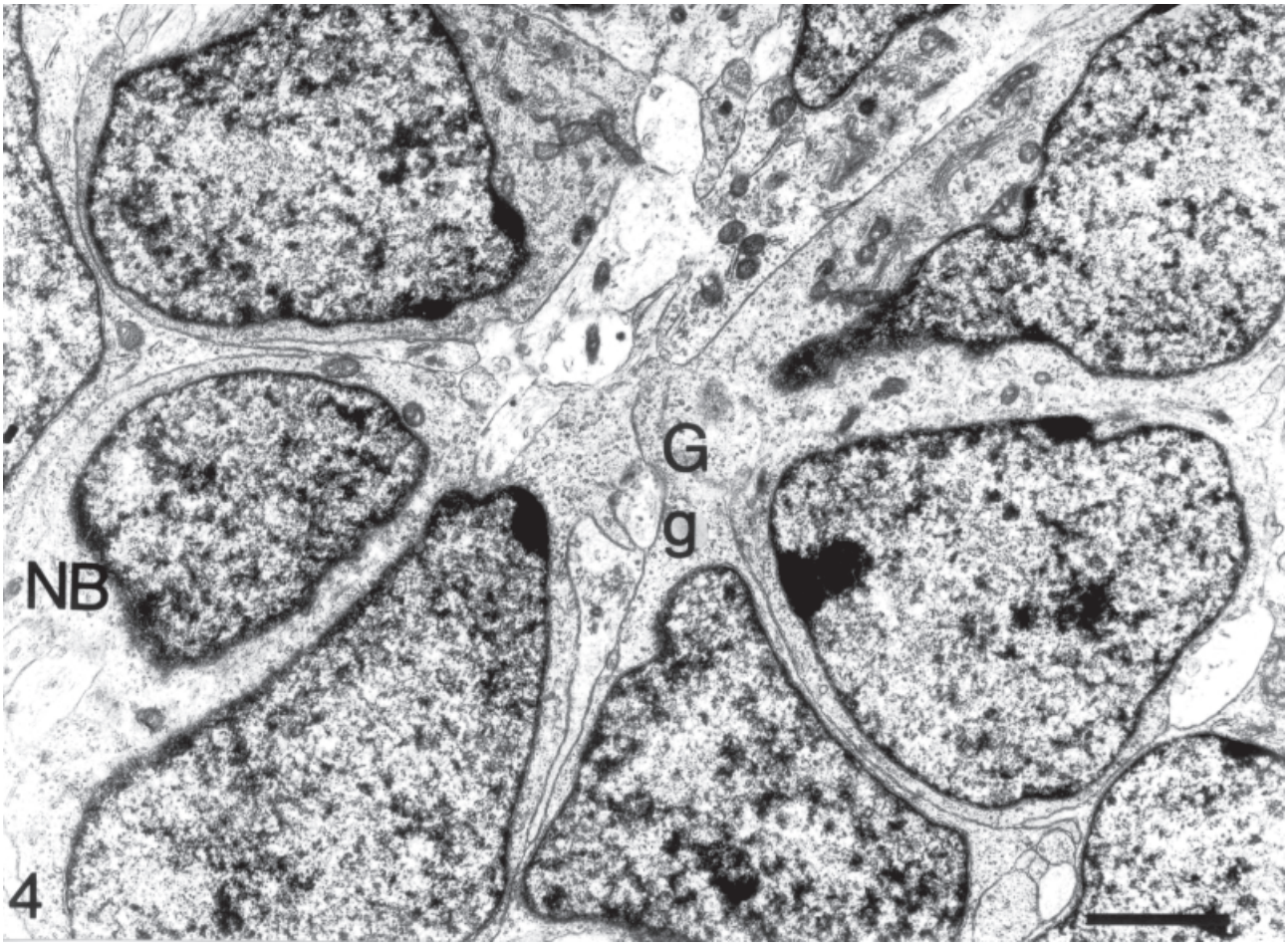


Figure 4. Neuroblasts (NB) and ganglion cells (G) compose a loose myenteric ganglion (g) in the gut wall of a 10-week-old human fetus. Bar: 1 μm.

as clearly separated entities at this stage (Fig. 6). The elements of the MP and CM are intermingled. Neurons, muscle cells, nerve plexuses and nerve terminals are in close contact with each other, without an intervening basal lamina. A similar arrangement was described by Gershon (Gershon et al. 1981) in the developing guinea-pig small intestine. In the absence of basal lamina, the elements of the MP can communicate with smooth muscle cells, providing an opportunity for nerve-muscle trophic interaction. To date, however, there is no evidence that enteric neurons are dependent on neurotrophic support for their survival during development (Ward et al. 1994). Nevertheless, there is evidence that the number of neurons, the density of the MP, and the average neuronal size are greater in areas where the smooth muscle layers are thicker (Gabella 1989). Intestinal smooth muscle has also been found to promote directional outgrowth from sympathetic ganglion explants in co-culture (Gintzler and Hyde 1983). The specific nerve-muscle interaction is reflected morphologically in the interdigitation of cellular processes which provide the cellular surface for the mutual metabolic activities. Desmosome-like contacts at the same time indicate the mechanical coupling between the developing CM layer and the MP. From week 18 of gestation onwards, the developing MP becomes increasingly ensheathed by different kinds of non-neuronal cells, collagen-filled spaces and basal lamina (Fekete et al. 1997). Meanwhile, new contacts are formed between the MP and the LM layer. Although some of these contacts appear permanent or at least long-lasting, intimate contacts between the MP and any part of the muscularis externa have practically disappeared by week 26 of gestation. Evidence has been provided that the microenvironment from which the neurons originate is critical in determining the ultimate pathway of differentiation (Le Douarin and Teilett 1971). The sequential appearance of the various types of enteric neurons (Gintzler and Hyde 1983) and the sequential changes in the nerve muscle contacts may be essential for morphogenesis or, more generally, for the functional maturation of the external

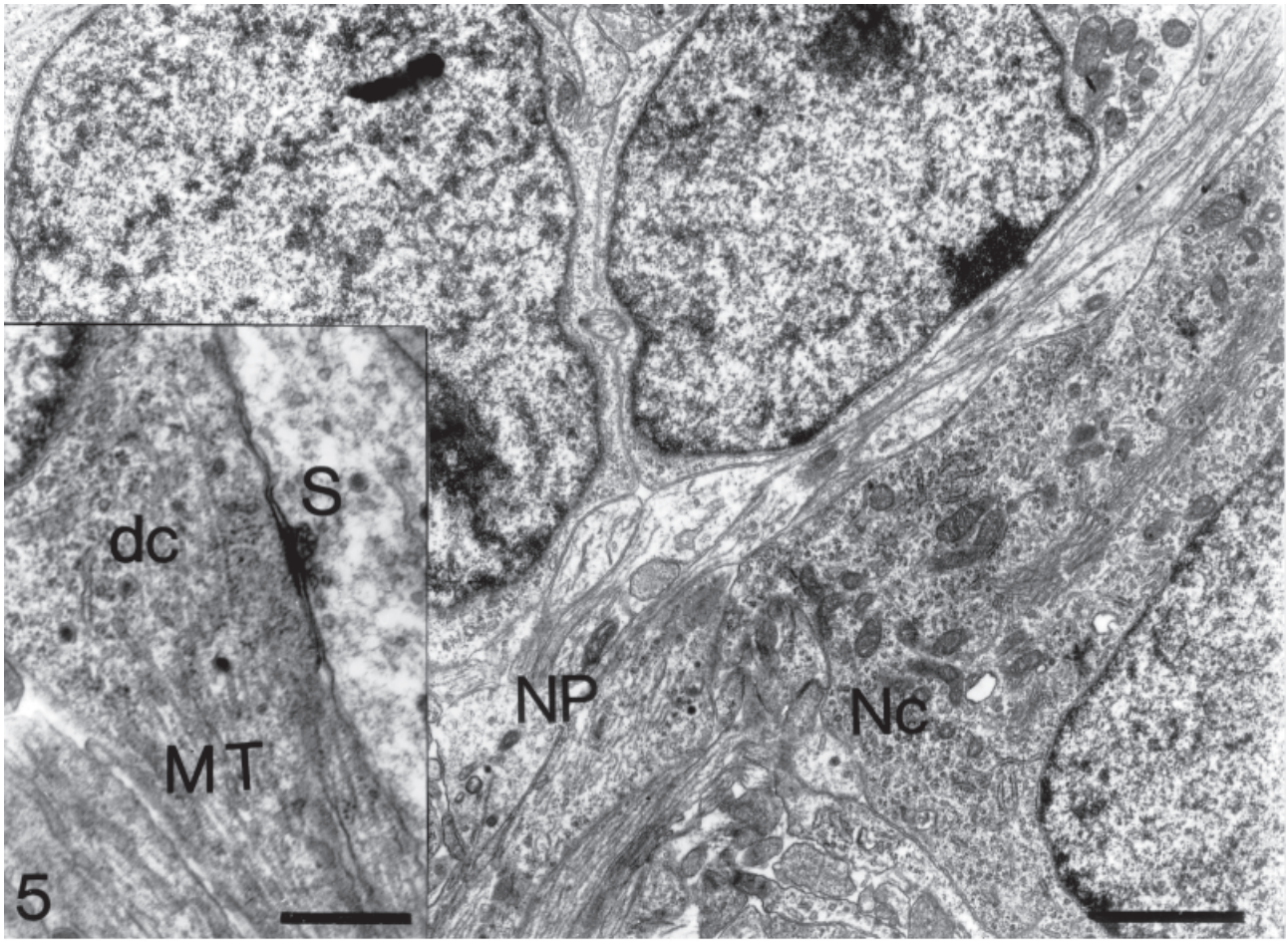
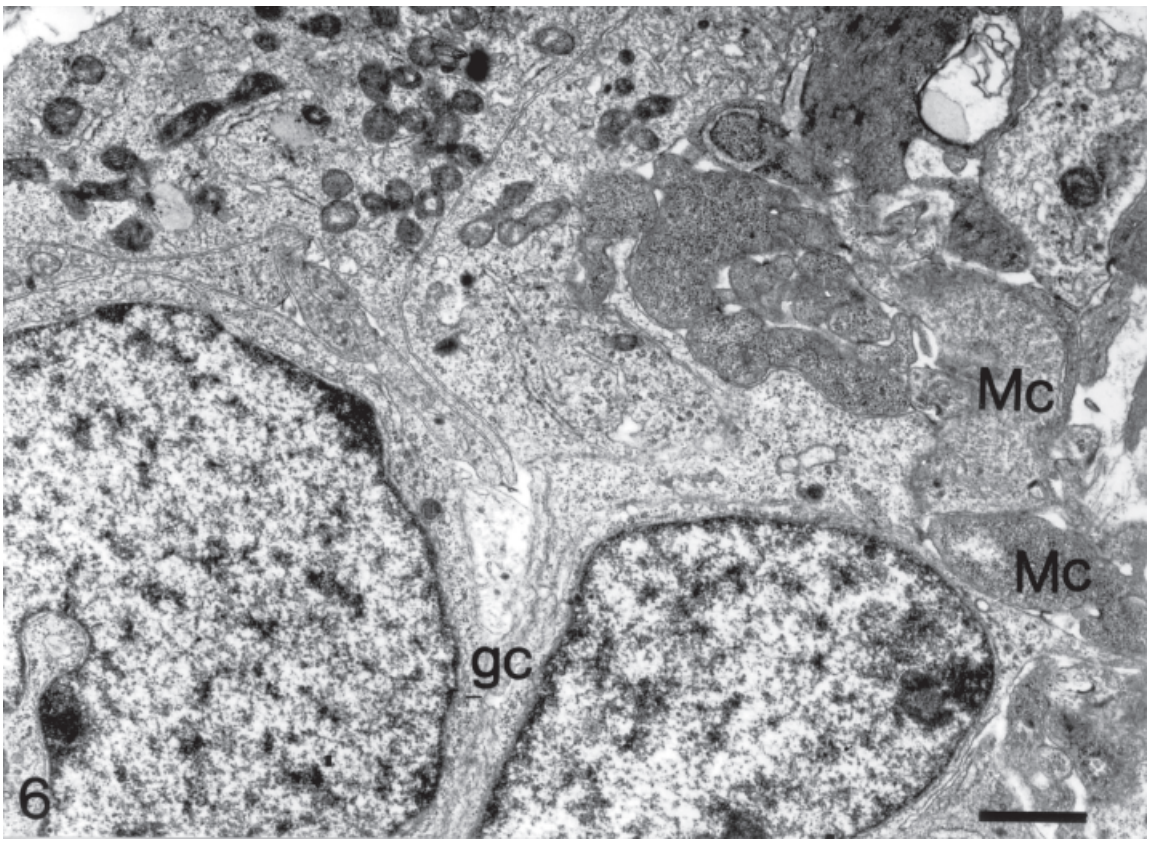


Figure 5. Simple neuropil (NP) exists among intestinal neurons in the 10-week-old fetus. Nc: nerve cell. Bar: 1 μm. Insert. S: axosomatic synapse; dc: dense core vesicle in the soma; MT: microtubules. Bar: 0.5 μm.

muscle coat and the interposed MP.

Recent electron microscopic and immunocytochemical investigations (Fekete et al. 1997) revealed two distinct types of NN cells in association with the developing MP. Although they both appear together around week 10 of gestation, their distributions already differ greatly by around week 14 of gestation. Two classes of morphologically distinct NN cells were found around the primordial ganglia in the 10-week-old human fetal intestine, when neither the MP nor the LM layer are well developed yet. There is a gradual change in the distribution of the NN cells during the fetal period. In week 10 of gestation, the two distinct types of NN cells, one spindle-shaped and the other with electron-dense cytoplasm and branching processes, are intermingled with the neuroblasts in the outer compartment of the intestinal wall. In week 12 of gestation, the spindle-shaped cells are frequently interposed between the MP and the LM layer, while in week 14, cells in this topographical position have long cytoplasmic processes and large, ovoid nuclei, rich in heterochromatin. NN cells with abundant surface caveolae and several short processes at the same stage are inter-connected with each other and with the ganglionic cells. Apart from these two distinct cell types, no NN cells are seen around or within the developing ganglia until week 17 of gestation. By this stage, the cells originally interposed between the developing ganglia and the muscle layer seem to change the topographical distribution and their slender processes penetrate deeply into the ganglia (Fig. 11). At the same time, some other NN cells appear in the extraganglionic space and their long, intervening processes definitely encapsulate the ganglia (Fig. 12). The presence of the highly ordered 10 nm thick filaments in some of these processes resembles the situation for the interstitial cells of Cajal (Thuneberg 1982). The other well-pronounced morphological change noticed in the 17-week-old fetuses is the appearance of the char-acteristic cellular networks in the vicinity of the MP (Figs.13 and 14). The ultrastructural features of the cells building these networks resemble those of the cells surrounding the primor-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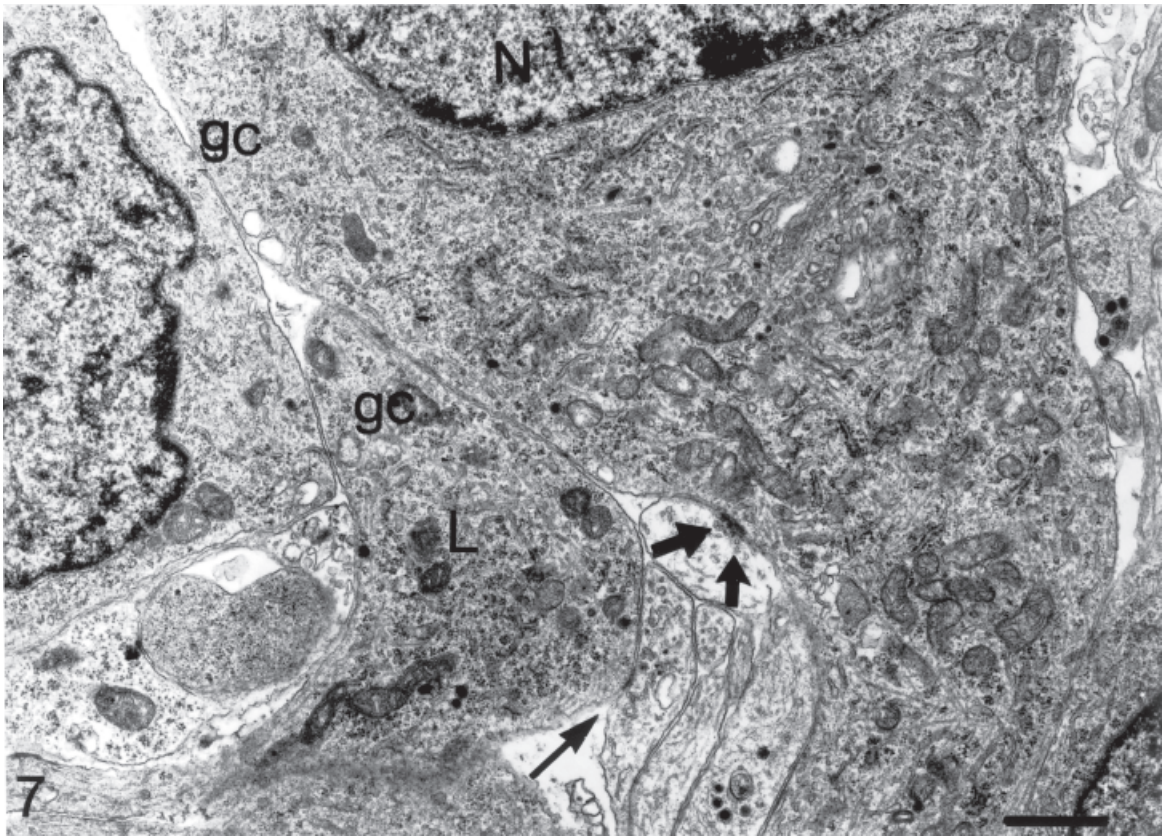
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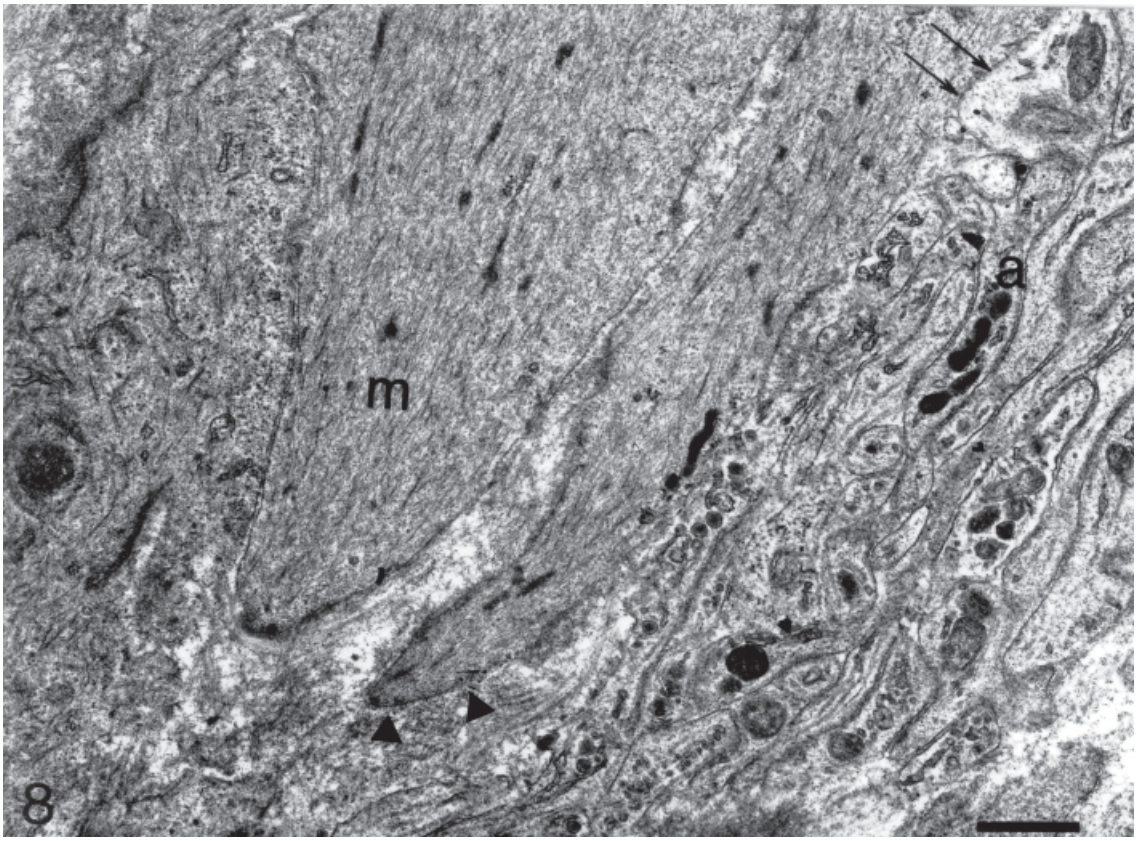
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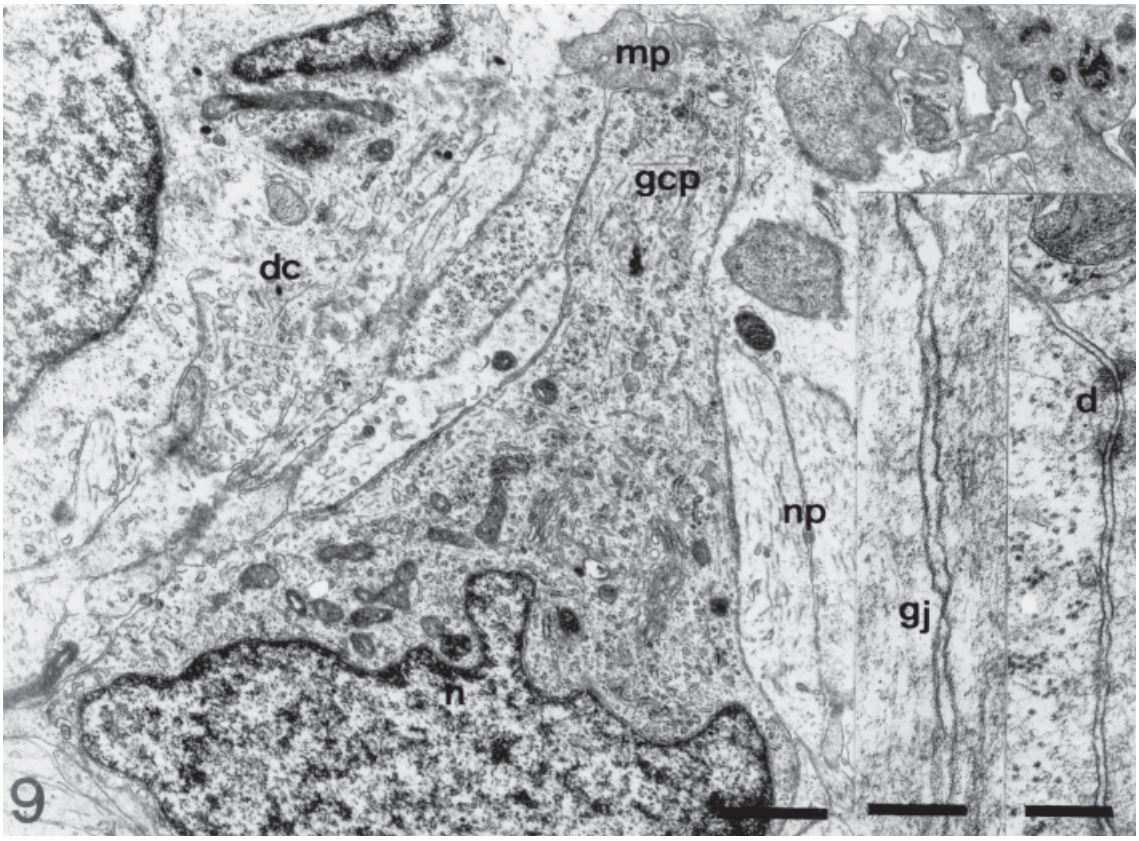
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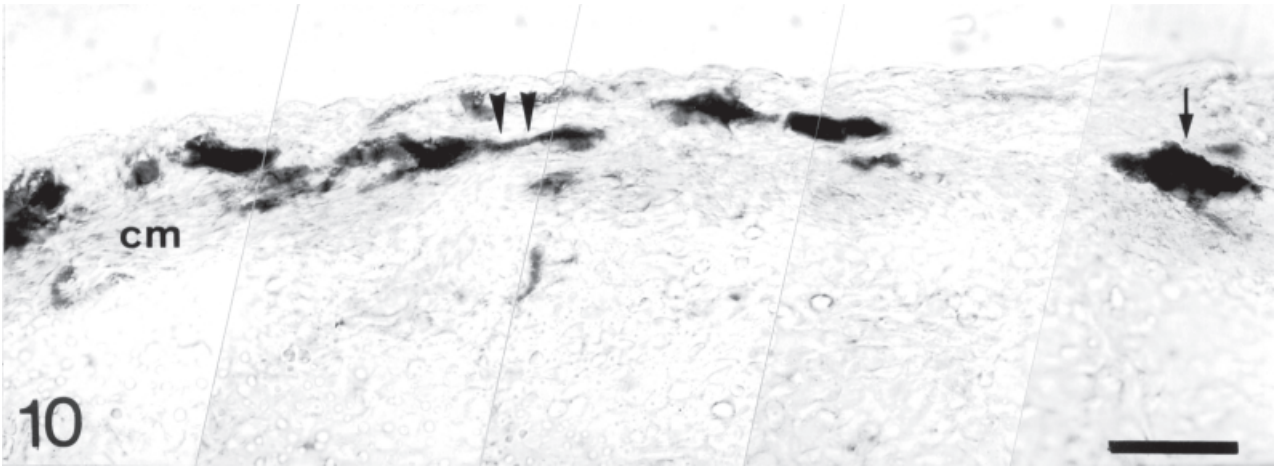


Figure 10. Cross-section of paraffin-embedded human embryonic small intestine at the week 10 of gestation, immunolabelled for neuron-specific enolase. Aggregates of enteric neurons (arrows) are situated on the outer surface of the circular muscle layer (cm). Arrowheads indicate the internodal segments. Bar: 50 μm.

dial ganglion cells, although their different origin cannot be excluded. The embryonic origin of these early-appearing cells in the human fetal intestine must be further investigated. It can be concluded, however, that the two populations of NN cells that appear before week 10 of gestation are among the first differentiated cells in the human fetal gut wall. They appear together with the primordial ganglion cells, long before the formation of the LM (Fekete et al. 1995). The random distribution of the neuroblasts and the presumptive NN cells at this early stage of development suggest that the neurons and the NN cells appear and develop together during the formation of the MP in the human fetal small intestine. These cells provide the first morphological elements of the ganglionic microenvironment and consequently play their role in the mechanical support, isolation and nutrition for the developing ganglia. In the subsequent development, when an array of enteroglial cells and different classes of ICCs appear around and within the MP, these early-appearing cells might be replaced by cells differentiating later in the intestinal wall.

The appearance, distribution and some histochemical features of NN cells have also been studied by means of S-100 protein and GFAP-immunocytochemistry between weeks 10 and 17 of gestation. In addition, double-labelling immunocytochemistry using antibody raised against the constitutive isoform of nitric oxide synthase (bNOS) in combination with an S-100 protein antibody has been applied to investigate the morphological relations between the NN cells and the nitrergic neurons in the developing gut wall. Light microscopic immunocytochemical techniques also revealed the two distinct types of NN cells of a glial phenotype and/or glial origin association with the developing MP (Fekete et al. 1997). They appear together around week 10 of gestation, but their distributions already differ considerably by around week 14 of gestation. Single cells with GFAP immunoreactivity are clustered to one side of the MP in the vicinity of the LM layer (Figs. 15, 19 and 20), while the cells with S-100 protein immunoreactivity are widely distributed in the intestinal wall and frequently form multilayered cellular networks both in the MP and in the submucous plexuses (Figs. 15, 17 and 18). On the basis of these results and other data (Ferri et al. 1982; Jessen and Mirsky 1983; Kobayashi et al. 1989), it can be concluded that two main populations of glial cells, one expressing S-100 protein,

Figure 6. Smooth muscle cells (Mc) are in close contact with nerve cell bodies (gc) in the gut wall of a 10-week-old human fetus. Bar: 1 μm.

Figure 7. Ganglion cells (gc) and axosomatic synapses (arrows) in the myenteric plexus of an 18-week-old human fetus. L: lysosome. Bar: 0.5 μm.

Figure 8. Close (arrow) and distant (arrowhead) neuromuscular contacts in the jejunum of an 18-week-old human fetus. m: muscle cell. Bar: 0.5 μm.

Figure 9. An electron micrograph showing extensive connections between the ganglion cell (gcp) and a muscle cell process (mp) from the circular muscle layer in the small intestine of a 14-week-old human fetus. Bar: 0.5 μm. The inserts show gap junction (gj) and adherent junction (d) between circular smooth muscle myoblast and nerve cell membranes (np). Bar: 0.2 μm.

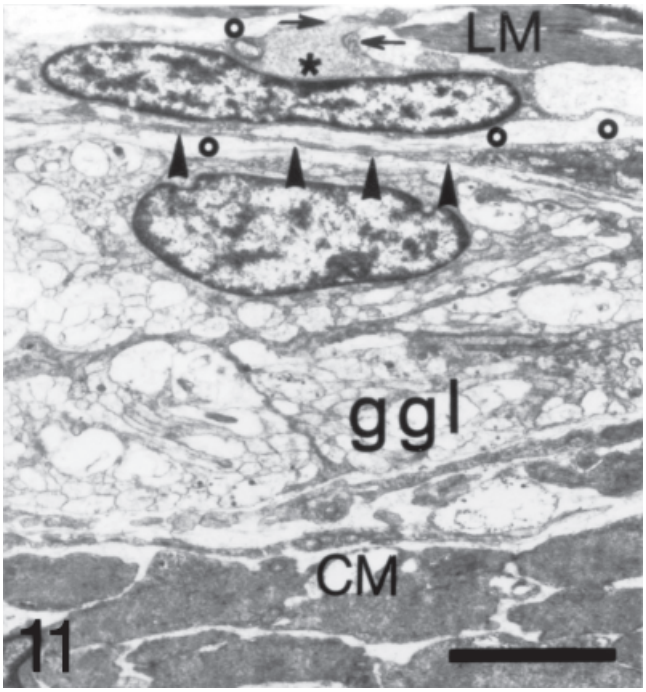


Figure 11. Extended thin processes (arrowheads) of non-neuronal cells penetrating into the ganglia (ggl) in the small intestine of the 17-week-old human fetus. A spindle-shaped cell (asterix) outside the capsule sends a process (arrows) to the longitudinal muscle layer (LM). Both fibroblast-like and spindle-shaped cells in a collagen setting (circles). CM: circular muscle layer. Bar: 1 μm.

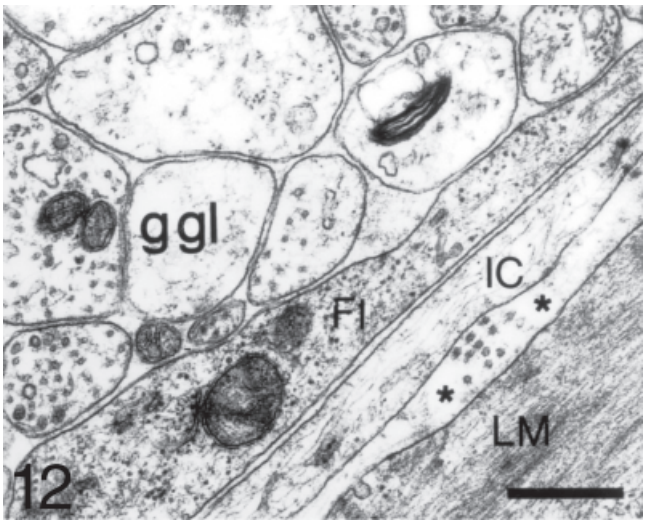


Figure 12. Overlapping processes of different NN cells between the longitudinal smooth muscle layer (LM) and the myenteric ganglia (ggl). Asterisks indicate the collagen-filled space between non-neuronal and smooth muscle cells. Bar: 0.3 μm.

and the other GFAP immunopositivity, appear within and/or around the MP at the very beginning of ganglionic morphogenesis. Thus, these cells provide the first morphological elements of the ganglionic microenvironment, necessary for the ganglionic morphogenesis. As glial cells, they might be involved in providing the mechanical support, isolation and nutrition for the developing ganglia. In the subsequent development or in the mature ganglia, enteroglial cells are mostly found within the ganglia. In other locations, they might be replaced by cells differentiating later in the intestinal wall.

Nitrergic neurons in the developing human small intestine

Nitric oxide (NO) research has expanded explosively in the past 10 years. There is growing evidence indicating the presence of abnormalities in the NO system in several pathological conditions (Stark and Szurszewski 1993). Changes in the density of NO-producing nerves, altered NO production and changes in smooth muscle cell sensitivity to endogenous NO could play roles in the pathophysiology of several neuromuscular disorders of the intestine. Alterations in muscular and neuronal NO production in the intestine result in sustained non-peristaltic contractions such as those

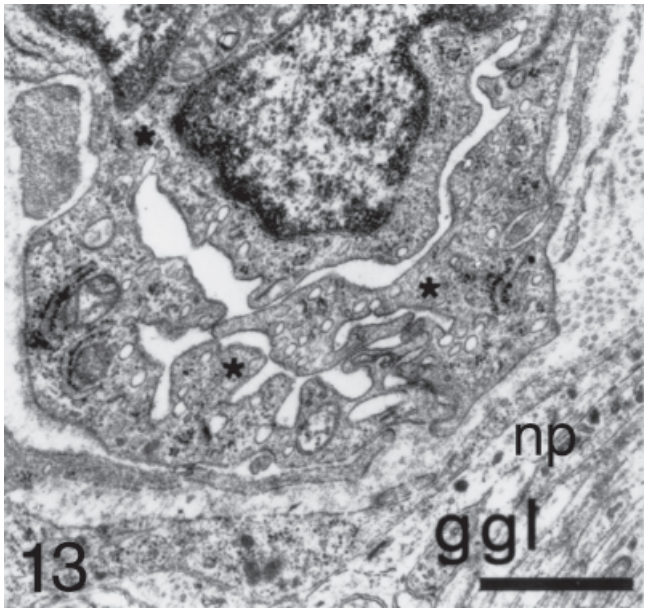


Figure 13. Cellular network (asterisks) formed by NN cells around the myenteric plexus (MP) at week 17 of gestation. Bar: 1 μm.

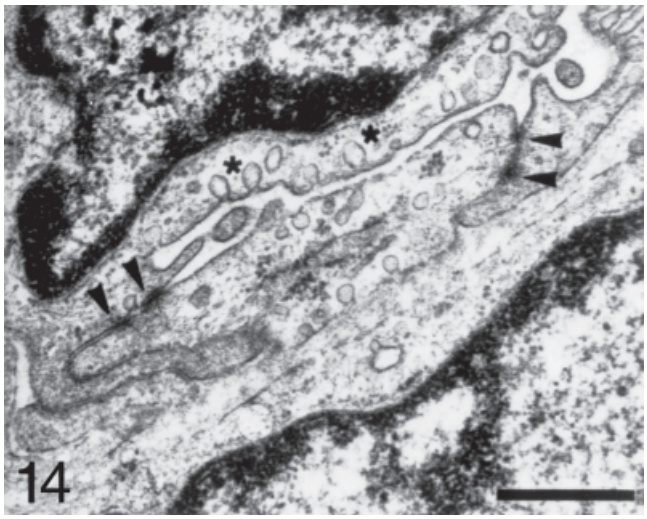


Figure 14. Higher magnification of the cellular network formed around the myenteric ganglia. Arrows point to adherent junctions between non-neuronal cells; asterisks indicate caveolae. Bar: 0.5 μm.

observed in patients with Hirschprung's disease. Hypertrophic pyloric stenosis in infants has also been found to be associated with a defect of NOS in the ENS (Vanderwinden et al. 1996). Hirschprung's disease and pyloric stenosis are both regarded as developmental malformations of the ENS, which has led to the implication that NO plays some role in the normal development of the ENS. Although the nature of this role is not clear, the trophic effect of NO has recently been reported (Ogura et al. 1996). Pharmacological and physiological studies have provided evidence that NO is involved in NANC relaxation of the gastrointestinal tract (Bult et al. 1990; Li and Rand 1990; Costa et al. 1991; Moncada and Higgs 1993; Sanders and Ward 1992). Following electrical field stimulation of NANC nerves, the inhibition of relaxation by L-arginine analogues, which are known to be inhibitors of NO synthesis, has led several authors to conclude that NO is involved in inhibitory NANC neurotransmission in the ENS of various mammalian species (Stark and Szuszewski 1993). There is pharmacological and physiological evidence that, in the normal human jejunum, exogenous NO evokes membrane hyperpolarization and inhibits mechanical activity in the CM (Stark et al. 1993). The presence of constitutive NOS in peripheral gut neurons was first identified through the use of immunohistochemical techniques (Bredt et al. 1990). Substantial activity was observed in the cell bodies and nerve fibres within the MP of the rat duodenum. Others have also demonstrated nitrergic nerves in different animal species (Costa et al. 1991; Llewellyn-Smith et al. 1992; Schmidt et al. 1992; Timmermans et al. 1993). The enzymic reduction of nitroblue tetrazolium to

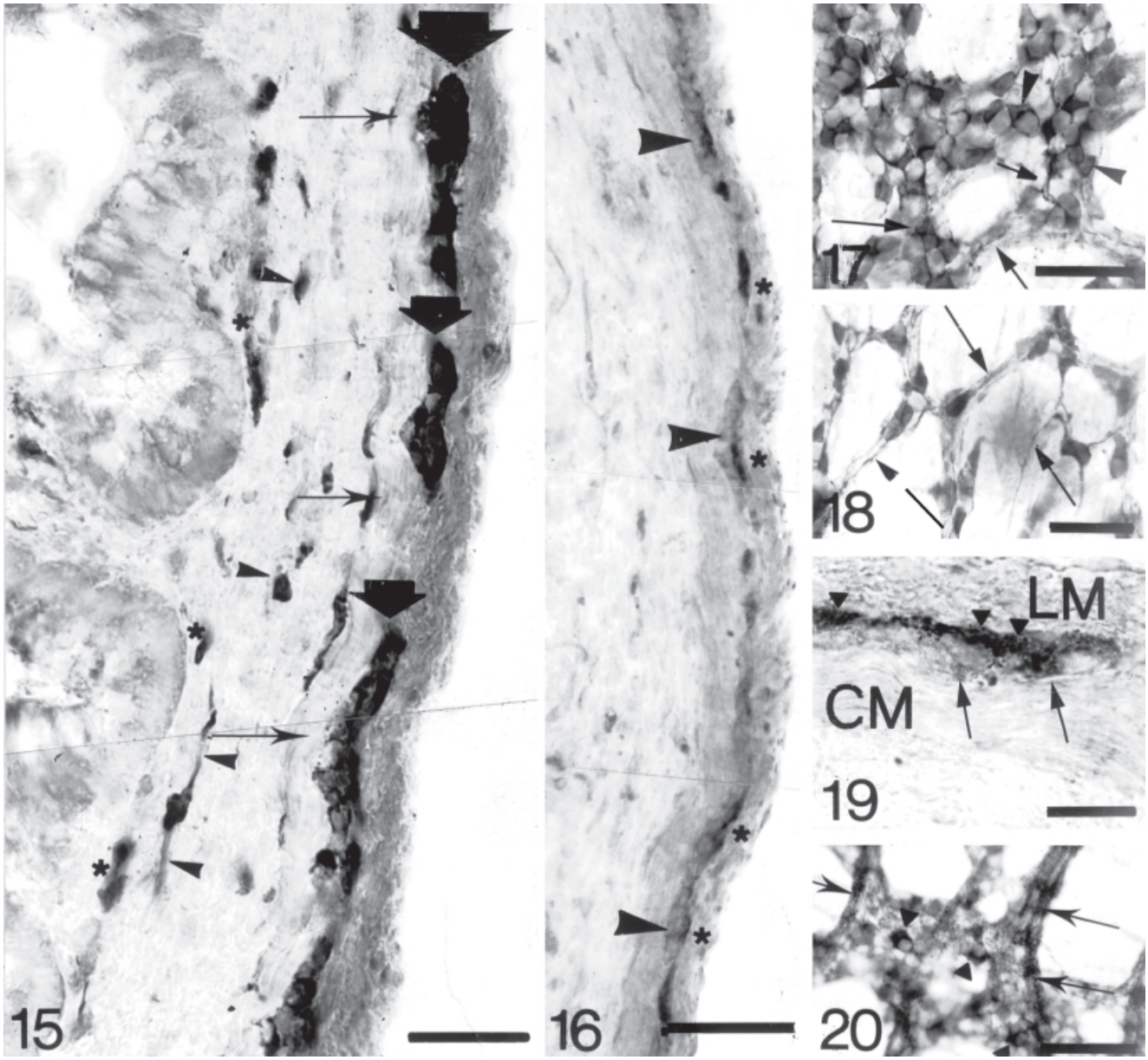


Figure 15. Immunostaining for S-100 protein in cryostat sections of human fetal small intestine in week 17 of gestation. Cells with S-100 immunoreactivity are distributed along the myenteric plexus (large arrowheads), within the circular muscle layer (arrows) and in the inner (asteriks) and outer (arrowheads) submucosal plexuses. Bar: 72 μm.

Figure 16. Immunostaining for GFAP in a cryostat section of the human fetal small intestine in week 17 of gestation. Immunoreactivity (arrowheads) was restricted to the side of the myenteric ganglia adjacent to the longitudinal muscle layer (asterisks). Bar: 44 μm.

Figure 17. Immunostaining for S-100 protein of a whole-mount preparation of human fetal small intestine in week 17 of gestation. Cells with S-100 protein immunoreactivity (arrowheads) interconnect and form a network in the plane of the myenteric ganglia (arrows). Bar: 35 μm.

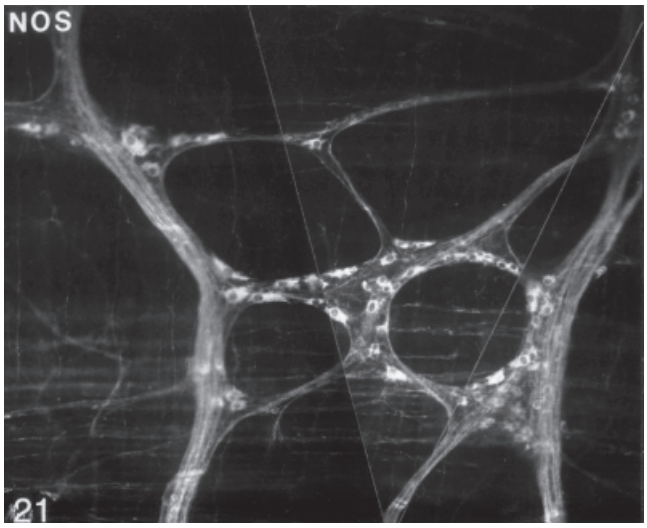
Figure 18. Immunostaining for S-100 protein of whole-mount preparations of human fetal small intestine in week 17 of gestation. Cells on the surface of the submucosal plexus interconnect (arrows) and form a network. Bar: 30 μm.

Figure 19. A higher magnification photograph of GFAP immunostaining in a cryostat section. CM: circular muscle layer; LM: longitudinal muscle layer; arrows point to the myenteric ganglia, and arrowheads to the GFAP reaction. Bar: 45 μm.

Figure 20. Immunostaining for GFAP in whole-mount preparations of human fetal small intestine in week 17 of gestation. Immunopositive cells (arrowheads) appear to be separated from each other and from the immunopositive plexuses (arrows). Bar: 40 μm.

a water-insoluble dye in an NADPH-dependent manner has long been the basis of a specific neuronal tissue histochemical marker. This NADPH-diaphorase activity has been demonstrated to colocalize with NOS in the brain and peripheral neuronal tissue (Dawson et al. 1991; Hope et al. 1991; Timmermans et al. 1993). Sequential application of NOS immunocytochemistry and NADPHd histochemistry in the human fetal small intestine revealed that the distributions of neuronal NOS and NADPHd are identical (Figs. 21 and 22). NADPH-diaphorase staining has therefore provided a useful tool with which to investigate the distribution of the nitrergic neurons during prenatal morphogenesis (Timmermans et al. 1993). In all gut regions investigated, i.e. the stomach and the small and large intestines, most of the NOS-immunoreactive (NOS-IR) or NADPHd-positive neurons are located in the MP. In the small intestine and colon, the outer submucous plexus accounts for only about 2% of the NOS-containing neurons, whereas the remaining intrinsic enteric neurons capable of synthetizing NO are located in the MP. Comparable to the situation in the guinea-pig intestine (Young et al. 1992), significant regional differences in the density of NOS-containing neurons can be observed between the small intestine and the colon. The latter contains about 2.0 to 2.5-fold more in both the outer submucous plexus and the MP. The near-absence of nitrergic neurons from the inner submucous plexus indicates that, in larger mammals, including humans, the two submucous plexuses have distinct functions (Timmermans et al. 1990; Crowe et al. 1992). NOS-IR varicose and non-varicose fibres are found within the three ganglionic nerve networks of the small intestine. As in the guinea-pig small intestine (Costa et al. 1991), a considerable number may be derived from submucous or myenteric interneurons. The dense NOS-IR fibre pattern in the outer muscle layer of the fundic and antral parts, in the pyloric sphincter and in the intestinal CM layer provides strong morphological support for a mediator role of NO in NANC inhibition of the gastrointestinal smooth muscle. The majority of these nerve fibres are presumably processes of motor neuronal cell bodies located in the MP. In the small intestine of the 2-month-old female, it has been established that NOS-IR nerve fibres progress from the outer submucous plexus into the most luminal part of the CM layer. Therefore, it seems likely that, in man as in the pig (Timmermans et al. 1993), this plexus is involved in NANC-mediated relaxation of gastrointestinal smooth muscle. The hypothesis that the outer submucous plexus is involved in the inhibitory innervation of the circular smooth muscle layer in the human gut can be correlated with an earlier report concerning the adult human colon, where VIP-ergic projections were described as running from the outer submucous plexus to the CM (Domoto et al. 1990), and with a study of the canine colon which provided electrophysiological data on submucous motor neurons innervating the CM (Sanders and Smith 1986). Detailed analysis of the inhibitory junctional potentials in the human jejunum seems to indicate that NO is not involved in the initial rapid hyperpolarization, but rather mediates the second part of the electrical response (Stark et al. 1993). Therefore, it is unlikely that NO is the only substance involved in nerve-mediated inhibition in the human ENS.

Other putative NANC neurotransmitters in the gut are ATP (Burnstock et al. 1970; Burnstock 1982) and VIP (Goyal et al. 1980; Furness and Costa 1982; Makhlouf 1982).



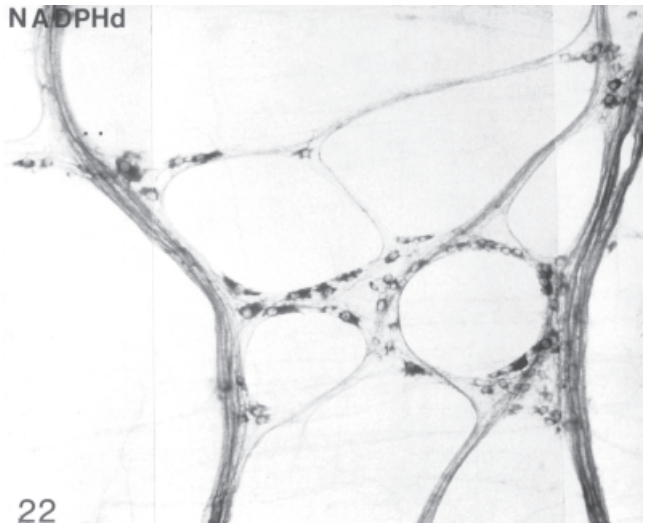


Figure 21 and 22. Paired micrographs of the myenteric plexus of the developing human small intestine (2 months postnatally) after sequential application of NOS immunocytochemistry (Fig. 21) and NADPHd histochemistry (Fig. 22). The distributions of neuronal NOS and NADPHd are identical. Bar: 40 μm.

Colocalization of VIP and NOS has been detected in nerve fibers of the taenia in the guinea-pig caecum (Furness et al. 1992). In man, *in situ* hybridization has demonstrated VIP-mRNA in the ganglion cells of the upper gut at 9 weeks of gestation, and VIP-IR enteric neurons have been observed from 15 to 18 weeks of gestation (Li and Rand 1990; Facer et al. 1992). Although immunocytochemistry in this study has revealed an extensive VIP-ergic fibre pattern in the ganglionic nerve networks and muscle layers, immuno-reactive nerve cell bodies have been seen only infrequently, which might result in part result from the lack of colchicine pretreatment or from a too low endogenous level. These neurons are distinct from NOS-IR neurons. On the other hand, the presence of VIP-ergic baskets around the myenteric NOS-IR neuronal aggregates in the small and large intestines of 21 to 26-week-old fetuses may argue in favour of the presynaptic action of VIP (Huizinga et al. 1992). The absence of NOS immunoreactivity and NADPHd activity from the smooth muscle fibres of the examined regions of the human gut does not lend support to the view (Grider et al. 1992) that NOS is produced in the gastrointestinal smooth muscle cells in response to putative inhibitory neurotransmitter VIP.

In the canine colon (Ward et al. 1994) and guinea-pig small and large intestines (Costa et al. 1991; Furness et al. 1992; Young et al. 1992), the great majority of NOS-IR neurons have been morphologically classified as Dogiel type-I neurons. Eighteen to 26 weeks after conception, NOS-IR or NADPHd-positive nerve cell bodies in whole-mounts of the developing human small intestine all have a round to oval appearance. The processes issuing from these cell bodies have only been traced over short distances, not allowing an unambiguous morphological identification. At a later stage of development, NOS-IR neurons with short processes display the Dogiel type-I morphology (Stach 1980), whereas others have both short and long processes. The particular distribution of NOS-IR fibres within the ganglionic plexuses and the muscle layers, together with the variety of cell sizes and shapes of the NOS-IR perikarya, are indicative of the existence of distinct NOS-containing neuron populations within the developing human ENS. As in the guinea-pig ENS (Costa et al. 1991), these neurons may act either as interneurons or as motorneurons.

NN cells such as the interstitial cells of Cajal (ICCs) have recently been postulated as another source of NO in relation to NANC-mediated relaxation (Daniel and Berezin 1992). VIP-ergic fibers have been found to innervate the ICCs (Berezin et al. 1990). The prospect of these fibres being capable of stimulating NO production in these or other NN cells, thereby indirectly exerting a relaxing effect on the smooth musculature, is an intriguing one, since it would mean that alternative mechanisms for inhibitory transmission exist in the ENS. The small NOS-IR and NADPHd-positive cells within the ganglia and nerve strands of the MP and in the CM layer are difficult to classify as enteric neurons. The overall morphological appearance, their extremely small size, and the fact that they do not stain for general neuronal markers such as PGP 9.5 and NSE favour an NN origin. They may be distinct types of ICCs (Thuneberg 1989) or macrophage-like cells (Mikkelsen et al. 1985, 1988) or glial cells (Aoki et al. 1991; Simmons and Murphy 1992).

Recent publications indicate the importance of the interrelationship between nitrergic neurons and astrocytes (Agullo et al. 1995; Murphy et al. 1995). There is evidence suggesting that glial cells serve as a potential reservoir of L-arginine (Kerwin and Heller 1994) and also as a main target of NO (Murphy et al. 1995). A double-labelling immunocytochemical method revealed a close morphological relation between NOS-IR fibres and S-100 protein-IR cells in the developing human fetal small intestine (Fekete et al. 1997). NOS-IR varicosities on the glial cell surfaces might function as communication sites between glial cells and nitrergic neurons. Although the nature of this communication is not clear, the glial cells closely related to nitrergic nerves might directly benefit from the trophic effect of NO, which has recently been reported (Ogura et al. 1996).

General conclusions from developmental studies and directions for further research

Evidence has been provided that the microenvironment from which neurons originate is critical in determining the ultimate pathway of differentiation. The sequential appearance of the various types of enteric neurons and NN cells suggests a changing microenvironment within the intestinal wall at the different stages of embryonic development. On the basis of ultrastructural and immunocytochemical studies, it was concluded that the period between week 10 and 18 of gestation is of paramount importance for both the morphological and functional maturation of the ENS. Several important questions regarding the nature of the signals that control the morphogenesis of the ENS remain unanswered. The development of methods to isolate relatively pure populations of ENS progenitors from the mammalian and avian fetal gut has already resulted several groups studing the mechanism of action of purified neurotrophic factors. Such studies will answer important questions regarding the normal and pathologic development of the ENS, and hence may promote the link between theory and clinical practice.

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