Spatial Pattern Analysis of Nitrergic Neurons in the Developing Myenteric Plexus of the Human Fetal Intestine

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Background: Enteric nervous system precursors derived from the neural crest migrate along defined pathways to colonize the bowel. The individual cells in different environments experience different growth, differentiation, and survival conditions. Hence, the spatial distribution of the neurons is determinant with regard to functional maturation. The question arises as to whether the distribution is random or nonrandom. Methods: Nitrergic cells were visualized by means of nicotinamide adenine dinucleotide phosphate diaphorase histochemistry. Stained specimens were photographed, and the borders of the myenteric plexus and the nuclei of the nitrergic neurons were digitalized. Plexus Pattern Analysis software was used to count the nuclei of nitrergic neurons, calculate the proportions of the areas covered by the plexus and the gut wall, and perform randomization analyses.

Results: The distribution pattern of the nitrergic neurons changed markedly between weeks 14 and 22 of gestation. The nitrergic neurons were randomly distributed at week 14 but were aggregated in the plexus and within the individual ganglia at week 19. The dynamics of these changes exhibited regional differences. Conclusions: The results suggest that, in addition to the gut wall and the plexus, other intraganglionic constituents may contribute to the aggregation of nitrergic cells and such examinations should be extended to other cell types in the future.

Key terms: enteric nervous system; spatial pattern formation; nicotinamide adenine dinucleotide phosphate diaphorase histochemistry; Plexus Pattern Analysis; randomization
and the weighted edge correction (8–10). Four main questions were considered in the present work: How does the spatial pattern of nitrergic neurons change between weeks 14 and 22 of gestation in the period when the functional maturation of the ENS is in progress (11)? Are there regional differences in the dynamics of the developmental processes in the small and the large intestine? Is the pattern of myenteric nitrergic neurons random or aggregated during the examined period? Is there any spatiotemporal organization of nitrergic neurons within the individual ganglia?

MATERIALS AND METHODS

Histochemical Procedure

Intestinal segments of human fetuses (from weeks 14, 19, and 22 of gestation) were obtained immediately after legally approved or spontaneous abortions. The crown-to-heel length was used to assign the gestational age. Three fetuses were examined at each gestational age. The experiments were performed in accordance with the declaration of the Medical World Federation proclaimed in Helsinki in 1964.

Intestines were ligated and distended with 4% paraformaldehyde solution buffered with 0.1 M phosphate buffered saline (PBS) at pH 7.4 and fixed overnight at 4°C. After washing with PBS, wholemount preparations were made from six selected segments of the small intestine and one segment of the colon. Nicotinamide adenine dinucleotide phosphate (NADPH) diaphorase histochemistry was performed according to the protocol of Scherer-Singler et al. (12). Wholemounts were incubated in a solution containing nitroblue tetrazolium (0.25 mg/ml; Sigma, Budapest, Hungary), β-NADPH (1 mg/ml; Sigma), and Triton X-100 (0.5%) in PBS (0.1 M, pH 7.4) for 1 h at 37°C.

Quantitative Methods

Five digital photographs of identical magnification, size, and resolution were taken from different areas of each stained wholemount via a Leica DMLB light microscope equipped with a Polaroid DMC camera. The outlines of the MP and the coordinates of the nuclei of the nitrergic neurons were digitalized by the PPAs developed in our laboratory. The PPAs counted the labeled nitrergic neurons and calculated the ratio of the area covered by the MP and the area of a viewfield. Pattern analysis was carried out with samples of 19-week-old embryos. The PPAs randomized digitalized data of histologic specimens by using Monte Carlo simulation and performed statistical analysis by using the nearest-neighbor method, univariate Ripley’s K function, the L function, and the weighted edge correction.

RESULTS

The density of nitrergic neurons decreased as the development progressed (Fig. 1). The density differed in the different gut segments: at weeks 14, 19, and 22 of gestation, it was significantly higher (P < 0.001) in the colon than in the small intestine.

The area of the viewfield covered by the MP decreased significantly between weeks 14 and 22 and displayed regional differences at each gestational age investigated (Fig. 2). It was always significantly smaller (P < 0.001) in the small intestine than in the colon.

The number of empty areas not covered by the MP differed in the different segments at different developmental stages (Fig. 3). Between weeks 14 and 19, the number of empty areas decreased significantly in the small and the large intestine (P < 0.001). However, the change between weeks 19 and 22 was significant only in the large intestine (P = 0.148 in the small intestine and P < 0.001 in the colon). The mean size of the empty areas increased with age in the small and the large intestine; it was higher in the small than in the large intestine at each gestational age.

Pattern analysis was carried out with samples of 19-week-old embryos. Randomization was generated by Monte Carlo simulation; the program redistributed the original number of neurons within the outlined MP in a random manner (Fig. 4). During randomization, the nuclei could be placed anywhere within the MP (Fig. 4C) and...
could even partly overlap, just as in the plexus in vivo (Fig. 4A,B). Total overlapping was excluded from the randomization. Randomization was also performed within the whole viewfield, when the program ignored the outlines of the MP and distributed the nitrergic neurons randomly. In both cases, 99 random patterns were generated. Means were taken of all such data.

The original and generated random patterns were subjected to statistical analysis by applying the nearest-neighbor method, univariate Ripley’s K function, and the L function, supported by the weighted edge correction. After calibration (determination of how many micrometers correspond to one pixel on the digitalized pictures), the program is also suitable for spatial scaling, which allows determination of the nuclear distance appropriate for statistical analysis. Further, the spatial scale was used as the abscissa in the coordinate system of the L function (Fig. 5). The uppermost curves in Figures 5A and 5B depict the L function derived from an analysis of the digitalized picture shown in Figure 4B. The number of neurons counted in Figure 1B can be randomized to the area of the MP alone (as in Fig. 4C), but the neurons also can be randomized to the overall viewfield. The maximum, median, and minimum values of these two forms of randomization are characteristic features of the corresponding randomization patterns. The curves of the L functions relating to these features are also depicted in Figure 5.

The area between the curves relating to the minimum and maximum values corresponding to the randomization restricted to the MP reflects the confidence interval corresponding to the given confidence level ($P = 0.01$). The nitrergic nuclei may be regarded as aggregated whenever the curve derived from the digitalized picture runs above the confidence interval. This part of the observed spatial scale is referred to as the aggregation interval. When the curve is within the confidence interval, the distribution of the nuclei is regarded as random.

The spatial scale value at which the L function for the digitalized picture was maximum is called the aggregation interval.
**FIG. 5.** Curves of the L function. Data derived from the pattern analysis of wholemount preparations prepared from the small (A) and large (B) intestine of a 19-week-old human fetus. Sample value, curve for the digitalized nitrergic neuron; median 1, minimum 1, and maximum 1, curves for randomization restricted to the MP; median 2, minimum 2, and maximum 2, curves for overall randomization.

**Discussion**

Many quantitative investigations have advanced our knowledge on the numerical properties of the nitrergic neurons in the developing MP (13,14), but the nature and the formation of spatial patterns of enteric neurons with a given neurochemical marker remain largely unknown. Literally, data suggest that remodeling of myenteric neurons in development (15), aging (16,17), and pathologic conditions (18) involves changes in the spatial pattern, and these changes result in motility disorders. As a consequence, experimental data concerning the nature and mode of formation of the cellular network help to develop new diagnostic tools and new therapeutic compounds for treatment of functional bowel disorders.

We have reported on the use of network analytical software that, in conjunction with histochemical or immunohistochemical staining, analyzes the distribution of neurons with different neurochemical markers in the developing MP. The PPAs we have developed can count the nuclei of any cell population visualized on wholemounts and can print out a randomized distribution pattern of this number of nuclei (randomized in various areas). With the application of nearest-neighbor analysis, univariate Ripley’s K function, the L function, edge correction, and Monte Carlo simulation, the program is capable of further statistical analysis. It is also able to answer questions concerning spatial pattern formation, such as whether the distribution of a cell population is random or nonrandom, aggregated or uniform.

As the development of the fetus proceeds, the pattern of the MP emerges and forces the nitrergic neurons to aggregate intrinsically by its anatomic construction, because the neurons must occupy the area covered by the MP. The fact that the MP has an intrinsic pattern-forming force is supported by the results that the overall and MP-restricted randomization intervals differed significantly in each intestinal segment. The means of the aggregational maximum were 75.5 ± 18.9 μm in the small intestine and 48 ± 4.47 μm in the large intestine. The most marked aggregation was found at 60–70 μm at week 19. Throughout the spatial scale, the maximal difference between the curve for the digitized picture and the curve for the median of the randomization restricted to the MP was significant in each observed segment of the gut. In contrast, throughout the spatial scale, the maximal difference between the curve for the digitized picture and the curve for the maximum of the randomization restricted to the MP was significant in each segment of the small intestine (P < 0.05), but not in the large intestine (P = 1.118).

The lower limit of the aggregation interval was the same in the small and large intestine (19.2 ± 2 μm). The upper limits of the aggregation interval were 140 ± 23 μm in the small intestine and 40 ± 11 μm in the large intestine. Thus, the size of the aggregation interval differed along the intestine: 122 μm on average in the small intestine and 21 μm on average in the large intestine.

**Fractional maximum.** This is the scale value in which the distribution of nitrergic cells is the most aggregated.

The statistical analysis of the L function data was performed by means of analysis of variance and the Student-Newman-Keuls test.

The values of the L function were analyzed statistically at week 19 of gestation, when the median values corresponding to the randomization restricted to the MP and to the overall randomization differed significantly (P < 0.001) in each intestinal segment.

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The curves for the digitized picture and the curve for the median MP-restricted randomization differed significantly in each intestinal segment, which means that at certain spatial scale values (in the intervals between 19 and 144 μm in the small intestine and between 19 and 40 μm in the large intestine) the nitrergic neurons were arranged in an aggregated, not a random, pattern. The lower limit of the aggregation interval in the small and large intestine was 19.2 ± 2 μm. This suggests that the nitrergic neurons form aggregated groups within each ganglion. This distance is much smaller than the average distance between neighboring ganglia (hundreds of micrometers). The same conclusion was drawn when the
randomization was performed without the outline of the MP. The nonrestricted randomization patterns were significantly different from the MP-restricted patterns in each observed case. All of the data pointed to the nitrergic neurons forming aggregated groups within the individual ganglia. At the same time, the spatial scale data indicated that the most expressed aggregation was observed at 60–70 μm at week 19 of gestation.

In summary, our results have revealed the formation of small aggregated groups of nitrergic neurons within the individual ganglia. This means that, in addition to the intrinsic force of the MP, there is a high probability that other factors influence the spatial pattern of neurons within the developing human fetal intestine. This is supported by data of Karaosmanoglu et al. (19), who found different densities of nitrergic neurons in different intestinal segments but a uniform density of the nitrergic neurons within the ganglia throughout the intestine. The present findings and the aggregation hypothesis necessitate further experiments to find out the intraganglionic constituents that contribute to the aggregation of neurons with different chemical coding. The PPAs developed in our laboratory makes it easy to quantify markers for the different neuronal phenotype and so provides an easy and fast tool for these investigations.

**LITERATURE CITED**