PH.D. THESES

QUALITATIVE AND QUANTITATIVE ANALYSIS OF THE DEVELOPING ENTERIC NERVOUS SYSTEM

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Introduction

The enteric nervous system (ENS) is a complex network of neurons and glial cells residing within the wall of the bowel, the gall bladder and the pancreas. The ENS coordinates several physiological functions on its own, independently of other regulatory systems. These physiological functions comprise peristalsis, water and ion secretion, blood supply, the growth of the epithelial cells in the mucosa, and the secretion of gall and pancreatic juice. The independent regulation is carried out by means of local reflex modules found repeatedly along the alimentary tract. These reflex modules contain sensory, motor and interneurons. The number of neurons in the ENS is in the millions: usually it is more than the number of neurons in the spinal cord of the given species.

Each tissue layer of the gut wall possesses its own nerve plexus. In higher vertebrates, neurons and glial cells reside within ganglia in two of these nerve plexuses. Ganglia are connected with internodal segments which are bundles of neuronal processes and supportive glial cells. The aforementioned two ganglionated plexuses are the submucous plexus of Meissner (SP) and the myenteric plexus of Auerbach (MP). The SP lies between the submucous connective tissue and the circular muscle layer, and comprises mainly sensory and secretomotor neurons. The MP can be found between the circular and the longitudinal muscle layers, and contains primarily motor neurons, and neurons which project to the gall bladder and the pancreas.

Similarily to other divisions of the peripheral nervous system, the ENS develops from the neural crest. The precursors of the enteric neurons and glial cells originate from the vagal and sacral neural crest. The vagal precursors enter the mesenchime of the gut wall at the level of the foregut, then they migrate anally and colonize the alimentary tract in its full length. The sacral precursors exclusively colonize the so-called postumbilical gut.

The ENS is remarkably complex in its neurochemistry. Almost all known neurotransmitters can be detected in the gut. Beside the classical transmitters, nitric oxide (NO) and glutamate is also present in the gut. NO is very important in the regulation of smooth muscle contractions in the gut. The gut musculature receives an excitatory cholinergic input, in this feature it is similar to other muscles. However, the inhibitory input to the musculature is special: it comes from so-called non-adrenergic non-cholinergic (NANC) neurons. These inhibitory neurons release NO as their major neurotransmitter. Recent research pointed out the importance of glutamate as a neurotransmitter in the ENS. Glutamate plays a role in the regulation of local reflexes, in the modulation of other neurons, and in signalling processes of vago-vagal reflexes.

Aims

During the past few decades various results have been published about the anatomy, development and functioning of the ENS, however, several questions have remained unanswered. This is true in particular for the embryonic development of the different neuronal populations and for their transmitter phenotype. Since there are basic interspecies differences in the structure of the ENS, it is impervious to carry out examinations on various species. By accomplishing comparative studies, we might be able to answer the question to which extent experimental data can be generalized among different species. Using chicken and fetal human materials three main questions have been raised.

I. The examination of nitrergic neurons in the ENS of developing chicken embryos

I.1. Are there region-specific changes in the nitrergic neuron population of the three segments of the gut on embryonic days 12, 13, 14, and 19?

I.2. Are there quantitative changes in the nitrergic neuron population along the entire gut on embryonic days 13 and 19?

II. Changes in the total number of enteric neurons in chicken embryos and hatched chickens

II.1. Is there a reliable staining method to label all neurons in the developing chicken ENS?

II.2. If so, what is the staining efficacy of this method compared to other histochemical and immunohistochemical techniques used for determining total neuronal numbers in the ENS?

II.3. Are there region-specific changes in the total number of neurons of four segments in the gut of chicken embryos and hatched chicken?

III. The examination of glutamatergic neurons in the developing human ENS.

III.1. Are there glutamate-positive neurons in the 18-week-old human fetal ENS?

III.2. Are there glutamate-receptive, NMDA receptor-immunopositive neurons in the 18-week-old human fetal ENS?

III.3. What is the neurochemical code of the functional NMDA receptor-expressing neurons?

Materials and methods

The chicken material:

In the present experiments, we used chicken embryos incubated at 38°C and hatched chickens. All animal experiments were carried out in strict compliance with the European Communities Council Directive (86/609/EEC) regarding the care and use of laboratory animals for experimental procedures. In experiment on the nitrergic system, the gastrointestinal tract was removed from decapitated embryos on day 12, 13, 14, and 19 of incubation. In the experiment on the total number of enteric neurons, the gastrointestinal tract was removed from 19-day-old chicken embryos and from chickens on posthatching days 1, 2, 4, and 7. Samples were fixed in paraformaldehyde, then several segments of the gut were isolated. From these segments, wholemount preparations were made. In order to obtain the wholemount preparations, guts were cut open along the mesenteric attachement, the mucosa and submucosa were scraped off, and the fibres of the circular musculature were peeled off. The wholemount preparations contained the MP freely exposed on the surface of the longitudinal muscle layer.

The human material:

Intestinal segments of three 18-week-old human fetuses were obtained immediately after legally approved or spontaneous abortions. The experiment was performed in accordance with the declaration of the Medical World Federation proclaimed in 1964. The crown-heel length and the last day of menstruation were used to assess gestational age. Wholemount preparations and paraffin sections were made from the human intestinal segments.

NADPH-diaphorase histochemistry:

Samples were processed for NADPH-diaphorase histochemistry following the protocol of Scherer-Singler et al. (1983). Samples were incubated in a solution containing nitroblue tetrazolium, NADPH, and Triton X-100 in PBS buffer for 1 h at 37°C. After the incubation, samples were rinsed in PBS.

Cuprolinic blue histochemistry:

Wholemounts were stained with cuprolinic blue according to Holst and Powley (1985) with some modifications. After preincubation in Triton X-100, wholemounts were rinsed in PB and destilled water. Cuprolinic blue was dissolved in sodium acetate buffer to which magnesium chloride was added. Small amounts of cuprolinic blue solution were filtered through a Millipore filter onto a glass slide. Rinsed wholemounts were placed in the drops of cuprolinic blue solution. Samples were then incubated at 37°C in a humid chamber. After the incubation, samples were rinsed and differentiated in sodium acetate buffer.

Immunocytochemical stainings:

Single and multiple immunocytochemical stainings were performed by using antibodies generated against the 200 kDa neurofilament (NF), NMDA receptor subunits, glutamate, NOS, and VIP. Stainings were done on paraffin sections and wholemount preparations. As normal serum goat or donkey serum was used. Preincubation was performed in normal sera. Later on, Cy3, AMCA, and FITC-conjugated as well as biotinilated species-specific secondary antibodies were applied. All staining steps were done at room temperature. For rinsing, PB was used. The terciary antibody was biotinylated streptavidine-conjugated horseradish peroxidase. In the case of labelling with horseradish peroxidase, diamino benzidine and hydrogen peroxide were used as chromogen and substrate, respectively.

Microscopy:

Preparations were mounted in glycerine/PBS 3:1, then viewed, counted and photographed with a Zeiss Axioscope light microscope. Ganglia and stained cells were counted in randomly selected areas. Care was taken to ensure that neurons in the full depth of the ganglia were counted. Fluorescent samples were studied by using a Zeiss fluorescent microscope. Cell body areas were measured with the AnalySIS Pro software.

Statistics:

Statistical analysis was done with two-way analysis of variance, the Newman-Keuls probe, and the Tukey's probe. A probability of $p<0.05$ was set as the level of significance in all analyses.

Results

Nitrergic neurons in the ENS of developing chicken embryos

A pronounced morphological change was found in the MP of chickens between embryonic days 12 and 19. On embryonic day 12, ganglia were interconnected by short internodal segments. The plexus was densely packed. However, by embryonic day 19, the distance between ganglia had grown, internodal segments had elongated and the plexus showed a looser morphology. These changes were apparent in the proximal small intestine and the colon on day 13. The same morphological changes were obvious in the distal small intestine only by day 14. During the examined period, not only the general morphology of the MP changed but also the shape, staining intensity, and size of the individual nitrergic neurons.

In our quantitative investigations a significant decrease in ganglionic density have been noticed during the entire examined period. By day 19, ganglionic density decreased to one-fifth of its value on day 12. Nitrergic neuronal density also decreased significantly between embryonic days 12 and 19. During the period between days 12 and 14, the number of nitrergic neurons per ganglion showed a wave-like pattern with higher values in the proximal small intestine and colon, and lower values in the distal small intestine. By day 19, the ratio changed, the number of nitrergic neurons per ganglion was higher in the three examined segments. In the proximal small intestine there were approximately 20 nitrergic neurons per ganglion, while in the distal small intestine and colon, this number was 14-15. The quantification of myenteric nitrergic neurons in the full length of the bowel on embryonic day 13 and 19 supported data, gained on selected intestinal segments.

Changes in the total number of enteric neurons in chicken embryos and hatched chickens

We found that cuprolinic blue selectively labelled neuronal cell bodies, whereas neuronal processes, glial cells, smooth muscle cells and blood vessels were poorly stained. The cuprolinic blue-positive neurons were uniform in shape and staining intensity. Consequently, cuprolinic blue staining made possible to gain exact data ont he numbers of neurons and draw correct statistical conclusions. NF immunostaining revealed a morphologically highly variable neuron population. Double staining with cuprolinic blue and NF resulted in an intensification of both stainings, allowing an accurate morphological classification of NF-stained myenteric neurons. NF staining labelled mainly uniaxonal and multidendritic neurons. In some cases, neurofilament-positive neurons were of the adendritic type. Double staining also revealed that only 30-35% of the myenteric neurons was double-labelled, and 65-70% of the neurons was stained exclusively with cuprolinic blue.

In the quantitative examination, we studied ganglionic and neuronal densities, and the number of neurons per ganglion. Ganglionic density significantly decreased in all segments during the first week after hatching. Neuronal densities were significantly different between embryonic day 19 and posthatching day 1, and between posthatching days 4 and 7. The number of neurons per ganglion showed a statistical tendency towards increase during the entire examined period, and increased significantly between embryonic day 19 and posthatching day 2.

Glutamatergic neurons in the ENS of developing human fetuses

The anti-glutamate antibody specifically labelled numerous myenteric neurons in the human fetal small intestine. The staining intensively labelled both neuronal cell bodies and processes which made morphological classification of glutamatergic neurons possible. Our semiquantitative estimations showed that the majority (approximately 80%) of glutamatergic neurons possesses large somata and lamellar dendrites, while the rest are small and smooth-surfaced.

The antibody developed against the NR1 subunit of the NMDA receptor also labelled myenteric neurons with great specificity. NR1 immunoreactivity was granular and linked to the surface of neurons. Occasionally, immunpositive granules were localized cytoplasmatically. NR2A immunopositivity was found exclusively in the cytoplasm, while NR2B immunoreactivity was localized both in the cell membrane and the cytoplasm. Besides neurons, the NR2A antibody also stained numerous cells resembling the interstitial cells of Cajal.

A small fraction of myenteric neurons displayed VIP immunopositivity. A small proportion of these VIP-positive neurons was also positive for NOS and NR1. NOS-positive neurons had rich glutamatergic and VIPergic innervation. Neurons immunostained exclusively with NOS, expressed NR1 subunits only occasionally, while the VIP/NOS neurons frequently showed NR1 immunoreactivity also.

Discussion

Nitrergic neurons in the ENS of developing chicken embryos

The expression of ncNOS changes dynamically during development. So it is an ideal parameter to investigate regional developmental alterations in the ENS. According to the literature NADPH-d histochemical staining can be used to label ncNOS-positive cells in the avian and mammalian ENS. Since no detailed regional investigation had been done on the chicken ENS, it was questionable as to what extent earlier results can be generalized among vertebrates. That is why the primary aim of this study was to determine the qualitative and quantitative changes that take place during the development of MP in chickens.

According to the results of the present study, not only the different neuronal populations develop from two directions, but the MP itself matures in the same way. The development of the MP starts from two initiation areas, the oral and anal part of the gut, towards the middle segments. This is supported by the fact that, on embryonic day 19 the duodenum and colon are far more matured than the jejunum or ileum. The more matured state of the duodenum and colon is manifested by lower ganglionic and neuronal densities in both segments if compared to the middle segments. Similarly, the wave-like changes in nitrergic neuron/ganglion ratio point to the two-initiation area hypothesis. This means that similarly to the colonization and the development of VIPergic neurons, the development of the MP and the gut wall might also be under differential regulation along the longitudinal axis of the gut.

In the present study, the nitrergic neuron per ganglion ratio showed substantial increase during the examined period. A similar tendency was found earlier in pigs, but this observed increase in nitrergic neuronal population took place postnatally. The higher NO levels due to a larger nitrergic neuronal population might play a role in the final maturation of other neuronal types or in the growth of the musculature. An alternative explanation for higher NO levels around hatching can be the physiological need for increased NANC innervation during posthatching life.

Changes in the total number of enteric neurons in chicken embryos and hatched chickens

In order to determine the total number of enteric neurons we applied cuprolinic blue histochemical and NF immunocytochemical staining. Both markers labelled enteric neurons selectively. Since cuprolinic blue did not label muscle cells, glial cells and neuronal processes, the counting of neurons could be easily and accurately done. The NF staining labelled neuronal processes and neuropil regions intensively, which made counting difficult. By applying double staining with the two markers, both labels became more intense and due to the brown-blue contrast, the morphological classification of NF-positive neurons was possible. The NF staining seemed to be a good marker for morphological studies but not for quantitative examinations. The semiquantitative analysis of double stained specimens showed that cuprolinic blue labelled far more neurons than NF did. This result points to the fact that NF cannot be used as a pan-neuronal marker, since it largely underestimates myenteric neuronal populations.

The number of neurons per ganglion showed increasing tendencies along the gut and during the entire examined period. Between embryonic day 19 and posthatching day 7, the number of neurons per ganglion increased three times in the proximal small intestine. In other regions, the number of neurons per ganglion were doubled. This finding raises the question that what is the source of these additional neurons. Based on literary data, we suppose that the

increased number of neurons demonstrated in present investigation at least in part is due to neurogenesis after hatching in the chicken ENS. The reason for higher neuronal numbers is probably the increasing innervational need of the growing musculature in the gut wall. On the other hand, regional differences in neuronal numbers per ganglion might be explained by the different function (secretion, absorption, immunresponse) of different gut segments.

Glutamatergic neurons in the ENS of developing human fetuses

The aim of this study was to obtain results which support the neurotransmitter function of glutamate in the human fetal ENS. Our semiquantitive estimations showed that the majority (80%) of glutamatergic neurons were large, uniaxonal and multidendritic. This cellular architecture corresponds best with the Dogiel type I morphology. The minority (20%) of glutamatergic neurons were smaller, smooth-surfaced and possessed one long process. This shape resembles to the Dogiel type II morphology.

Earlier studies have shown NMDA receptor positivity present in almost all enteric neurons of the rat. In contrast to this, the expression of NMDA receptors in human fetal ENS was more selective and restricted to certain neuronal populations. The NR1 and 2B subunits were localized mostly in the cell membrane, while subunits NR2A, 2C, and 2D were cytoplasmatic. Since ionotropic receptors can function only when in the plasma membrane, we suppose that in the developing human ENS the NR1 and 2B subunits are the main mediators of glutamatergic transmission.

According to our semiquantitative results, approximately 20% of the VIPergic neurons receives glutamatergic input on gestational week 18 in human fetuses. VIPergic neurons in the MP are interneurons, inhibitory motoneurons and secretory neurons. A subpopulation of VIPergic neurons expresses NOS, this population functions as inhibitory motoneurons. Since these VIP/NOS neurons are also present in the human fetal ENS, it is plausible, that glutamate can be an important stimulatory input to these inhibitory neurons. Beside its effects on motility, glutamate might also be important in the developmental regulation of the gut, for instance, in the embryonic development of the aforementioned VIP/NOS neurons.

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