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SPATIAL PATTERN ANALYSIS OF NITRERGIC NEURONS IN THE MYENTERIC PLEXUS OF THE DUODENUM OF DIFFERENT MAMMALIAN SPECIES

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Nitrergic myenteric neurons are especially susceptible to the development of neuropathy in functional gastrointestinal disorders. Investigations of the similarities and dissimilarities in the organization of nitrergic neurons in the various mammalian species are therefore important in an effort to determine the extent to which the results obtained in different animal models can be generalized. In the present work, the density and the spatial organization of the nitrergic neurons in the myenteric plexus of the duodenum were investigated in 7 mammalian species. After nicotinamide adenine dinucleotide phosphate diaphorase (NADPH-d) histochemistry, the Plexus Pattern Analysis software (PPAs) was applied to count the nuclei of nitrergic neurons, calculate the proportions of the areas covered by the plexus and perform randomization analysis. All 7 species exhibited a large population of nitrergic myenteric neurons, with densities in the range 12–56 cells/mm^2. The distribution patterns of these neurons differed markedly in the different species, however, the rat was the only species in which the nitrergic neurons appeared to be randomly distributed. The PPAs in conjunction with NADPH-d histochemistry proved to be a simple and fast tool with which to reveal similarities and dissimilarities in the spatial arrangement of the nitrergic neurons in the different species.

Keywords: Nitrergic myenteric neurons – NADPH-diaphorase histochemistry – duodenum – Plexus Pattern Analysis software – comparative study

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INTRODUCTION

Nitric-oxide (NO) has been demonstrated to be a major inhibitory non-adrenergic, non-cholinergic neurotransmitter in the gastrointestinal (GI) tract [4, 6, 19–21, 23]. Neuronal NO synthase (nNOS), an enzyme capable of synthesizing NO is identical to neuronal nicotinamide adenine dinucleotide phosphate diaphorase (NADPH-d) in the brain [14] and also in the enteric nervous system [3, 36]. Under defined fixation conditions [3, 29, 36], therefore, NADPH-d histochemistry provides a specific histochemical marker for neurons producing NO.

Numerous reports in the literature have suggested that nitrergic myenteric neurons are especially susceptible to the development of neuropathy in digestive tract diseases [5, 7, 32]. Nitrergic myenteric neurons have therefore been the targets of detailed morphological and functional investigations. Many quantitative investigations with clear species variations have advanced our knowledge on the numerical properties of the nitrergic neurons in different mammals [1, 9, 11–13, 16, 29, 34]. However, it is often quite difficult to compare the data obtained by different investigators under different experimental conditions. The literature suggests that the remodelling of myenteric neurons in development [30, 31], aging [2, 10, 22, 33] and pathological conditions [8, 21, 26, 28] involves changes in the spatial pattern, and these changes result in motility disorders. Accordingly a quantitative method suitable for following the changes in the spatial pattern in pathological environments might help towards an understanding of the pathomechanism of GI diseases related to nitrergic neuropathy. We have therefore introduced network analytical software (the Plexus Pattern Analysis software; PPAs), which is able to answer questions concerning spatial pattern formation, such as whether the distribution of a cell population is random or aggregated. This software has proved to be a reliable tool for analysing the quantitative and spatial pattern changes of nitrergic neurons in the myenteric plexus (MP) during human foetal development [24] and under pathological conditions [15, 18]. When pathological animal models are used, the question frequently arises of the extent to which the results obtained in a given model can be generalized and applied to human conditions, e.g. the extent to which the spatial organization of the enteric plexuses in different animal species are similar or dissimilar. In the present work therefore, we adapted the PPAs so as to be able to compare the spatial distributions of the nitrergic myenteric neurons in the duodenum of rat, mouse, guinea pig, rabbit, dog, cat and monkey.

MATERIALS AND METHODS

Animals

Healthy young adult animals kept on standard laboratory chow were used, with free access to food, pellets and water (Table 1). The handling and the sacrifice of the animals were in accordance with the University of Szeged Guidelines for the Care and

Table 1
Species, number, body weight, sex and manner of sacrifice of the animals used in these investigations. Body weights are expressed as means ± SD. m = male, f = female

Species	*n*	Body weight	Sex	Manner of sacrifice
Rat (*Rattus rattus)* (Wistar)	3	329.13±27.01 g	m	cervical dislocation
Mouse *(Mus musculus)* (Flp)	4	30±0.82 g	m	cervical dislocation
Guinea pig *(Cavia porcellus)* (Crl:(HA)BR)	3	263±4.24 g	m	overdose of 30% urethane
Rabbit *(Oryctolagus cuniculus domesticus)*	3	3±0.87 kg	m	stunning by a blow to the head and severing of the carotid artery
Dog *(Canis familiaris)* (mongrel dog)	4	17±0.82 kg	3 m, 1 f	overdose of sodium-pentobarbital
Cat *(Felis catus)*	3	3.23±0.55 kg	2 m, 1 f	overdose of sodium-pentobarbital
Monkey *(Macaca mulatta)*	3	6.1±0.2 kg	m	overdose of sodium-pentobarbital

Use of Laboratory Animals. The animals in the various species were sacrificed by different methods (Table 1).

Histochemical procedure

Immediately after sacrifice, segments of the duodenum were excised from distally to the pylorus. The dissected duodenal segments were washed in 0.05 M phosphate buffer (PB), pH 7.4, cut along the mesentery, pinched flat, and fixed overnight at 4 °C in 4% paraformaldehyde solution buffered with 0.1 M PB (pH 7.4). The samples were then washed in 0.05 M PB and whole-mount preparations were made of the entire circumferential axis.

NADPH-d histochemistry was performed according to the modified protocol of Scherer-Singler et al. [25]. Whole-mount preparations were incubated in a solution containing nitroblue tetrazolium (0.25 mg/ml; Sigma, USA) and β-NADPH (0.25 mg/ml; Sigma, USA) in PB (0.1 M, pH 7.6) for 1 h at 37 °C. The whole-mounts were mounted on gelatin-coated slides in glycerol-PB, observed and photographed randomly with an Olympus DP70 camera attached to an Olympus BX51 light microscope.

Quantitative methods

Twenty digital photographs with identical magnification, size and resolution were taken of each stained whole-mount from the duodenum of the different animals. The stained cells in each digital photograph were counted by means of the PPAs devel-

oped earlier [24]. Care was taken to ensure that neurons in all focal planes were counted.

The nitrergic neuronal density was calculated as the number of stained cells per mm^2 of whole-mount area. We also used the PPAs to follow and compare the spatial distributions of the nitrergic neurons during the formation of the MP in the different species [24]. The PPAs counted the labelled nitrergic neurons and calculated the ratio of the area covered by the MP and the area of a viewfield. The program redistributed the original number of neurons within the outlined MP in a random manner. During randomization, the nuclei could be placed anywhere within the MP and could even partly overlap, just as in the plexus *in vivo*. The PPAs randomized the digitalized data of histological specimens by using Monte Carlo stimulation, and performed statistical analysis by using the nearest-neighbour method, the univariate Riplay K function, the L function, and the weighted edge correction [24]. The original and generated random patterns were subjected to statistical analysis.

Statistical analysis

The statistical analysis was carried out by using one-way ANOVA and the Newman-Keuls test. All analyses involved use of the GraphPad software for Windows. A probability of $p < 0.05$ was set as the level of significance. Data were expressed as means ± SEM.

RESULTS

In all 7 mammalian species investigated in this study, a substantial proportion of the duodenal myenteric neurons were positive for NADPH-d. However, there were marked differences in nitrergic cell density between the duodenal MP of the rat and the mouse, or the cat and the dog. The density was the highest in the cat (56 cells/mm^2), but lower than average in the dog (28 cells/mm^2); it was again relatively high in the rat (46 cells/mm^2), and lower than average in the mouse (24 cells/mm^2) (Fig. 1). The density of the nitrergic neurons in the duodenum of the cat was significantly higher than those in the other species ($p < 0.001$), and it was significantly lower in the rabbit than in all the others ($p < 0.001$). It was significantly higher in the rat than in the dog, mouse, guinea pig or monkey ($p < 0.001$) and it was significantly higher in the dog than in the mouse ($p < 0.01$), guinea pig ($p < 0.01$) or monkey ($p < 0.001$) (Fig. 1).

All 7 duodenal plexuses were well developed, but the shapes of the MPs varied considerably from species to species (Fig. 2). The ganglia in the duodenal MP in the rat were arranged in a regular manner, mostly aligned parallel with the longitudinal axis of the gut. In the other species, the ganglia were distributed circumferentially, resulting in a regular meshwork of the MP. Pattern analysis was carried out on stained duodenal whole-mount samples of each animal species (Fig. 2).

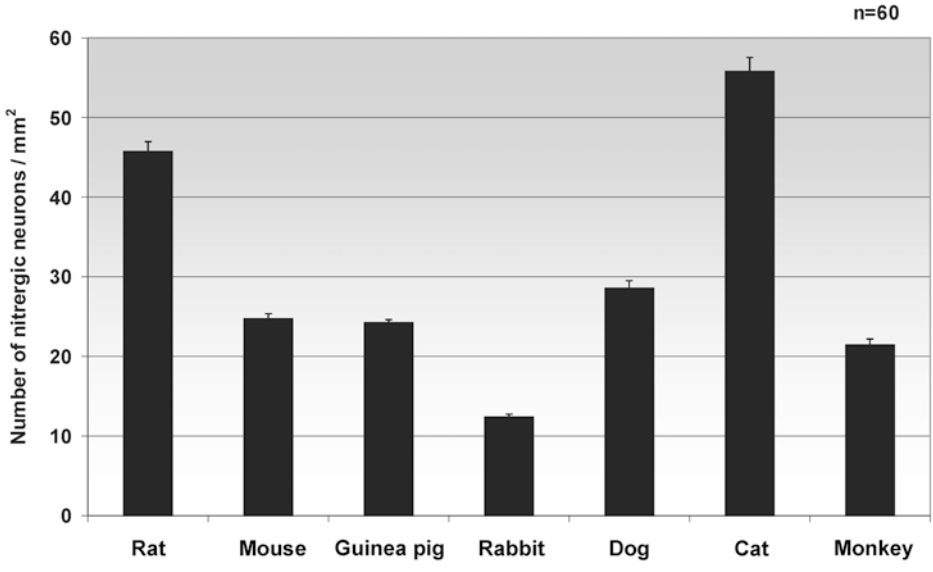
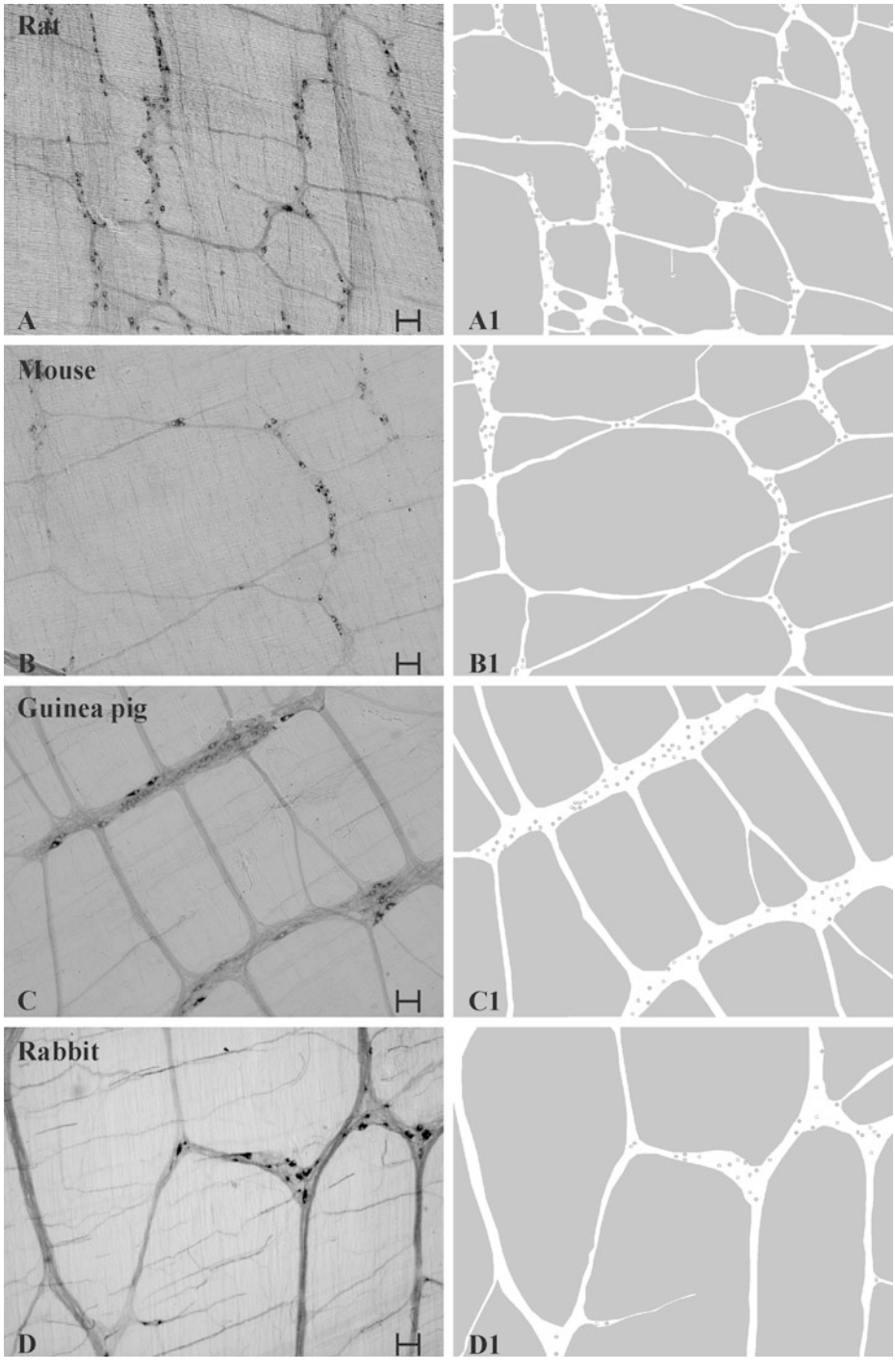


Fig. 1. Histogram showing the density of the nitrergic neurons in the duodenal myenteric plexus of the rat, mouse, guinea pig, rabbit, dog, cat and monkey. The density was the highest in the cat (56 cells/mm^2), but lower than average in the dog (28 cells/mm^2); it was again relatively high in the rat (46 cells/mm^2), and lower than average in the mouse (24 cells/mm^2). The density of the nitrergic neurons in the duodenum of the cat was significantly higher than those in the other species ($p < 0.001$), and it was significantly lower in the rabbit than in all the others ($p < 0.001$). It was significantly higher in the rat than in the dog, mouse, guinea pig or monkey ($p < 0.001$) and it was significantly higher in the dog than in the mouse ($p < 0.01$), guinea pig ($p < 0.01$) or monkey ($p < 0.001$). Data are expressed as means ± SEM

The nitrergic nuclei may be regarded as aggregated whenever the curve of the L function derived from the digitalized picture is situated above the confidence interval (determined by the random maximum and the random minimum). When the curve is within the confidence interval, the distribution of the nuclei is regarded as random.

In the present study, all the spatial patterns of the duodenal nitrergic neurons were aggregated (representative examples in Fig. 3A and B), with the exception of the duodenum of the rat, where the spatial pattern of the MP was random (Fig. 3C).


Rat
A
A1
Mouse
B
B1
Guinea pig
C
C1
Rabbit
D
D1

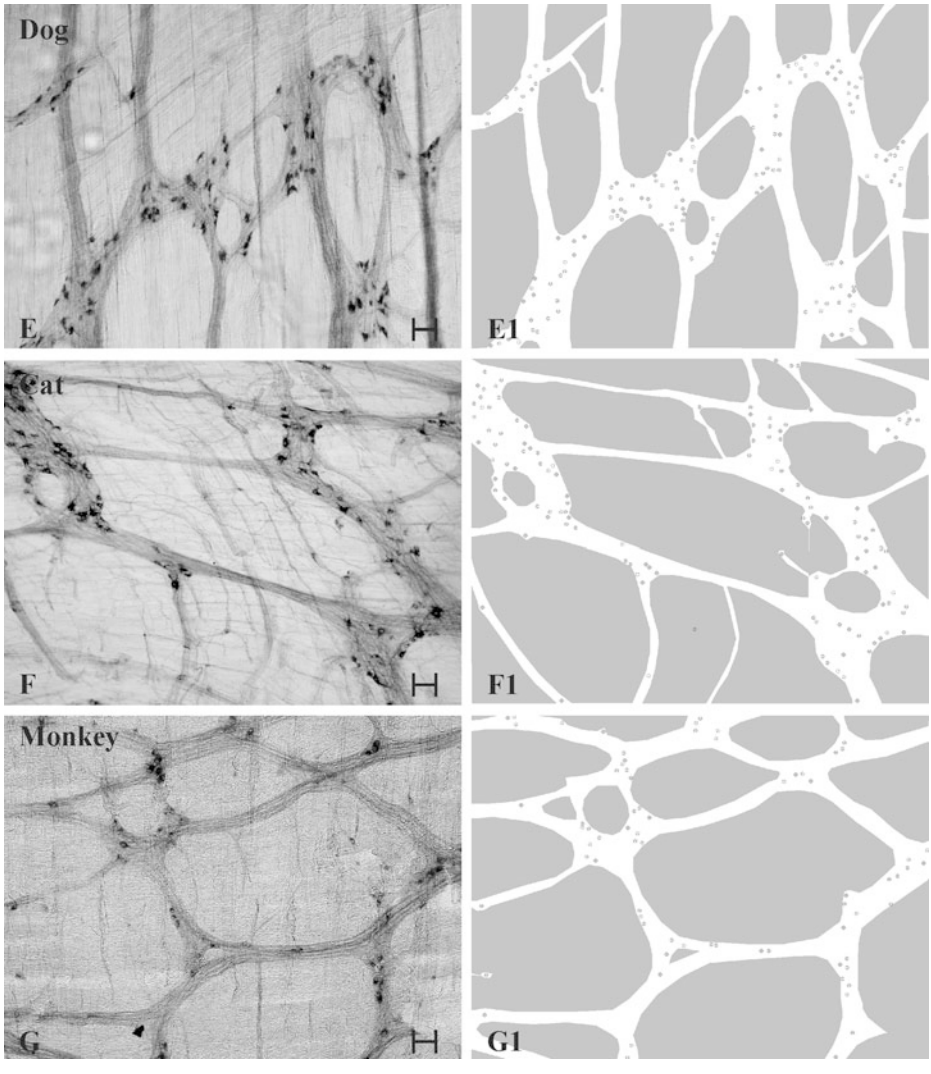
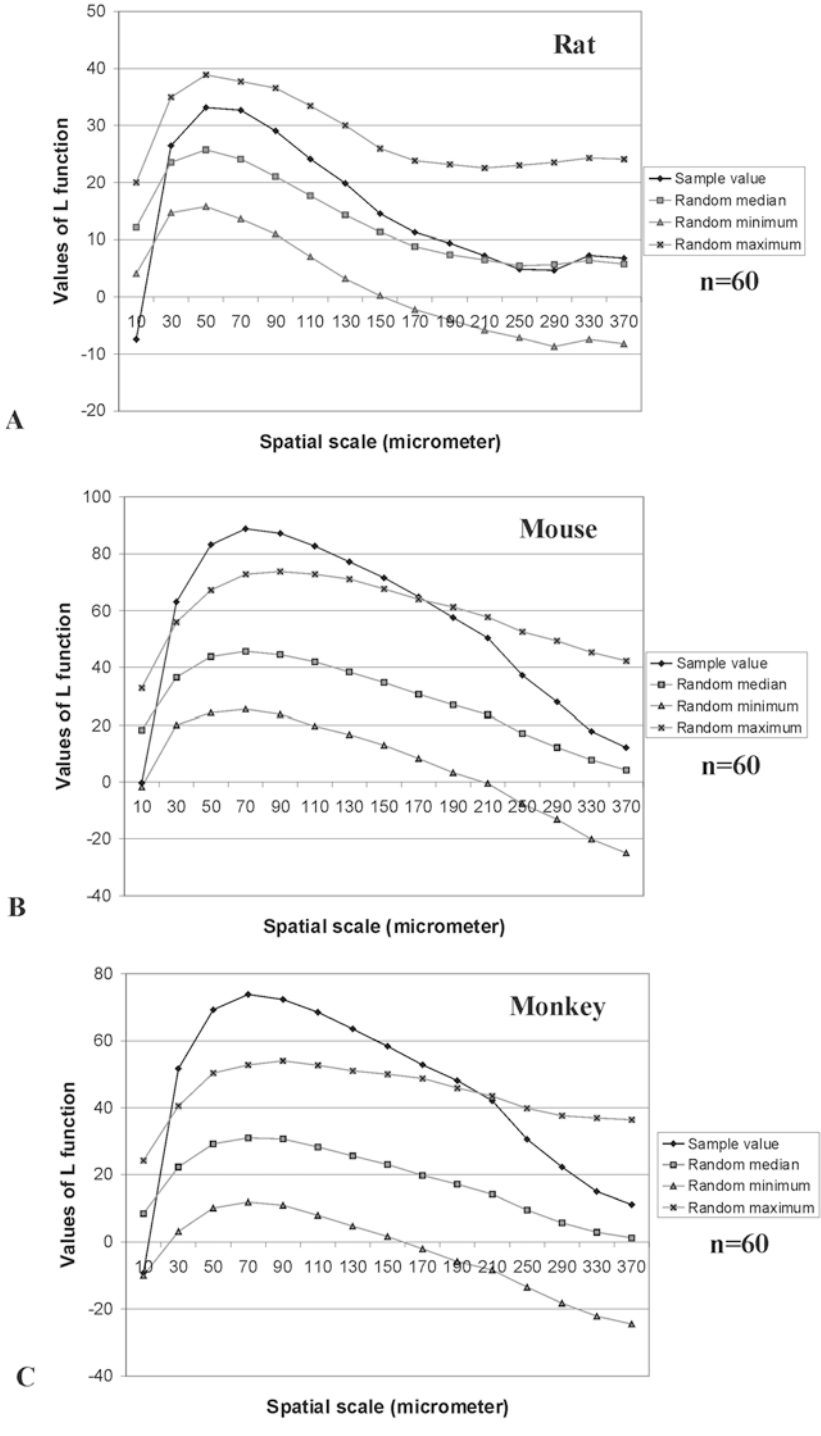


Fig. 2. Representative light micrographs (A–G) and the corresponding digital pictures (A1–G1) used for cell counting and pattern analysis. The circles in the digital pictures denote the nuclei of the nitrergic neurons. The light micrographs were recorded on the duodenal myenteric plexus of the rat, mouse, guinea pig, rabbit, dog, cat and monkey after NADPH-d histochemistry. The calibration bar denotes 100 μm in each picture


Rat
Values of L function
Spatial scale (micrometer)
Sample value
Random median
Random minimum
Random maximum
n=60
A
Mouse
Values of L function
Spatial scale (micrometer)
Sample value
Random median
Random minimum
Random maximum
n=60
B
Monkey
Values of L function
Spatial scale (micrometer)
Sample value
Random median
Random minimum
Random maximum
n=60
C

DISCUSSION

In the present work, the densities and spatial distributions of the nitrergic neurons in the MP of the duodenum of rat, mouse, guinea pig, rabbit, dog, cat and monkey were investigated by means of PPAs after NADPH-d histochemistry [24]. There have already been numerous investigations of the spatial density of nitrergic myenteric neurons [13, 16] and various reports have described the plastic remodelling of these neurons during development [30, 31], aging [10, 22] and pathological conditions [8, 21, 26, 28]. However, the counts of nitrergic myenteric neurons were found to vary significantly, depending on the staining protocols or the sampling strategy applied [16]. Studies attempting to quantify nitrergic cell counts in pathological environments with the aim of revealing the pathomechanisms of nitrergic neuropathies in GI diseases, must therefore standardize the protocols so as to minimize the sources of variability. The completeness of staining of myenteric neurons with different protocols has long been a subject of controversy [17, 35], and underestimates of the total pool of myenteric neurons could lead to incorrect estimates of the percentage of nitrergic neurons, as would incomplete labelling. In our adaptation of the protocol of Scherer-Singler et al. [25] to allow study of the spatial densities of nitrergic neurons in human GI whole-mounts, we considered the preliminary conditions optimum when there was a one-to-one correlation between the NADPH-d histochemical staining and the immunoreactivity for nNOS, even though intermediate degrees of positivity have frequently been reported [29]. In whole-mounts after NADPH-d histochemical staining, the outlines of cells are frequently obscured by the dense staining of the ganglionic neuropil; makes it difficult to distinguish cell boundaries and is a potential source of variability in counts. Since the PPAs counts the nuclei of nitrergic neurons, the level of overlapping can be neglected, which facilitates simple, fast and reproducible automated cell counting. In addition to making the neurons easy to count, the PPAs is able to determine the total area occupied by the ganglia in any given gut region, which (in contrast with the counts of neurons per ganglion) provides a good measure of the density of the innervation of the GI tract [17]. Since myenteric ganglia are capable of fusing with one another and are quite variable in size and shape, a count of neurons per ganglion provides only a rough approximation of the innervation density [12, 27]. Using the PPAs, we demonstrated marked species differences in the nitrergic neuronal densities in the MP of the duodenum of rat, mouse, guinea pig, rabbit, dog, cat and monkey. In general, the results of the present

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Fig. 3. Representative curves of the L function showing the results of pattern analysis of whole-mount preparations, after NADPH-d histochemistry, from the duodenum of the rat, mouse and monkey. The confidence interval was determined by the random maximum and the random minimum. The curves of the L function run above the confidence interval in the mouse and monkey; the patterns of nitrergic myenteric neurons were therefore regarded as aggregated in these species. In contrast, the curve of the L function is within the confidence interval in the rat, meaning that the distribution of the duodenal myenteric nitrergic neurons here is random

calculation of nitrergic cell densities are in the range observed by others [34], although our counts were always higher than the previous estimates [15, 18, 24]. Assuming that undercounting is a more common source of error than overcounting [17] we consider that the PPAs is an adequate tool with which to achieve true counts.

With the application of nearest-neighbour analysis, univariate Riplay's K function, the L function, edge correction, and Monte Carlo simulation, the program is capable of further statistical analysis and is able to answer questions concerning spatial pattern formation, such as whether the distribution of a cell population is random or aggregated.

Randomization analysis revealed that the nitrergic neurons are arranged in an aggregated, non-random pattern in the MP of adult mammals. The only exception was the rat duodenum, where nitrergic neurons were interspersed throughout the myenteric ganglia. This difference in the network topology may imply different functions of the nitrergic neurons and might predict the response of a cell to environmental perturbations, e.g. different pathological conditions. Aggregation might suggest more localized operations of neurons synthesizing NO and the injury of a single neuron within the aggregates would tend to have a considerable effect on the network function. In contrast, the random distribution could suggest, that the nitrergic subpopulation of myenteric neurons are sparsely connected to the plexus and individual neurons could be removed without any appreciable phenotypic effect.

In view of these findings, the PPAs, which is able to follow even minor changes in the topographic distribution of myenteric neurons [24], can be used as a diagnostic tool with which to reveal nitrergic neuropathies in a pathological environment in numerous mammalian species and also in human [24]. When rat models are used, however, the unique distribution of nitrergic neurons in the MP of the rat duodenum must be considered.

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