SUMMARY OF THE PH.D. DISSERTATION

DEVELOPMENT OF THE GLUTAMATERGIC NEURONS IN THE HUMAN FETAL ENTERIC NERVOUS SYSTEM

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INTRODUCTION

The enteric nervous system (ENS) is situated between the histological layers along the whole length of the intestine from the oesophagus to the inner anal sphincter, but it can be also found in the pancreas, the wall of the gall bladder and the bile passage. ENS is built up of the complex network of enteric neurons and glial cells.

Among the neurons building up the ENS we distinguish sensory, motor and interneurons, which form their own reflex arches. These reflex arches control the gut-peristalsis, the secretory function of the mucosa, the water and ion transport processes and the blood flow of the intestine.

Each histological layer of the gut wall has its own plexus. Mammalian ENS is built up by two larger ganglionic plexuses: myentericus plexus (MP) and submucosus plexus (SP), and several smaller, not ganglionic plexuses.

Similarly to the rest of the peripheral nervous system, the neurons and glial cells of the ENS arise from neural crest cells. Neurons building up the ENS migrate to the gut wall from the region of vagal and sacral neural crest cells. Through the formation of the ganglionic plexuses the number and regional distribution of the neurons building the ganglia change significantly.

Similarly to the central nervous system (CNS), there are various kinds of neurotransmitters and neuromodulators in the ENS. In the CNS glutamate is primarily a neurotransmitter, but it also has a significant role in regulating the development of the CNS. Components of the glutamate neurotransmission, like glutamate itself, VGLUT isoforms or neurons expressing different glutamate receptors, can be found in the ENS as well. It is proved that glutamate has a role in directing the local reflexes, in controlling the function of the enteric neurons and in transmitting the vagovagal reflexes. However, we do not have data about the developmental role of the enteric glutamatergic system.

Although numerous data have been collected about the build-up, development and functioning of the ENS in recent decades, many questions have not yet been answered. Especially, little is known about the embryonic development of neuron populations of the ENS. Available literary data are mainly from studying model animals. However, the morphology and neurochemical characteristics of the plexus building up the ENS vary basically in different species. Thus, development of the human ENS can be known only through examining human fetal tissue samples. Investigating the development of the human ENS is very important from clinical aspects, since several congenital gastrointestinal diseases, such as Hirschprung disease, is closely related to the development of the ENS.

Aims

During our study we followed the spatiotemporal changes of the four components of glutamatergic neurotransmission, which can be examined with immunohistochemical methods, in the human fetal ENS. We focused our analysis on the fetal period between the 12^{th} and the 23^{rd} weeks and examined the development of the glutamatergic system from four main aspects.

1. The investigation of the glutamate immunoreactive neurons
2. The investigation of the VGLUT immunoreactive neurons
3. The investigation of the glutamate-receptive myenteric neurons
4. The investigation of the chemical code of the neurons containing NMDA receptors

MATERIALS AND METHODS

The human samples that we used during our experiments came from legally approved spontaneous or induced abortion from the Department of Obstetrics and Gyneacology, University of Szeged. The experiments were performed in full accordance with the decleration of the Medical World Federation proclaimed in Helsinki in 1964.

1. Tissue preparation

Intestinal segments from 14, 15, 18, 20, 22, 23-week-old human fetuses were obtained immediately after legally approved abortions. The crown-heel length was used to assign gestational age. Three fetuses were analysed per gestational age. Intestinal segments were ligated and distended, then fixed with 4% paraformaldehyde, buffered with phosphate-buffered saline (PBS, 0.1M, pH 7.4). After rinsing with PBS, tissues pieces were further processed for paraffin embedding and/or whole-mount preparations.

2. Immunohistochemistry

All immunocytochemical incubations were carried out at room temperature. Antibodies against glutamate, VGLUT1, VGLUT2, VGLUT3, NMDA receptor subunits, NOS, VIP, neurophilament 200 (NF200) and neuronal HuC/HuD, were used throughout these studies. For single-labelling with VGLUT1, biotinylated donkey anti-rabbit immunoglobulin (Amersham, UK) and streptavidin biotinylated horseradish peroxidase (Amersham, UK) were used as secondary and tertiary antibodies, and immunoreaction was detected with diaminobenzidine. For double and multiple-labelling experiments fluorescent secondary antibodies were used.

Control preparations were performed by omission of either the primary or the secondary antibodies and/or by blocking the primary antibodies with the relevant control proteins.

3. Microscopic analysis

Single-labelled whole-mounts were viewed and photographed via an Olympus BX60 light microscope equipped with an Olympus DP70 digital camera.

Multiple-stained specimens were analysed with an Olympus confocal laser scanning microscope equipped with an argon and helium laser. Images were processed by means of 3D reconstruction facilities.

Stained whole-mounts were used for quantitative and semiquantitative analysis.

4. Quantitative and semi-quantitative analysis

Ten digital photographs of identical magnification, size and resolution were taken from randomly selected areas ($0.188mm^2$) at 400-fold magnification of each stained whole-mount via an Olympus BX60 light microscope equipped with an Olympus DP70 digital camera. The coordinates of the nuclei of the VGLUT-immunoreactive (IR) neurons were digitalized by the Plexus Pattern Analysis software developed in our laboratory, which counted the labelled VGLUT-immunoreactive neurons.

Statistical analysis was performed by using one-way ANOVA with GraphPadPrism software. A probability of $P<0.05$ was set as the level of significance in all analysis.

For semiquantitative analysis VGLUT-IR puncta were counted in randomly selected ganglia and interganglionic fibers. Data from 15 ganglia and 15 interganglionic fibers per intestinal segment per fetal intestine per age group were included in the present study.

RESULTS

1. Glutamate immunoreactive neurons in the developing human fetal enteric nervous system

Anti-glutamate antibody specifically labelled several myenteric and submucosus neurons already in the 15-week-old human fetal intestine. After studying the 15-week-old human fetal intestine, we found that glutamate-IR myenteric neurons do not occur in the

whole length of the intestine, but they concentrated only in a few ganglia. Glutamate immunoreactivity made not only the cell body but also the cell processes visible, so the characterisation of the cells on the basis of size and morphology became possible. By means of our semi-quantitative morphometric investigations we divided the glutamate-IR neurons into two groups from the aspect of their morphological nature. One of the groups, which was 80% of the glutamate-IR cells, possessed a big cell body, some large, lamellar dendrites and one long axon. The other group of the glutamate-IR neurons was much smaller, the surface of the cells was smooth and possessed one long axon. This cell type was around 20% of the glutamate-IR cells. Besides the immunoreactive cell bodies, we often saw glutamate-immunopositive varicose fibres, too, in both the ganglia and between the muscle cells.

2. VGLUT immunoreactive neurons in the developing human fetal enteric nervous system

To study the spatiotemporal pattern of the three VGLUT isoforms in the MP of the human fetal small intestine, we analysed their immunoreactivity between weeks 14 and 23 of gestation. No specific immunreactivity was found in control specimens that were processed without primary or secondary antibodies or with antisera preadsorbed with the fusion protein used as antigen. Immunoreactivity for all three VGLUT isoforms was revealed in the MP of the developing human fetal small intestine. Specific immunoreactivity for each transporter isoform appeared as early as gestational week 14. VGLUT-IR perikarya and even perisomatic puncta appeared frequently at this age, although the premature ganglia were separated from each other. From this age on there was a gradual increase in the intensity of immunoreactivity and in the number of immunopositive nerve structure in the MP. From week 22 of gestation onwards, VGLUT-IR cell bodies and interganglionic fibers also appeared in the SP. Wholemounts prepared from intestinal segments of fetuses at between 18 and 23 weeks of gestation were suitable for the quantitative analysis of stained cell bodies and a

semiquantitative estimation of the immunoreactive puncta. Characteristic age-related developmental patterns were revealed for each transporter. However, the quantitative predominance of VGLUT-IR nerve cell bodies and puncta was unambiguous at all fetal ages and in both intestinal segments.

3. The morphochemical characterisation of the VGLUT1-IR myenteric neurons

After double-labelling with the panneuronal marker HuC/HuD and neuronal marker nNOS, all VGLUT1-IR cells were also labelled for HuC/HuD, but none of them were stained for nNOS. Using confocal laser scanning microscopy after double-labelling for VGLUT1 and NF200, we attempted a morphological characterization of the VGLUT1-IR neurons. It appeared that only 35-40% of them were also labelled for NF200. The double-labelled neurons were of at least three different morphological types. 80% of the double-labelled neurons were uniaxonal, multidendritic neurons. They had short dendrites with lamellar endings and one axon, which ran predominantly orally in the plane of the MP. Around 10% of the double-labelled neurons were non-dendritic and uni- or multiaxonal. The remainder of the double-labelled neurons had a smooth-contoured spindle-shaped cell body, from which two projections emenated.

4. The investigation of the NMDA-receptive myenteric neurons

The antibody against the C-terminal of the NR1 subunit of NMDA receptors stained many myenteric neurons. NR1 immunoreactivity showed point-like staining, which localised mainly to the surface of the cell, although we often detected grainy, cytoplasmatic labelling as well. NR2 A, C and D subunits of the NMDA receptor showed cytoplasmatic staining exclusively, whilst we often found point-like immunoreactive grains on the surface of the myenteric neurons, referring to the presence of NR2B subunit. Besides myenteric neurons, we also found intensive cytoplasmatic staining in several smaller cells, reminders of interstitial cells of Cajal after the NR2A staining.

5. The chemical code of the NMDA receptive neurons

After the NMDA receptor-VIP double-labelling we observed VIP-IR varicose fibres and baskets around some NMDA receptor immunopositive cells. A small group of the myenteric neurons was VIP-IR, and we detected point-like NMDA-NR1 immunoreactivity on the surface and grainy, cytoplasmatic colouring on some 10% of the VIP-IR neurons. A few VIP-IR neurons showed both NMDA receptor subunit and nNOS-immunoreactivity, too. The exclusively nNOS-IR neurons did not, while the VIP/NOS double-labelled myenteric neurons did often express NMDA receptor NR1 subunit.

DISCUSSION

In the present work we studied the presence of the different components of the glutamatergic neurotransmission and the spatiotemporal distribution of these components in the developing human ENS. The aim of our work was to collect immunohistochemical data, from which we can conclude the functional and the developmental role of the glutamatergic neurons.

The anti-glutamate antibody specifically labelled several myenteric and submucosus neurons already in the 15-week old human fetal intestine. On the basis of our morphometric experiments we could label 80% of the glutamatergic neurons as members of the group Dogiel I, the rest (20%) as members of the Dogiel II group. Dogiel II-type neurons in pigs are considered to be intrinsic primary afferent neurons (IPAN), hence our results are in accordance with the literary data, according to which a part of the glutamatergic neurons is IPAN, and so has a determining role in developing the peristaltic reflex.

For detailed developmental investigation we used a much more specific marker of the glutamatergic neurons. We examined the spatiotemporal distribution of VGLUT1-3-immunoreactivity in the MP of the developing human fetal small intestine between weeks 14 and 23 of gestation. The quantitative predominance of VGLUT1-immunoreactivity over

VGLUT2-immunoreactivity was characteristic at all fetal ages in all intestinal segments. A quantitative predominance of VGLUT1 was similarly demonstrated previously as concerns the mRNA levels in the cerebellar granule cells in vitro, and also in neocortical circuits. The main findings of the present study were that all three VGLUT-IR are present in the different ganglionic compartments of the human fetal MP, and each displays a unique and characteristic spatiotemporal distribution.

Despite our lack of knowledge concerning the functional differences between the VGLUT isoforms, the unique developmental pattern of nerve elements immunoreactive for the three VGLUTs revealed in the present study suggests functional differences between them in the developing MP.

To get closer to understandig the function of the VGLUT-IR neurons found in the human fetal PM, we started the morphochemical characterisation of the VGLUT1-IR cells. We made single and multiple labelling immunohistochemical investigations with the panneuronal marker HuC/HuD, a neurofilament protein NF200 and a neuronal marker nNOS. Our preliminary results clearly show that VGLUT1 immunoreactivity in the MP of the human fetal small intestine labels exclusively neurons with non-nitrergic phenotype. The NF200-immunoreactivity data indicate that the VGLUT-IR neurons belong in different neuronal subclasses.

According to former investigations almost all enteric neurons in the rat ENS proved to be NMDA receptor-positive. We found several NMDA receptor-positive neurons in the developing human PM as well, but the distribution of the NMDA receptor-immunoreactivity is much more restricted in the human fetal ENS than in the rat gut.

It is also known from former immunohistochemical investigations that VIP and NOS occur together in the inhibitory motorneurons both at the end of the fetal life and in the adult

human ENS, so we were eager to know whether neurons consisting only VIP or only NOS or rather both of these materials possess NMDA receptors.

We did not observe coexistence of nNOS- and NR-immunoreactivity at the investigated fetal ages, however, around the NMDA receptor-positive cells we often found baskets of nNOS-IR and VIP-IR varicose fibres. This results indicate that, at least at early fetal ages, both NO and VIP somehow control the glutamatergic neurotransmission, which goes through NMDA receptors.

LIST OF PUBLICATIONS RELATED TO THE DISSERTATION

Full papers

1. M. Krecsmarik, F. Izbéki, M. Bagyánszki, **N. Linke**, N. Bódi, J. Kaszaki, Z. Katarova, Á. Szabó, É. Fekete, T. Wittman (2006) *Chronic ethanol exposure impairs neuronal nitric oxide synthase in the rat intestine.* Alcoholism: Clinical and Experimental Research 30(6):967-73 **IF: 3.175**
2. F. Izbéki, T. Wittman, A. Rosztóczy, **N. Linke**, N. Bódi, É. Fekete, M. Bagyánszki (2008) *Inmediate insulin treatment prevents gut motility alterations and loss of nitrergic neurons in the ileum and colon of streptozotocin-induced diabetic rats.* Diabetes Research and Clinical Practice 80(2):192-8 **IF: 1.823**
3. **N. Linke**, N, Bódi, B.A. Resch, É. Fekete, M. Bagyánszki (2008) *Developmental pattern of three vesicular glutamate transporters in the myenteric plexus of the human fetal small intestine.* Histology and Histopathology 23(8):979-86 **IF: 2.007**
4. **N. Linke**, Z. Novák, M. Krecsmarik, É. Fekete, H. Orvos, A. Pál, M. Bagyánszki (2006) *Identification of c-kit-positive cells in the human umbilical vein*. Placenta (submitted)

Abstracts

1. **N. Linke**, N. Bódi, V. Balázs, Zs. Feltóti, F. Izbéki, É. Fekete, M. Bagyánszki (2006) *Quantitative changes in the nitrergic neurons in diabetic rat.* International IBRO Workshop, Budapest, Hungary, 2006, Clinical Neuroscience 2006, 59(S1):42
2. **N. Linke**, N. Bódi, V. Balázs, Zs. Feltóti, F. Izbéki, É. Fekete, M. Bagyánszki (2006) *Quantitative changes in the nitrergic neurons in diabetic rat.* Wind Spring 2006, 9th International Meeting for Hungarian Scientists, PhD Students and Researchers, Kaposvár, Hungary, 2006

3. **N. Linke**, Z. Novák, M. Krecsmarik, É. Fekete, H. Orvos, A. Pál, M Bagyánszki (2006) *Identification of C-kit positive cells in the human umbilical vein.* Europien Academy of Paediatrics Congress, Barcelona, Spain, 2006

4. **N. Linke**, N. Bódi, B. Á. Resch, B. E. Resch, É. Fekete, M. Bagyánszki (2007) *Prenatal development of glutamatergic neurons in the human enteric nervous system.* Magyar Idegtudományi Társaság XI. Konferenciája, Szeged, Hungary, 2007

5. **N. Linke**, N. Bódi, B. Á. Resch, É. Fekete, M. Bagyánszki (2007) *Glutamáterg neuronok prenatális fejlődése a humán bélidegrendszerben.* Wind Spring 2007, 10th International Meeting for Hungarian Scientists, PhD Students and Researchers, Budapest, Hungary, 2007

6. N. Bódi, **N. Linke**, F. Izbéki, M. Bagyánszki, É. Fekete (2007) *Loss of nitrergic myenteric neurons in diabetic rats coincides with structural alterations in capillaries supplying the myenteric plexus.* 14th International Student Congress of Medical Sciences, Groningen, The Netherlands, 2007

7. **N. Linke**, F. Izbéki, M. Bagyánszki, N. Bódi, A. Rosztóczy, É. Fekete, J. Lonovics, T. Wittmann (2007) *Early insulin treatment prevents the loss of nitrergic neurons in the ileum and colon and restores altered gut motility in streptozotocin-induced diabetic rats.* 49th Annual Meeting of the Hungarian Society of Gastroenterology, Tihany, Hungary, 2007, Gastroenterologie 2007, 5: 434

8. **N. Linke**, N. Bódi, Á. B. Resch, É. Fekete, M. Bagyánszki (2007) *Prenatal development of glutamatergic neurons in the human enteric nervous system.* XVI. Nemzetközi Semmelweis Szimpózium és VI. Magyar Sejtanalitika Konferencia, Budapest, Hungary, 2007

9. N. Bódi, **N. Linke**, F. Izbéki, M. Bagyánszki, É. Fekete (2007) *Loss of nitrergic myenteric neurons in diabetic rats coincides with structural alterations in capillaries supplying the myenteric plexus.* XVI. Nemzetközi Semmelweis Szimpózium és VI. Magyar Sejtanalitika Konferencia, Budapest, Hungary, 2007