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Sequential pattern of nerve-muscle contacts in the small intestine of developing human fetus. An ultrastructural and immunohistochemical study

E. Fekete[1], I. Benedeczky[1], J.-P. Timmermans[2], B.A. Resch[3] and D.W. Scheuermann[2]

[1]Department of Zoology and Cell Biology, Jozsef Attila University, Szeged, Hungary,

[2]Laboratory of Cell Biology and Histology, University of Antwerp (RUCA), Belgium and

[3]Department of Obstetrics and Gynaecology, Albert-Szent-Györgyi Medical University, Szeged, Hungary

Summary. The developing enteric nervous system of the human fetus has been studied by means of electron microscopy and neuron-specific enolase immunocytochemistry between the 10th and 26th week of gestation, with special reference to the development of nerve-muscle contacts. In the 10th week of gestation the circular muscle layer is formed, followed by the appearance of a primitive myenteric plexus, and the longitudinal smooth muscle layer in the 12th week of gestation. Adherens-type junctions between the smooth muscle cells and the elements of the myenteric plexus, interdigitation of nerve and muscle processes, and also contacts without any morphological specialization are frequent until the 18th week, when the mechanical points of attachment are relocated from the circular to the longitudinal muscle layer. By this time the developing myenteric plexus becomes ensheathed by non-neuronal cells, disrupting the direct contacts between smooth muscle cells and the primary strands of the myenteric plexus. The possible functional significance of these changing nerve-muscle contacts is discussed in the present paper.

Key words: Changing pattern, Nerve muscle contacts, Myenteric plexus, Human fetus

Introduction

The innervation of the mammalian gut is relatively developed at birth (Gershon et al., 1981; Furness and Costa, 1987), so in order to study the development of the enteric nervous system the use of fetal tissue is required. Several ultrastructural studies have already been performed on the developing myenteric plexus (MP) in small laboratory animals (Gershon et al., 1981; Furness and Costa, 1987). Generally the MP is strictly isolated from the musculature by fibroblast-like interstitial cells already in the early embryonic period (Gershon et al., 1981), yet nerve-muscle contacts have frequently been detected in certain developmental stages (Daikoku et al., 1975; Gabella, 1981). Komuro (1988) has described direct contacts between the neuropil and ganglion cells of MP and smooth muscle cells of the longitudinal layer in adult rat. A transient connection between the developing MP and the circular smooth muscle cells was observed in the embryonic guinea pig small intestine (Gershon et al. 1981). It has been revealed that muscle cell processes penetrate into the capsular layer composed of non-neuronal cells and that they make close contacts with the neural elements of the MP during the late embryonic development in the chicken midgut (Boros and Fekete, 1993). Since the results for small laboratory animals cannot simply be extrapolated to other mammalian species, including humans (Hoyle and Burnstock, 1989; Scheuermann et al., 1989; Timmermans et al., 1992; Ibba-Manneschi et al., 1995), detailed investigation of human fetal material is necessary to deepend our understanding of the functional development of the human gastrointestinal tract. By means of neuron-specific enolase (NSE) immunocytochemistry the present paper reports the changing topographic relation between the elements of MP and the muscle coat in the human fetal small intestine from the 10th through the 26th week of gestation. In addition, the ultrastructural changes of MP muscle contacts are described.

Offprint requests to: Dr. Eva Fekete, Ph.D., department of Zoology and Cell Biology, Jozsef Attila University, H-6722 Szeged, Egyetem u. 2. POBox 659, Hungary

Materials and methods

Tissues

Intestinal segments were taken from autopsies on human fetuses (10-26 weeks of gestation) obtained

after legally approved or spontaneous abortions. The crown-heel length was used to assign gestation age. Three fetuses of all ages were used for each examination. The experiments were approved by the local ethical committee and performed in accordance with the ethical standards laid down in the declaration of Helsinki.

NSE immunohistochemistry

Depending on the embryonic age studied, paraffin sections or whole-mounts were used for immunohistochemistry. Either small pieces of small intestine were fixed overnight in a modified Zamboni fixative (Scheuermann et al., 1987), dehydrated and embedded in paraffin, or segments of the small intestine were ligated and distended with the same fixative. Both sections and whole-mount preparations were processed for NSE immunocytochemistry. They were incubated in the primary antiserum for 48 hours at room temperature. The primary antiserum was raised against NSE in rabbits (Dakopatts, diluted at 1:400 in PBS). The immunoreactivity was visualized using the biotin-streptavidin bridge system. Subsequently, a biotinylated goat anti-rabbit (Dakopatts, diluted 1:100) and a streptavidin-biotinylated-horseradish peroxidase complex were applied (Amersham, diluted 1:100). As a substrate for the peroxidase reaction, 4-Cl-naphtol was used (Scheuermann et al., 1987).

Electron microscopy

Pieces of ileum were fixed in 2% paraformaldehyde, 2% glutaraldehyde, 0.5% acrolein and 0.5% dimethylsulphoxide in 0.1M cacodylate buffer (pH 7.4) overnight at 4 °C. After washes in the same buffer, the samples were postfixed in 2% OsO_4 at 4 °C for 2 h. Samples were dehydrated through graded ethanol solutions, and stained en bloc with saturated uranyl acetate in 75% ethanol for 30 min. Small samples were embedded in Durcupan ACM resin (Fluka). Ultrathin sections were cut on a Reichert ultramicrotome and contrasted with lead citrate according to Reynolds (1963). Sections were examined with a Philips CM 10 electron microscope.

Results

NSE immunocytochemistry

Prior to the appearance of cells recognizable as neurons at the 8th week of gestation, the embryonic gut consisted of mesenchyme lined by epithelia on the luminal side. Demarcation of layers within the mesenchyme first became apparent around the 10th week of gestation, when myoblasts formed the circular layer of muscle (Fig. 1). Immunocytochemistry applied to paraffin sections clearly localized aggregates of enteric neurons on their outer surface (Fig. 1). The NSE-positive nerve strands deeply penetrated and sometimes even traversed the circular muscle layer. At 14 weeks of gestation, the MP was firmly attached to the circular smooth muscle cells, whereas muscles strips of the longitudinal layer could easily be removed, thereby enabling visualization of the MP (Fig. 2). Strips of circular muscle with the adhering MP could be separated from the submucosal layer, where a dense array of NSE-positive fibres could be seen (Fig. 2). The mechanical points of attachment between the muscularis externa and the MP were relocated by the 18th week of gestation, when strips of the circular muscle layer could be peeled off from the longitudinal layer, to which the MP was now adhered (Fig. 3). At 26 weeks of gestation a well-developed MP could be recognized with NSE immunocytochemistry. The primary, secondary and tertiary plexus could already be distinguished. Some of the fine fibres entered the smooth muscle layers (Fig. 4).

Electron microscopy

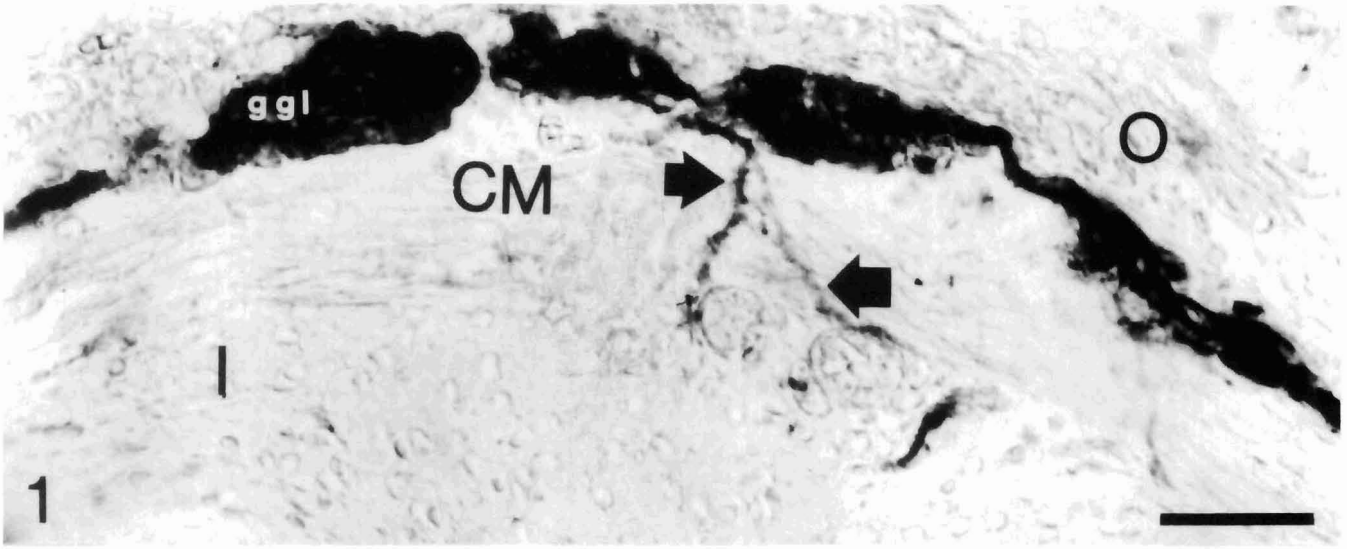
Ultrastructural investigation of human fetal intestine revealed a changing pattern of MP/muscularis externa contacts between weeks 10 and 26 of gestation (Figs. 5-10). Although both the circular muscle layer and the MP appeared by the 10th week of gestation they could not be recognized as clearly separated entities at this stage. The border of the MP was unsharp, the smooth muscle myoblast often possessed long thick processes which deeply invaginated into the perikarya (Fig. 5). The developing axons and dendrites frequently established close contacts with muscle processes (Fig. 6). In the 14-

Fig. 1. Cross section of paraffin-embedded human embryonic small intestine at the 10th week of gestation, immunolabelled for NSE. The circular muscle layer (CM) demarcates the inner (I) and outer (O) part of the mesenchyme. Aggregates of enteric neurons (ggl) are on the outer surface of the muscle. Nerve strands traverse through the circular muscle layer (arrows). Bar: 95 μm.

Fig. 2. Whole-mount preparation from the small intestine of a 14-week-old human fetus processed for NSE immunocytochemistry. The myenteric plexus (MP) lies over the circular muscle layer. Stripping off the circular muscle layer, the underlying submucosal plexus is exposed (SM). Bar: 85 μm.

Fig. 3. Whole-mount preparation from the intestine of an 18-week-old human fetus processed for NSE-immunocytochemistry. The myenteric plexus (MP) attaches to the surface of the longitudinal muscle layer. Strips of the circular muscle layer (CM) are stripped off. Bar: 65 μm.

Fig. 4. Whole-mount preparation from the intestine of a 26-week-old human fetus immunolabelled for NSE showing the myenteric plexus (MP) sandwiched between the longitudinal muscle layer (LM) and the circular muscle layer. The secondary and tertiary branches of the myenteric plexus (arrows) enter into both muscle layers. Asterisks: remnants of fibres of the circular muscle layer not peeled off during the whole-mount preparation. Bar: 50 μm.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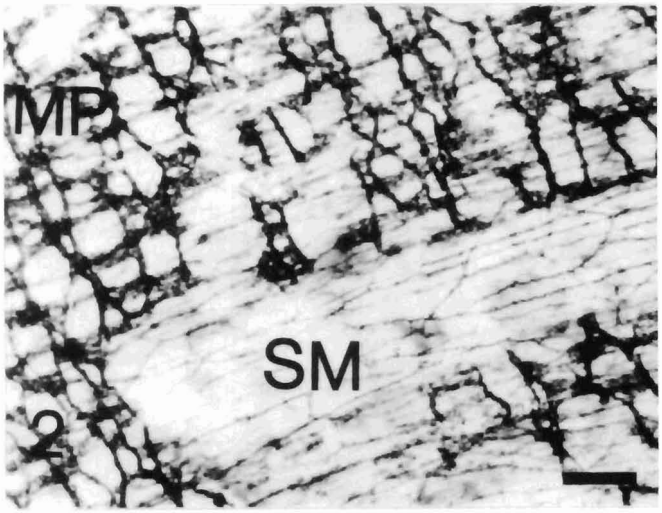
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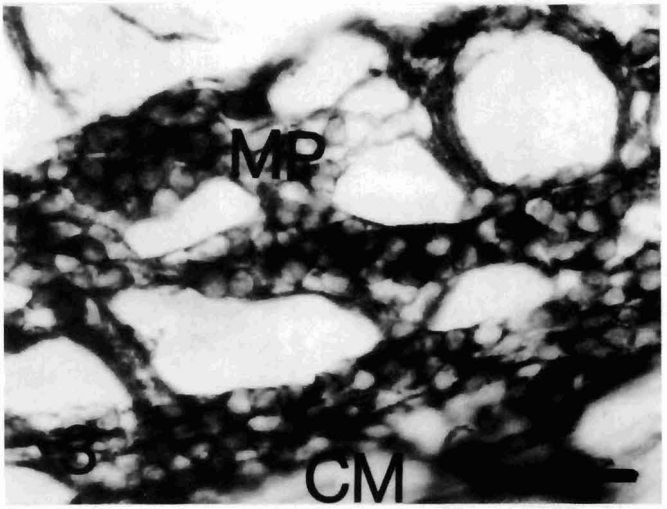
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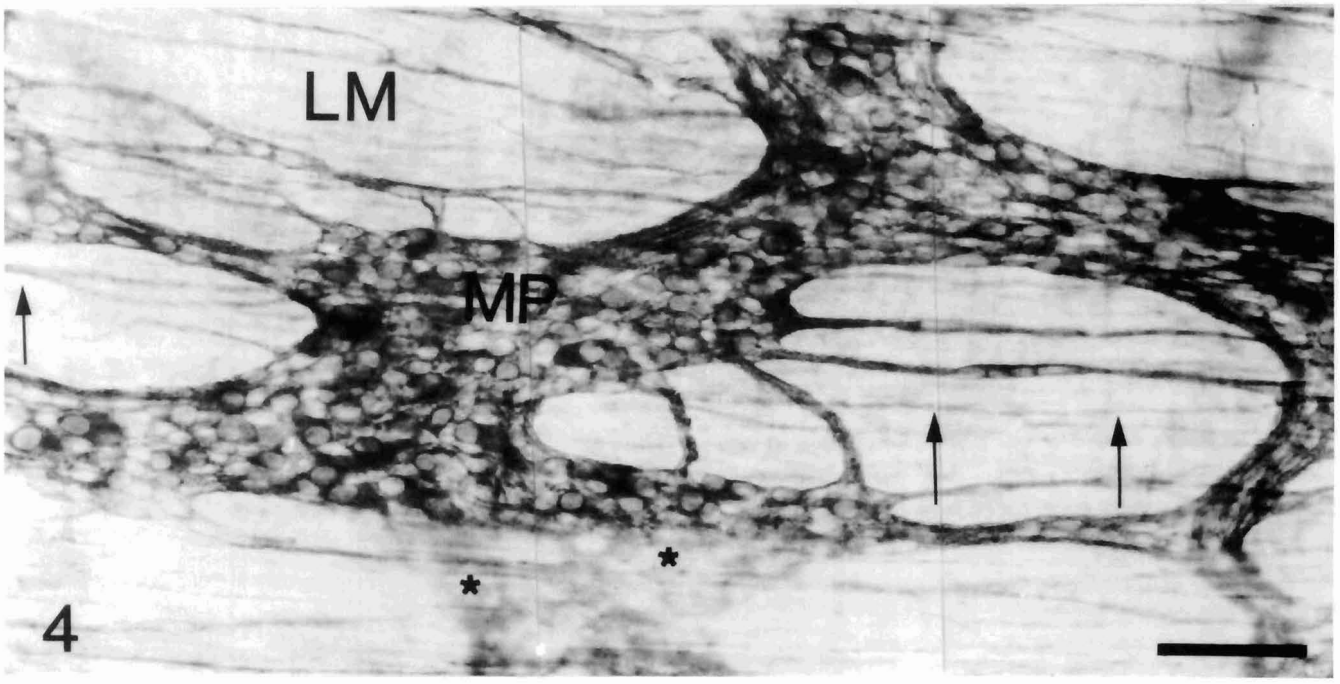
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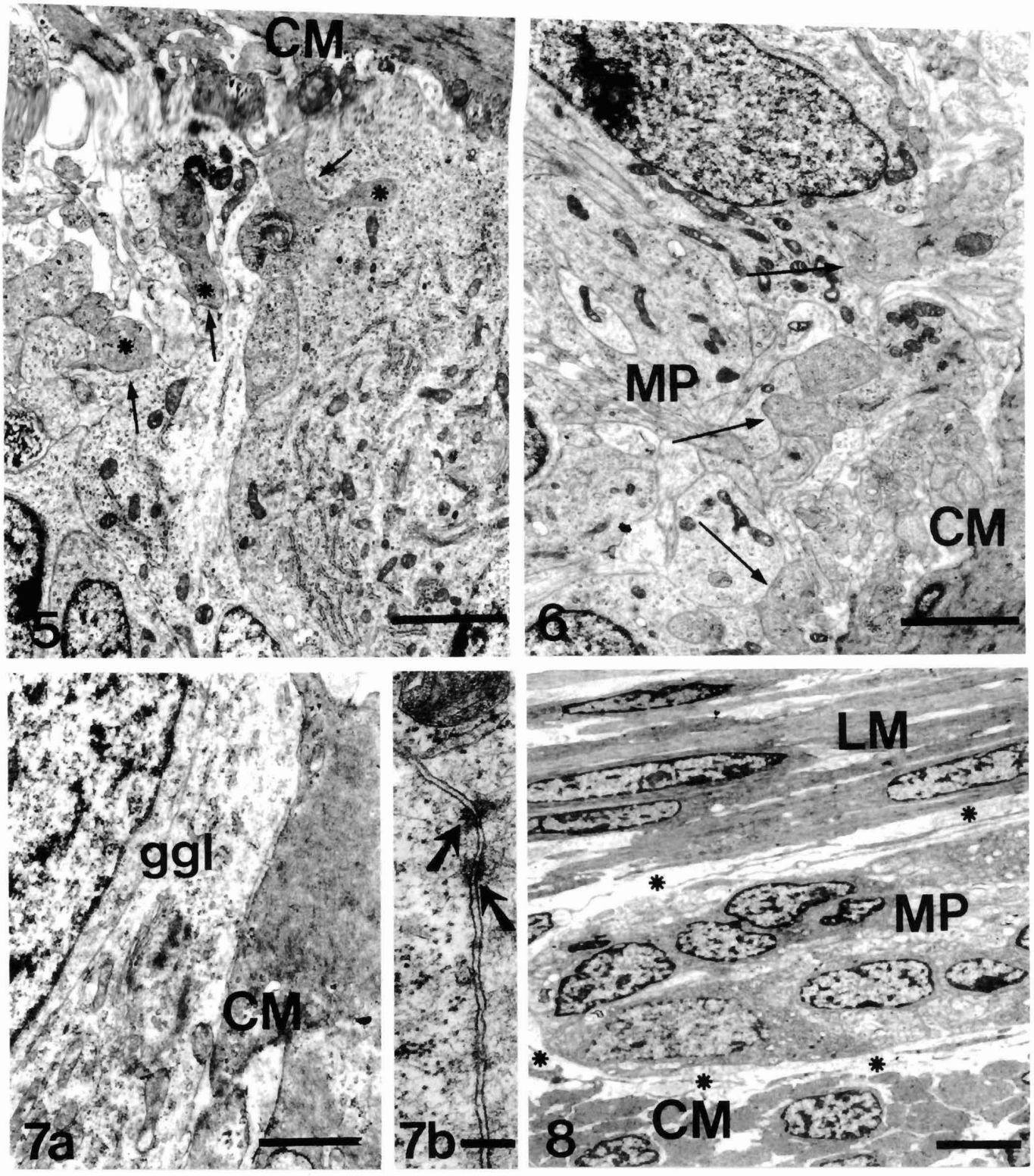


Fig. 5. An electron micrograph showing interdigitation of myoblast processes (asterisks) from the inner circular layer (CM) of the muscle coat in the small intestine of a 10-week-old human fetus. Arrows indicate close contacts between the muscle and nerve elements. Bar: 0.5 μm.

Fig. 6. Close contact (arrows) between myoblast from the circular muscle coat (CM) and a growing nerve strand of myenteric plexus (MP) in the small intestine of a 10-week-old human fetus. Bar: 0.5 μm.

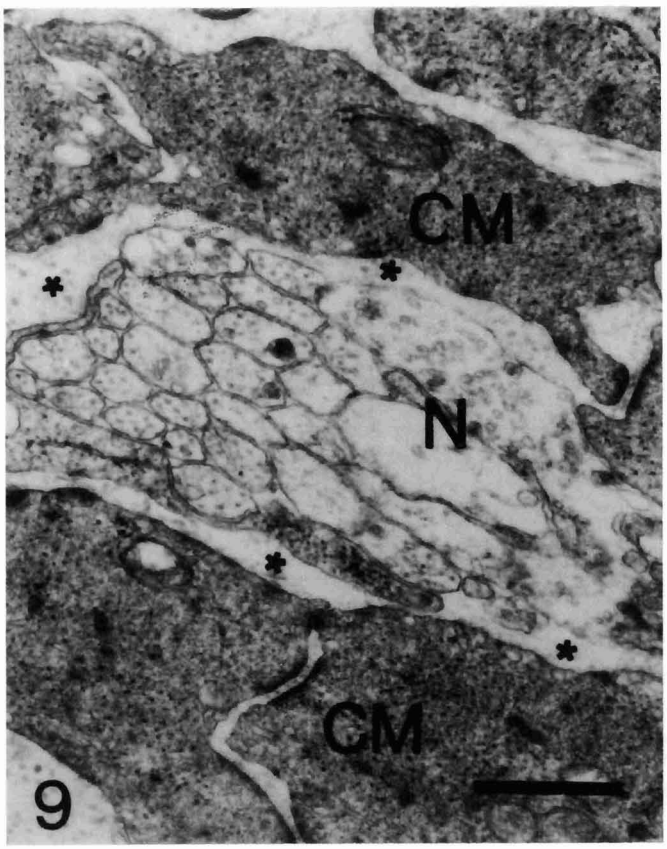
Fig. 7. a. An electron micrograph shows extensive connections between the myenteric ganglion (ggl) and the circular muscle layer (CM) in the 14th week of gestation. Bar: 0.75 μm. **b.** Adherents junction between circular smooth muscle myoblast and nerve cell membranes (arrows). Bar: 0.25 μm.

Fig. 8. Low magnification electron micrograph shows that the myenteric plexus (MP) is separated from both circular (CM) and longitudinal (LM) muscle layers by a collagen-filled space (asterisks) in the small intestine of the 26-week-old human fetus. Bar: 1.5 μm.

week-old human embryo the circular smooth muscle myoblast frequently connected through adherents junctions with nerve cell membranes (Fig. 7). At 26 weeks of gestation the developing collagen-filled space isolated the MP from the circular and longitudinal muscle layer (Fig. 8). Although nerve branches penetrating into the circular muscle layer were always embedded in collagen (Fig. 9), the nerves within the longitudinal layer frequently established close contacts with the membranes of muscle cells (Fig. 10).

Discussion

In paraffin sections and whole-mount of human fetal small intestine processed for NSE immunocytochemistry a changing pattern of MP-muscle contacts can be revealed between the 10th and the 26th week of gestation. 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 layer. Through out the 18th week of gestation the CM provides the mechanical surface for the developing MP, which attaches firmly to this muscle layer. Around the 18th week of gestation, the mechanical points of attachment shift from the circular to the longitudinal muscle layer. Concomitantly, the MP adheres to the longitudinal muscle 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 (Moscona, 1976). The changing pattern of nerve-muscle contact is also reflected at the ultrastructural level. In the 10-week-old human fetal intestine the elements of MP and CM are intermingled, they cannot be recognized as clearly separate entities. 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 et al. (1981) in the developing guinea-pig small intestine. In the absence of a basal lamina, the elements of MP can communicate with smooth muscle cells, providing an opportunity for nerve-muscle trophic interaction. To date, there is no evidence that enteric neurons are dependent on neurotrophic support for their survival during development (Saffrey and Burnstock, 1994). Never-



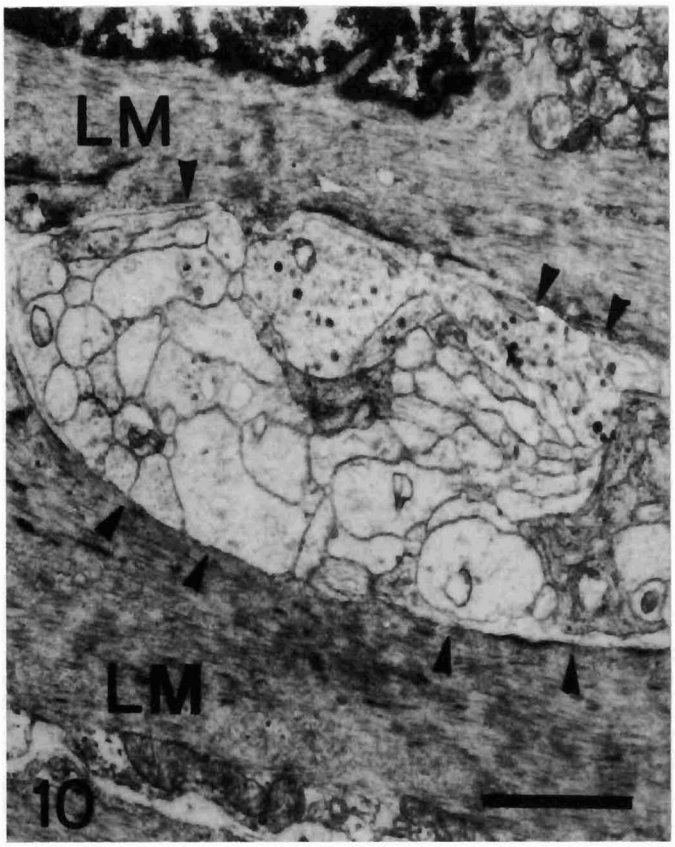


Fig. 9. A nerve branch (N) within the circular muscle layer (CM) separated from the muscle cells by collagen-filled spaces (asterisks) in the small intestine of the 26-week-old human fetus. Bar: 0.4 μm.

Fig. 10. An electron micrograph of a nerve bundle penetrating into the longitudinal muscle layer (LM). Some of the nerve fibres establish close appositions with the muscle cells (arrowheads) in the small intestine of the 26-week-old human fetus. Bar: 0.4 μm.

theless, there is evidence that the numbers of neurons and the density of the myenteric plexus, as well as average neuronal size, are greater in areas where the smooth muscle layers are thicker (Gabella and Trigg, 1984). Intestinal smooth muscle has also been found to promote directional outgrowth from sympathetic ganglion explants in co-culture (Rawdon and Dockray, 1984). 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 circular muscle layer and the MP. From the 18th week of gestation onwards, the developing MP becomes more and more ensheathed by different kinds of non-neuronal cells, collagen-filled spaces and basal lamina. Meanwhile, new contacts are formed between the MP and the longitudinal muscle 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 the 26th week of gestation. It is very probable that during development, changing micro-environmental conditions cause these sequential patterns of nerve-muscle contacts. These changes may be essential for morphogenesis or, more generally, for the functional maturation of the external muscle coat and the interposed MP.

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