Volume 46(1-2):17-23, 2002
Acta Biologica Szegediensis
http://www.sci.u-szeged.hu/ABS

ARTICLE

Individual distribution and colocalization of nitric oxide synthase with vasoactive intestinal polypeptide and neuropeptide Y in the developing human fetal small intestine

Mária Bagyánszki, Viktor Román, Mónika Krecsmarik, Béla A Resch[1], Éva Fekete*

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

ABSTRACT The appearance, the individual distribution and the pattern of colocalization of nitric oxide synthase (NOS-immunoreactivity, NOSi), vasoactive intestinal peptide (VIP-immunoreactivity, VIPi) and neuropeptide Y (NPY-immunoreactivity, NPYi) immunoreactivity were examined in the developing human fetal small intestine at weeks 12 and 18 of gestation. Neurons expressing VIPi, NPYi and NOSi were observed in the small intestine of the 12-week-old human fetuses and from this age on a gradual increase in the immunoreactivities appeared until week 18 of gestation when a dense network of immunopositive fibres and cell bodies were observed both in the submucous (SmP) and in the myenteric plexuses (MP). The double-labelling immunocytochemistry showed different pattern of the overlapping immunoreactive structures within the myenteric and submucous plexuses. The cellular colocalization of VIPi and NOSi in submucous ganglia were revealed around week 12 of gestation while in the myenteric ganglia cells with overlapping immunoreactivity appeared around week 18. A limited cellular colocalization of NPYi and NOSi were noticed before week 18, and NOSi neurons in the MP of the 12-week-old fetuses were preferentially innervated by NPYi varicosities. These results suggest that VIP, NPY and NO may exert a cooperative action in human fetal gastrointestinal motility.

Acta Biol Szeged 46(1-2):17-23 (2002)

KEY WORDS

human intestine
immunocytochemistry
NOS
VIP
NPY

Detailed studies about the ontogeny of peptide-containing neurons in the human enteric nervous system (ENS) were performed (Bryant et al. 1982; Larsson et al. 1987) and it is known that neuronal elements expressing VIP or NPY appear during early fetal development. Many studies have suggested that VIP containing neurons are important mediators of the descending inhibitory phase of peristalsis (Larsson et al. 1976; Hata et al.1990; Furness et al. 1992; Grider and Jin 1993; Allesher and Daniel 1994). Other evidence indicates that the mediation of descending inhibition is not limited to VIP and suggests that nitric oxide (NO) may also serve as an inhibitory neurotransmitter (Giorgio et al. 1994; Yuan et al. 1995; Young et al. 1995). Recent evidence indicates that NPY is present in inhibitory motor neurons of guinea pig mye/nteric ganglia (Uemura et al. 1995). The wide distribution of neurons with NPYi suggests that just like VIP, NPY is also involved as an inhibitory neurotransmitter in all regions of the fetal gut. There are two populations of NPY immunoreactive nerve fibres in the gut. One major population of fibres is of intrinsic origin, distributed in all layers of the gut (Sundler et al. 1993). A minor population of NPY fibres, with extrinsic origin, is identical with the adrenergic NPY fibres distributed mainly around blood vessels and in the myenteric ganglia (Lundberg et al. 1982; Browning et al. 1999). Colocalization studies showing that NO is produced in enteric neurons that express neuropeptides including VIP (Costa et al. 1992) and NPY (Kirchgessner et al. 1994) make VIP, NPY and NO viable candidates as parallel neurotransmitters.

The early appearance of neuronal elements with VIP, NPY and NOS immunoreactivity is well documented in human fetal intestine (Bryant et al. 1982; Chayvialle et al. 1983; Larsson et al. 1987; Timmermans et al. 1994). Since most of these investigations were performed on sections, they do not give informations about the distribution of nerve fibres and cell bodies within the different compartments of the human fetal ENS. Studies as concerns the possible co-localization of VIPi with NOSi or NPYi with NOSi during the development of the human ENS do not exist. The first aim of these investigations was therefore to determine the individual distribution of VIPi, NPYi and NOSi within the human fetal ENS using wholemount preparations of the intestinal wall. The second aim was to investigate in what extent these

Accepted March 27, 2002
*Corresponding author. E-mail: efekete@bio.u-szeged.hu

substances are colocalized in the developing human fetal small intestine. The third aim was to examine changes in the distribution of the overlapping immunoreactive neuronal elements between weeks 12 and 18 of gestation.

The present study provides the first evidence on the simultaneous appearance of NOSi with VIPi and NOSi with NPYi in different neuronal populations in the developing human fetal small intestine.

Materials and Methods

Tissues

Intestinal segments of human fetuses (weeks 12 and 18 of gestation) were obtained immediately 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 performed in accordance with the declaration of the Medical World Federation proclaimed in Helsinki in 1964.

Immunocytochemistry

Segments of small intestine were ligated and distended using a modified Zamboni fixative (Scheuermann et al. 1987) and fixed overnight at 4°C. After washing with phosphate buffered saline (PBS) at pH 7.4, tissue pieces were used for wholemount preparations and cryosections. Double-labelling immunofluorescence histochemistry was performed applying simultaneous incubation using a monoclonal mouse anti-NOS antiserum (Affinity, Menasha, USA; final dilution 1:200) in combination with either a rabbit anti-VIP (Affinity; final dilution 1:200) or a rabbit anti-NPY antiserum (Amersham; final dilution 1:500) overnight at room temperature. After incubation with primary antisera wholemounts were washed with PBS and exposed for 6 h to a mixture of species-specific secondary antibodies conjugated to FITC (Jackson, Baltimore, USA; final dilution 1:100), Cy3 (Sigma, Budapest, Hungary; final dilution 1:200) or biotin (Amersham, Backinghamshire, England; final dilution 1:100). After incubation in secondary antiserum, tissues were washed and incubated for overnight with streptavidin-Texas Red (Amersham; final dilution 1:100) or streptavidin-biotinylated horseradish peroxidase (Amersham; final dilution 1:100). Specimens were mounted in PBS-buffered glycerol. Preparations were viewed and photographed with a Zeiss Axioscope 2 MOT fluorescent microscope equipped with a Zeiss AxioCam digital camera.

Results

The individual distribution and the pattern of coexistence of NOSi, VIPi and NPYi were examined in cryosections and in wholemount preparations of human fetal small intestine between weeks 12 and 18 of gestation. NOS, VIP and NPY immunoreactive structures were observed in each part of the small intestine at week 12 of gestation (Figs. 1 and 2). From this age on there was a gradual increase in the intensity of immunofluorescence, and also in the number and the diversity of the immunopositive nerve structures until week 18 of gestation, when dense networks of immunopositive fibres and large number of immunopositive cell bodies were seen (Fig. 3). Most of the peptide-containing intraganglionic fibres expressing either VIP or NPY were distributed within the myenteric plexus (MP) as varicose fibres and freqently formed baskets around non-immunopositive cell bodies (Figs. 1D, F and 2D, 2F). NOSi fibres within the MP showed a dense, less structured pattern of fibres with rare varicosities which never formed baskets around non-immunopositive cell bodies (Fig. 2A). Both peptidergic and nitrergic neuronal structures were less densely distributed in the submucosus plexus (SmP), although varicose fibres and cell bodies with VIPi are frequently seen at 12 week of gestation (Figs. 1C and E). NOS was also expressed in the submucous fibres from week 12 on (Fig. 1B). Double-labelling experiments revealed a limited coexistence of VIPi with NOSi and NPYi with NOSi depending on the embryonic age examined. NOSi with VIPi or NOSi with NPYi were never expressed together in the varicosities of the embryonic ENS. The dense varicosities of peptidergic fibres expressed only VIP or NPY, whereas the scarce varicosities containing NOS never expressed peptides. Three populations of immunoreactive cell bodies were revealed after double-labelling with VIP and NOS: a population of myenteric neurons co-expressing VIP with NOS (Figs. 2C and D) and another two populations containing VIP or NOS alone (Figs. 2C, D and Figs. 3E, F). A population of nerve cell bodies in submucous ganglia were observed that express VIPi and NOSi together from week 12 of gestation. Nerve cells after double-labelling with NPY and NOS also fall into three classes: a population containing NPY with NOS (Figs. 2A and B) and another two populations expressing NPY or NOS alone (Figs. 2E and F). The number of cell bodies expressing NPY alone or NPY and NOS together were limited to one or two cells per ganglia.

Discussion

Our present investigations covered the localization of three substances: NO, VIP and NPY, known to occur in neuronal elements of human adult intestine. VIP-containing nerve fibres occur abundantly in all layers of the human gut and VIP nerve cell bodies are regularly observed in intramural ganglia (Bishop et al. 1982). The presence of the regulatory peptides and NO has been proved in the early human fetal intestine (Bryant et al. 1982; Timmermans et al. 1994). Most of the peptides studied throughout the fetal development had an adult-like distribution pattern by weeks 20 and 24 of gestation (Bloom et al. 1983). As well as having a regulatory role in intestinal motility, relaxing smooth muscle and mediating inhibitory non-adrenergic non-cholinergic

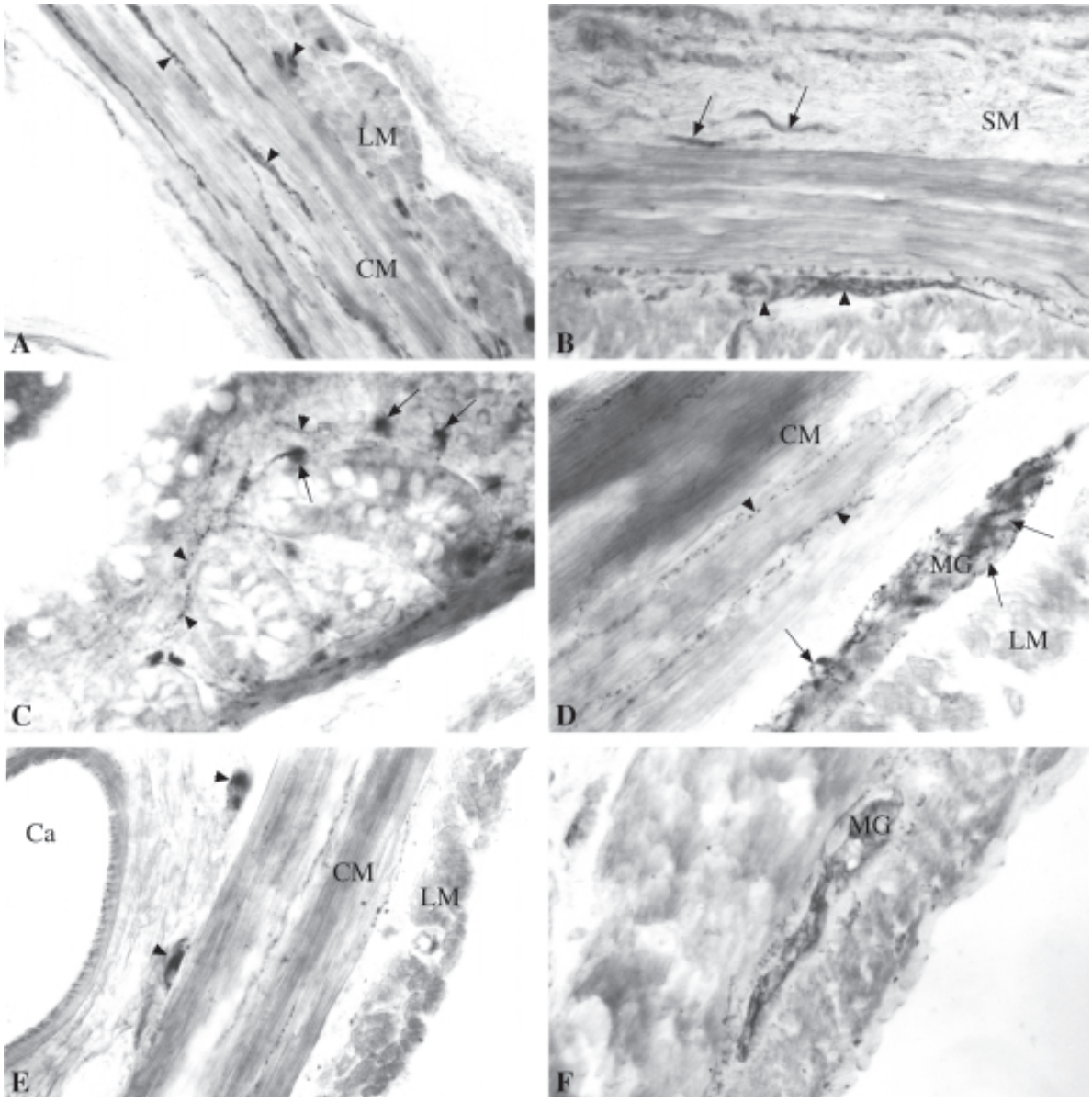

Figure 1. Light photomicrographs of cross-sections of the human fetal gut wall after NOS (A, B), VIP (C, D, E) and NPY (F) immunocytochemistry on week 12 of gestation.
A. NOS-immunopositive neuronal elements (arrowheads) are abundant both in the longitudinal (LM) and circular muscle (CM) layers. x200
B. NOS-immunopositive neuronal elements are present in myenteric ganglia (arrowheads). Smooth individual fibres in the submucous layer (SM) with NOS-immunopositivity appear (arrows). x400
C. VIP-immunoreactive varicose fibres (arrowheads) and cell bodies (arrows) around glandular epithelia in the submucosal layer on week 12 of gestation. x650
D. VIP-immunoreactive varicose fibres form baskets around non-immunoreactive cell bodies (arrows) within a myenteric ganglion. Dense array of VIP-immunoreactive fibres (arroheads) was also revealed in the circular muscle layer (CM). LM: longitudinal muscle layer, MG: myenteric ganglion. x400
E. VIP-immunoreactive cell bodies (arrowheads) in the submucous plexus. CM: circular muscle layer, LM: longitudinal muscle layer, Ca: capillary, x200
F. NPY-immunopositive neuronal elements in a myenteric ganglion (MG). x400

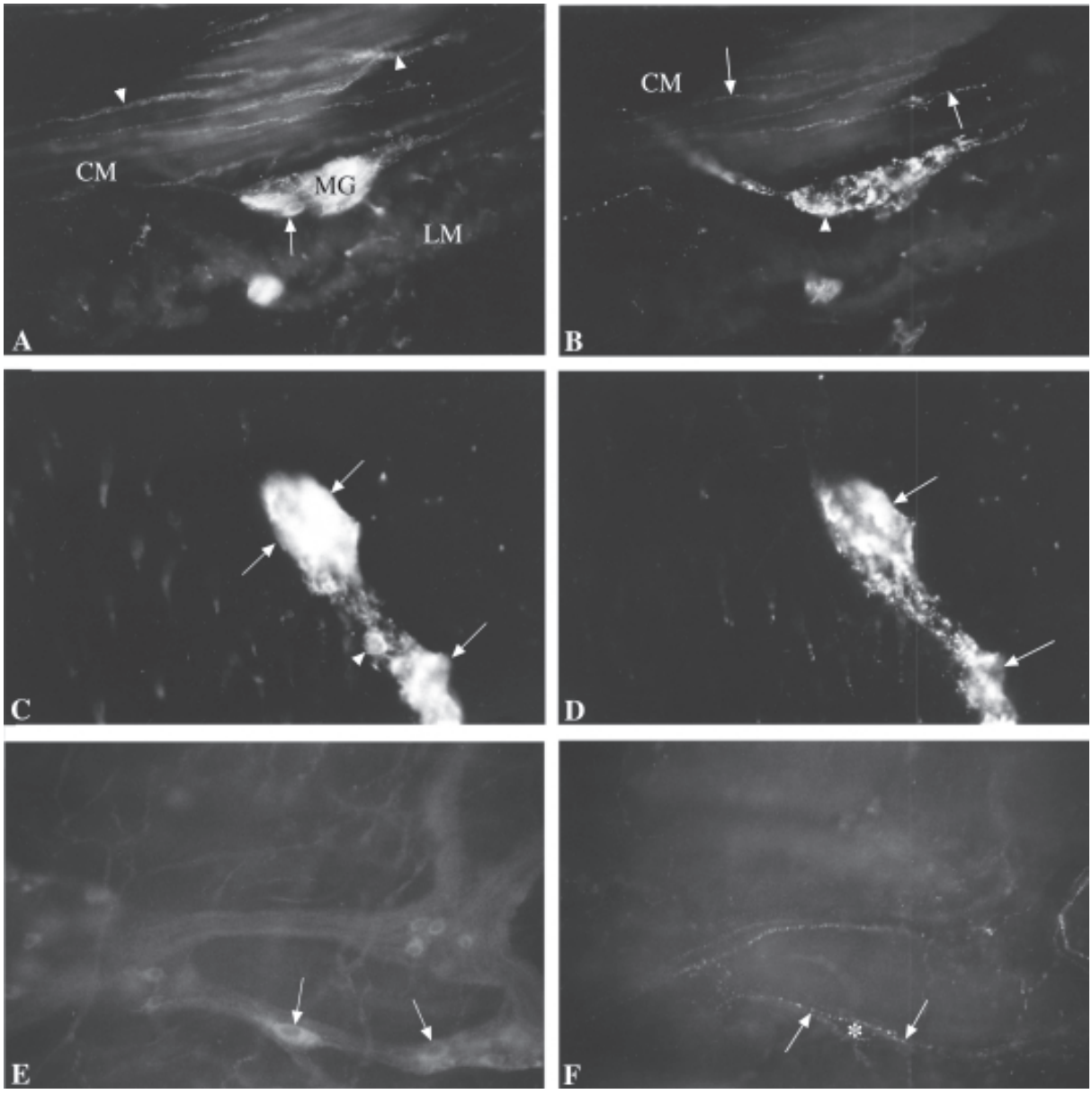

Figure 2. Fluorescent micrographs of cross-sections (A-D) and wholemounts of the human fetal gut wall after double-labelling immunocytochemistry for NOS, NPY (A, B, E, F) and NOS, VIP (C, D) on week 12 of gestation.
A. NOS-immunopositive neurons (arrow) in a myenteric ganglion (MG) and dense arrays of fluorescent fibres (arrowheads) in the musculature. CM: circular muscle layer, LM: longitudinal muscle layer, x400
B. NPY-immunoreactive varicose fibres (arrows) in the circular muscle layer (CM) and in a myenteric ganglion. Some of the ganglion cells coexpress NOS and NPY (arrowhead). The NPY-positive fibres form baskets around non-immunreactive myenteric neurons. x400
C. NOS-immunopositive neuronal elements in a myenteric ganglion. Most of the cells express NOS and VIP together (arrows), while others express only NOS. x650
D. VIP-immunopositive neuronal elements in a myenteric ganglion. Most of the ganglion cells co-express NOS and VIP (arrows). x650
E. NOS-positive neurons in the myenteric plexus (arrows). x200
F. NPY-positive varicose fibres in the myenteric plexus. A nitrergic neuron (asterisk) is surrounded by NPY immunopositive fibres (arrows). 200x

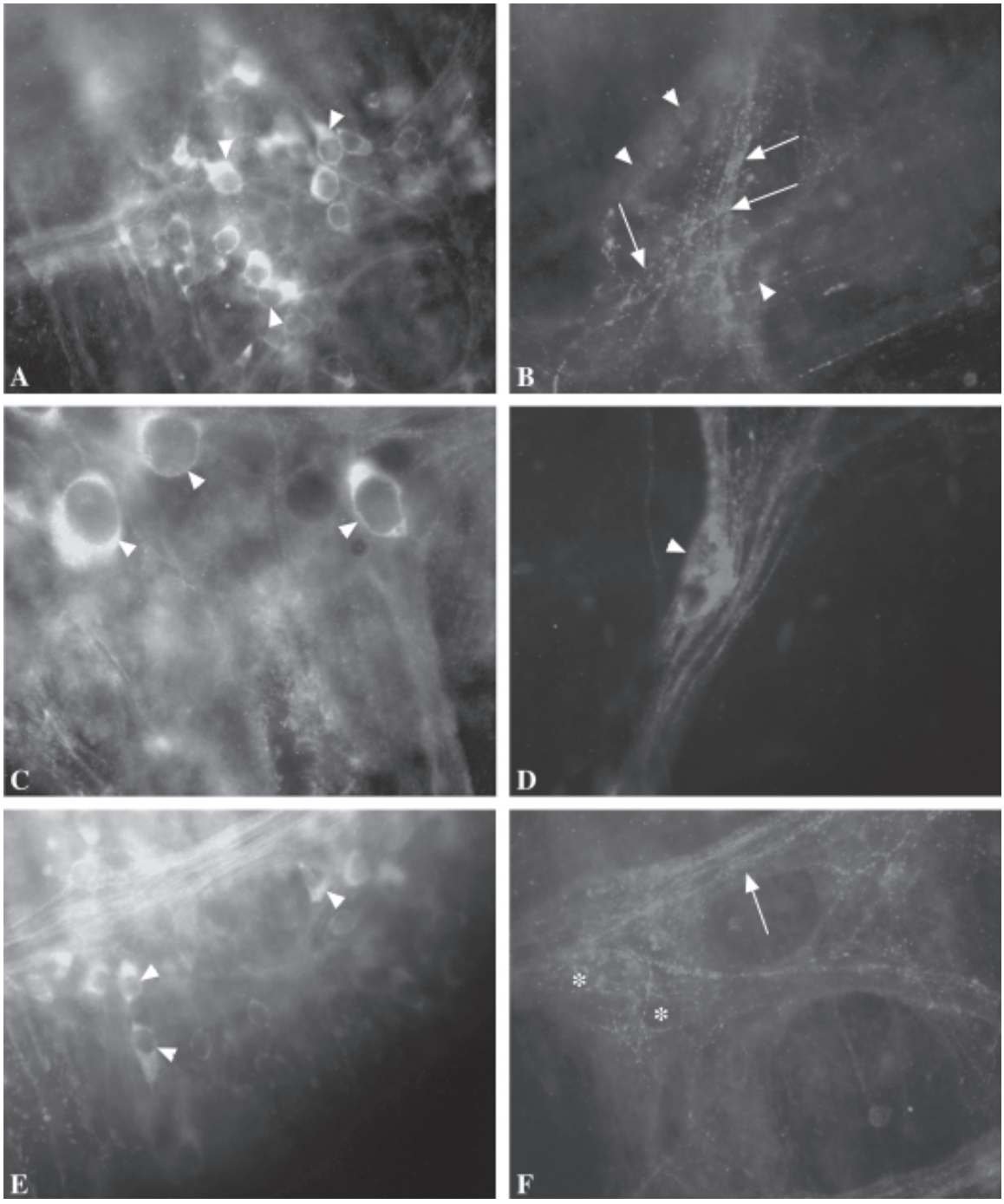

Figure 3. Fluorescent micrographs of wholemount preparations of the human fetal small intestine after single labelling immunocytochemistry for NOS (A, C), VIP (B, D), and double labelling immunocytochemistry for NOS, VIP (E, F) on week 18 of gestation.
A. NOS-immunopositive neurons (arrowheads) in a myenteric ganglion. x400
B. Dense array of VIP-immunopositive varicose fibres (arrows) and cell bodies (arrowheads). x400
C. NOS-immunopositive neuronal cell bodies (arrowheads) in a myenteric ganglion. x1000
D. VIP-immunopositive neuronal cell body (arrowhead) in a myenteric ganglion. The immunoreactivity is unevenly distributed within the perikaryon. x1000
E. NOS-immunopositive neuronal cell bodies (arrowheads) in a myenteric ganglion. x400
F. Dense array of the VIP-immunopositive varicose fibres (arrow) in the myenteric plexus. Varicose fibres frequently form baskets (asterisks) around non-immunoreactive cell bodies. x400

(NANC) neurotransmission (Larsson et al.1976; Furness et al. 1992; Grider and Jin 1993; Allesher and Daniel 1994), the ability to act as growth factors has also been demonstrated both for NO (Ogura et al. 1996) and VIP (Gressens et al. 1993). In order to evaluate the potential interactions of NO, VIP and NPY in the developing human fetal small intestine double-labelling immunocytochemistry was used and simultaneous appearance of NOSi with VIPi and NPYi with NOSi in the different neuronal structures was followed from week 12 of gestation, when immunoreactive neuronal elements were widely distributed in the fetal ENS. While both VIP and NPY were localized mainly to varicose fibres and they frequently formed baskets around cell bodies, the majority of fibres with NOSi were smooth and never formed baskets around cell bodies. The number of cells expressing NOS was higher in the MP than those expressing VIP, while in submucous ganglia the number of VIPi neuronal cell bodies overwhelmed the one or two NOSi cells per ganglia. Due to the high number of cells expressing VIP it can be assumed that most of the fibres displaying VIPi are intrinsic of the fetal gut. Both the peptidergic and the nitrergic neuronal structures were less densely distributed in the SmP. However, varicosities within the SmP with VIPi were not seen before week 18 of gestation, cells expressing VIP and NOS alone or together appeared already at week 12. On the contrary, cellular colocalization of NOSi and NPYi was not revealed within the SmP; however, the scarce NOS immunreactive neurons frequently received NPY immunreactive fiber terminations suggesting a modulatory role for NPY (Cox et al. 1998; Feletou et al. 1998).

The coexistence of both VIPi with NOSi and NPY with NOSi was most pronounced in the 18-week-old fetus, when the pattern of colocalization is similar using either VIP or NPY antibody in combination with NOS antibody. The neurons which simultaneously expressed either VIP and NOS or NPY and NOS fall into five groups, two populations contained NOS and VIP or NOS and NPY together, another populations contained NOS, VIP or NPY alone. The NOS immunreactive neurons which have a dominant NPY innervation might represent a subgroup of NOS neurons. The physiological significance of this pattern of coexistence is still a subject of debates. Due to the early expression of these substances (Bryant et al. 1982; Chayvialle et al. 1983; Larsson et al. 1987; Timmermans et al. 1994), they might have their individual action during the early development, relaxing smooth muscle (Costa et al. 1992; Grider and Jin 1993; Kirchgessner et al. 1994) and acting in NANC neurotransmission. Later in development, when oro-anal peristalsis starts (Grand et al. 1976) and the motility of the gut needs a more sophisticated regulation, a complementary role might be presumed to these substances. NO may mediate the same type of response as VIP or NPY but with a different time course. The different classes of neurons suggest that in some cases NO is the final transmitter but most of the cases it probably serves as a modulator to another NANC neurotransmitter or it has an effect on the regulation of development. The lack of varicosities in submucous fibres expressing VIP or NOS suggests that NANC inhibition is not an important part of the submucous function in the early development of human ENS. The early appearance of cellular colocalization at the same time suggests a regulatory effect of these substances either in neurotransmission or in neuronal differentiation.

In conclusion, the distribution and the pattern of coexistence revealed in our present investigation using double-labelling experiments with NOS and VIP or NOS and NPY antibodies, strongly support the concept that VIP with NOS and NPY with NOS are parallel neurotransmitters in the human fetal small intestine, although the neurotrophic effect of NO and VIP should be a subject of further investigation.

References

Allescher HD, Daniel EE (1994) Role of NO in pyloric, antral and duodenal motility and its interaction with other inhibitory mediators. Dig Dis Sci 39:73S-75S.

Bishop AE, Ferri GL, Proberg L, Bloom SR, Polak JM (1982) Peptidergic nerves. Scand J Gastroenterol 17:43-59.

Bloom SR, Christofides ND, Delamarter J, Buell G, Kawashima E, Polak JM (1983) Diarrhea in VIPoma patients associated with co-secretion of a second active peptide (peptide histidine isoleucine)-explained by a single coding gene. Lancet 2:1163-1165.

Browning KN, Cunningham SM, Duncan L, Timmermans JP, Lees GM (1999) Regional differences in the sympathetic innervation of the guinea pig large intestine by neuropeptide Y- and tyrosine hydroxylase-immunoreactive nerves of divergent extrinsic origin. J Comp Neurol 410:515-530.

Bryant MG, Buchan AMJ, Gregor M, Ghatei MA, Polak JM, Bloom SR (1982) Development of intestinal regulatory peptides in the human fetus. Gastroenterology 83:47-54.

Chayvialle JA, Paulin C, Descos F, Dubois PM (1983) Ontogeny of vasoactive intestinal peptide in the human fetal digestive tract. Regul Pept 5:245-256.

Costa M, Brookes SJH, Waterman SA, Mayo R (1992) Enteric neuronal circuitry and transmitters controlling intestinal motor function. In: Holle GR, Wood JD eds., Advances in the innervation of the gastrointestinal tract, Elsevier Scientific Publisher, London. 115-121.

Cox HM, Tough IR, Ingenhoven N, Beck-Sickinger AG (1998) Structure-activity relationships with neuropeptide Y analogues: a comparison of human Y1-, Y2- and rat Y2-like systems. Regul Pept 75-76:3-8.

Feletou M, Rodiguez M, Beauverger P, Germain M, Imbert J, Dromaint S, Macia C, Bourrienne A, Henlin JM, Nicolas JP, Boutin JA, Galizzi JP, Faucherre JL, Canet E, Duhault J (1998) NPY receptor subtypes involved in the contraction of the proximal colon of the rat. Regul Pept 75-76:221-229.

Furness JB, Bornstein JC, Murphy R, Pompolo S (1992) Roles of peptides in transmission in the enteric nervous system. Trends Neurosci 15:66-71.

Giorgio de R, Parodi JE, Nicholas C, Brecha F, Brunicardi C, Becker JM, Go VLW, Sternini C (1994) Nitric oxide producing neurons in the monkey and human digestive system. J Comp Neurol 342:619-627.

Grand RJ, Watkins JB, Torti FM (1976) Development of the human gastrointestinal tract. Gastroenterology 70:790-810.

Gressens P, Hill JM, Gozes I, Fridkin M, Brenneman DE (1993) Growth factor function of vasoactive intestinal peptide in whole cultured mouse embryos. Nature 362:155-158.

Grider JR, Jin JG (1993) Vasoactive intestinal peptide release and L-citrulline production from isolated ganglia of the myenteric plexus: Evidence for regulation of vasoactive intestinal peptide release by nitric oxide. Neuroscience 54:521-526.

Hata F, Ishi T, Kanada A, Yamano N, Kataoka T, Takeuchi T, Yagasaki O (1990) Essential role of nitric oxide in descending inhibition in the rat proximal colon. Biochem Biophys Res Comm 172:1400-1406.

Kirchgessner AL, Liu MT, Gershon MD (1994) NADPH-diaphorase (nitric oxide synthase) containing nerves in the enteropancreatic innervation. Sources, co-stored neuropeptides and pancreatic function. J Comp Neurol 342:115-130.

Larsson LI, Farhenkrug J, Schaffalitzky de Muckadell O, Sundler F, Hakanson R, Rehfeld JF (1976) Localization of vasoactive intestinal polypeptide (VIP) to central and peripheral neurons. Proc Natl Acad Sci USA 73:3197-3200.

Larsson LT, Helm G, Malmfors G, Sundler F (1987) Ontogeny of peptide-containing neurones in human gut - an immunocytochemical study. Regul Pept 17:243-256.

Lundberg JM, Terenius L, Hokfelt T, Martling CR, Takemoto K, Mutt V, Polak J, Bloo SR, Goldstein M (1982) Neuropeptide Y (NPY) like immunoreactivity in peripheral noradrenergic neurons and effects of NPY on sympathetic function. Acta Physiol Scand 116:477-480.

Ogura T, Nakayama K, Fujisawa H, Esumi H (1996) Neuronal nitric oxide synthase expression in neuronal cell differentiation. Neurosci Lett 204:89-92.

Scheuermann DW, Stach W, De Groodt-Lasseel MHA, Timmermans JP (1987) Calcitonine gene-related peptide in morphologically well-defined type II neurons of the enteric nervous system in the porcine small intestine. Acta Anat 129:325-328.

Sundler F, Bottcher G, Ekblad E, Hakanson R (1993) PP, PYY and NPY occurence and distribution in the periphery. In Colmers WF, Wahlestedt C, eds., The biology of neuropeptide Y and related peptides. 157-196.

Timmermans JP, Barbiers M, Scheuermann DW, Bogers JJ, Adriaensen D, Fekete E, Mayer B, Van Marck EA, De Groodt Lasseel MHA (1994) Nitric oxide synthase immunoreactivity in the enteric nervous system of the developing human digestive tract. Cell Tissue Res 275:235-245.

Uemura S, Pompolo S, Furness JB (1995) Colocalization of neuropeptide Y with other neurochemical markers in the guinea-pig small intestine. Arch Histol Cytol 58:523-536.

Young HM, Furness JB, Powley JM (1995) Analysis of connection between nitric oxide synthase neurons in the myenteric plexus of the guinea-pig small intestine. J Neurocytol 24:257-263.

Yuan SY, Bornstein JC, Furness JB (1995) Pharmacological evidence that nitric oxide may be a retrograde messenger in the enteric nervous system. Br J Pharmacol 114:428-432.