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How big is the little brain in the gut? Neuronal numbers in the enteric nervous system of mice, Guinea pig, and human

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Abstract

Background: Despite numerous studies on the enteric nervous system (ENS), we lack fundamental knowledge on neuronal densities or total neuron numbers in different species. There are more anecdotal than actual figures on nerve counts.

Methods: We used standardized preparation techniques and immunohistochemistry with validated panneuronal markers (human or mouse anti-HuD/C) to determine neuronal densities in specimen from the entire gastrointestinal tract of mice, guinea pig, and humans. In parallel, we measured the dimensions of the gastrointestinal regions in mouse and guinea pig. For humans, we had to rely on literature data.

Key Results: The average neuronal densities along the gastrointestinal tract were $35,011 \pm 25,017$ $1/cm^2$ for the myenteric and $16,685 \pm 9098$ $1/cm^2$ for the submucous plexus in mice, $24,315 \pm 16,627$ and $11,850 \pm 6122$ $1/cm^2$ for guinea pig myenteric and submucous plexus, respectively, and $21,698 \pm 9492$ and $16,367 \pm 5655$ $1/cm^2$ for human myenteric and submucous plexus, respectively. The total number of neurons in the ENS was 2.6 million for mice, 14.6 million for guinea pig, and 168 million for human.

Conclusions & Inferences: This study reports the first comprehensive nerve cell count in mice, guinea pig, and human ENS. Neuronal densities were comparable between the three species and the differences in the total numbers of enteric neurons are likely due to body size and intestinal length. The number of enteric neurons is comparable to the number of neurons in the spinal cord for all three species.

KEYWORDS
enteric nervous system, gastrointestinal tract, Guinea pig, human, immunohistochemistry, mouse

1 | INTRODUCTION

The autonomic nervous system is composed of three distinct functional parts: sympathetic, parasympathetic, and enteric nervous system (ENS).[1] The neuronal structures of the ENS are located in the gut wall and were first described around 1860 (see[2]). Up to now, numerous studies revealed basic functional and neurophysiological properties of the ENS (see[3]). From the very beginning, researchers were impressed by the independence of the ENS from external influences and its ability to regulate most vital gut functions in isolation.

Moreover, it seems that the ENS was the first network type of nervous system in evolution, which makes the brain the second only.[4,5] This prompted pioneers of the field to refer to the ENS as the second brain or the little brain of the gut.[6,7] They even attributed some smartness to the ENS, and recently, strong evidence to perform higher functions such as learning and memory was presented.[5] With all the excitement around the neurochemical complexity, the brain-like extent in synaptic and receptor repertoire, trivial things like reliable results on the real number of enteric neurons are still missing.

The ENS forms two continuous ganglionated networks that span from the esophagus to the rectum, in the case of the myenteric plexus and from the duodenum to the rectum in the case of the submucous plexus.[2] It has been recognized early that the plexus contains a large number of neurons. Nerve cell counts have been done mostly in single regions of the gastrointestinal tract and only few studies made direct comparisons between different species.[8–10] Because of different methodologies, the results from these studies are not comparable. For obvious reasons it is not possible to count all neurons in the gastrointestinal tract. Therefore, the neuronal density in smaller preparations is determined, and the total number of neurons in one region is then extrapolated to the complete region. However, because the tissue of the gastrointestinal tract is very elastic, the determination of neuronal density and total area depends strongly on the experimenter. Still, these studies have shown that neuronal density and total number of neurons may vary considerably and depend on various factors such as species, intestinal region, and age: Based on earlier publications, Furness and Costa estimated the number of neurons in the ENS to be in the range of 10^7 and 10^8 and the number of neurons in the small intestine of mice, guinea pig, and sheep has been determined as 403,000, 2,750,000, and 31,500,00, respectively.[8,11] The density of neurons in the human submucous plexus has been found to increase by a factor of approximately six from duodenum to ileum and three from ascending to sigmoid colon and similar variations have been demonstrated for myenteric neurons in guinea pigs.[9,12] The results for changes of neuronal density or total number with age are equivocal: While the number of neurons seems to be maintained in mouse and guinea pig colon during aging, number of nerve cells decrease with age in guinea pig small intestine and throughout the rat gut.[10,13–15]

The aim of this study was to provide reliable figures on neuronal density and total number of neurons in both plexus and all regions of the gastrointestinal tract in two laboratory animals (mice and guinea pig) and in humans.

2 | MATERIAL AND METHODS

2.1 | Mice

Experiments with tissue from mice were done according to the German guidelines for animal care and welfare (Deutsches Tierschutzgesetz) and approved by the Bavarian state ethics committee (Regierung Oberbayern, which serves as the Institutional

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Key points

- We report the first comprehensive nerve cell count in mice, guinea pig, and human enteric nervous system using validated panneuronal markers.
- Average neuronal densities in the myenteric plexus of mice, guinea pig and human were 35,011, 24,315 and 16,627 1/cm2, respectively; the corresponding numbers in the submucous plexus were 16,685, 11,850 and 16,367 1/cm2, respectively. The total number of neurons in the ENS was 2.6 million for mice, 14.6 million for guinea pig, and 168 million for human.
- Neuronal densities were comparable between the three species and the differences in the total numbers of enteric neurons are likely due to intestinal length. The number of enteric neurons is comparable to the number of neurons in the spinal cord for all three species.

Care and Use Committee for the Technische Universität München) according to Töten von Tieren und Zucht, Halten von Tieren, Handel mit Tieren Deutsches Tierschutzgesetz under the reference number 32-568-2.

We used tissue from male, 6-week-old C57BL6 mice (weight 24.5 ± 3.4 g, $n = 4$). The mice were killed by cervical dislocation and the whole gastrointestinal tract was quickly removed and placed in ice cold Krebs buffer (in mM: 117 NaCl, 4.7 KCl, 1.2 $MgCl_2$ 6 H_2O, 1.2 NaH_2PO_4, 25 $NaHCO_3$, 2.5 $CaCl_2$ 2 H2O, 11 glucose, bubbled with 95% O_2/5% CO_2). The gastrointestinal tract was then divided into nine regions (esophagus, forestomach, glandular stomach, duodenum, jejunum, ileum, cecum, proximal colon, distal colon), and total length (l_{total}) and diameter ($d_{unstretched}$) of each region were measured. Pieces of 1–2 cm length ($l_{unstretched}$) of each region were taken, opened along the mesentery, and washed from any contents with Krebs solution. The pieces were then pinned with mucosa side up under maximal stretch in silicon lined (Sylgard 184, Dow Corning Midland) petri dishes and length (in the direction of longitudinal muscle, $l_{stretched}$) and width (in the direction of circular muscle, $d_{stretched}$) were measured. Lengths and diameters allowed us to calculate an area stretch factor for each region: $\text{ASF} = \left(l_{stretched} \times d_{stretched} \right) / \left(l_{unstretched} \times d_{unstretched} \right)$. The stretch factor for the stomach was determined differently: The shape of the freshly excised organ was sphere-like. Thus, we could calculate the surface area from its diameter $d_{stomach}$ as $A_{stomach} = \pi * d^2_{stomach}$. The stomach was then opened along the greater curvature, washed from its contents, and pinned under maximal stretch on Sylgard 184. The surface of the whole, stretched stomach was measured with a microscope and a motorized cross table (BX61 WI, Olympus). Finally, the stretch factor was calculated as the quotient of the surface of the tissue in stretched and unstretched state. To avoid further changes in size, the preparations were fixed (overnight at 4°C, 4% paraformaldehyde and 0.0024% picric acid

in 0.1 M phosphate buffer) in this stretched state. After fixation, tissue pieces were washed in phosphate buffer (3×10 min), and all further preparations were done in phosphate buffered saline (PBS). As a ganglionated submucosal plexus is nearly absent in the esophagus and stomach, we removed the mucosa/submucosa and circular muscle in these regions.[2,16] In all other regions we removed the mucosa and divided the submucosal layer from the muscle layers. Finally, the myenteric plexus was exposed by removing the circular muscle layer.

2.2 | Guinea pig

All guinea pig work was conducted according to the German guidelines for animal care and welfare (Deutsches Tierschutzgesetz) and approved by the Bavarian state ethics committee (Regierung Oberbayern), which serves as the Institutional Care and Use Committee for the Technische Universität München) according to §4 and §11 Deutsches Tierschutzgesetz under the reference number 32-568-2. We used tissues from three male guinea pigs (Dunkin-Hartley, Harlan GmbH) with an average weight of 347.8 ± 17.8 g. The guinea pigs (Dunkin Hartley) were killed by cervical dislocation followed by exsanguination. The gastrointestinal tract was quickly removed and placed in ice-cold carbogenated Krebs buffer. Samples were taken from the following regions: esophagus, proximal stomach (fundus and corpus), pylorus, duodenum, jejunum, ileum, cecum, proximal colon, distal colon, and rectum. Total length and diameter of each region were recorded, and further preparation procedures were identical to the procedures used for mouse tissue.

2.3 | Human

Human intestinal samples were collected from patients undergoing surgery in the Hospital Rechts der Isar of the Technical University of Munich and at the Department of Surgery of the Hospital in Freising. The collection and use of human surgical specimens for staining were approved by the Ethics Commission of the TUM (Project number 504/16S). We used tissues from 21 patients (eight female, 13 male) with a mean age 66.2 ± 18.2 years. After removal, macroscopically normal surgical specimens were immediately placed in cold oxygenated sterile Krebs solution (117 mM NaCl, 4.7 mM KCl, 1.2 mM $MgCl_2$ $6H_2O$, 1.2 mM NaH_2PO_4, 25 mM $NaHCO_3$, 2.5 mM $CaCl_2$ $2H_2O$, 11 mM glucose) containing 1% antibiotics/antimycotics. The tissue samples were kept at 4°C during transport. After arrival, the tissue specimens were transferred into a Sylgard (Dow Corning, Midland) lined Petri dish and superfused with fresh, carbogen-bubbled (95% O_2, 5% CO_2) Krebs solution. For obvious reasons we did not receive complete bowel regions and were thus not able to determine the total length of the region for each patient. These values were therefore taken from the literature (see results). To calculate the stretch factors, we used only specimen from intact intestinal tubes. First, diameter and length of the tissue sample were measured. The

specimen was opened and pinned without stretch into the Petri dish. A small piece suitable for further processing was cut out and length and width were measured. Under a stereomicroscope, mucosal and muscle layers were carefully separated with fine preparation instruments and pinned with maximal stretch. Both layers were also measured in stretched condition to calculate the stretch factors for myenteric and submucous plexus. After fixation in stretched condition (4% formalin, overnight) and washing (3×10 min in phosphate buffered saline), the final preparation steps were done in phosphate buffered saline. The mucosa was removed from the mucosa/submucosa layer and circular and longitudinal muscles were removed to expose the myenteric plexus. The submucosal layer was not divided further. We used samples from the following regions (number of patients in parentheses): Duodenum ($n = 3$), jejunum ($n = 3$), ileum ($n = 3$), coecum ($n = 1$), ascending colon ($n = 2$), transverse colon ($n = 3$), descending colon ($n = 2$), sigmoid colon ($n = 3$), rectum ($n = 1$).

2.4 | Immunohistochemistry

For immunohistochemistry, the preparations were first incubated for 1h at room temperature in a blocking solution that contained 4% horse serum and 0.5% Triton X-100 (Sigma Aldrich) in phosphate buffered saline/sodium azide (0.1%). Mouse and guinea pig tissues were then incubated overnight at room temperature in a solution of a human serum in phosphate buffered saline/sodium azide (0.1%), Triton X-100 (0.5%), and horse serum (4%). The human serum ("patient #27") that we used contains highly specific antibodies against HuD and was used at a dilution of 1:6000 for guinea pig tissue and 1:10000 for mouse tissue.[17] After washing (3×10 min in phosphate buffered saline), the tissues were incubated for 90–120min in the secondary antibody (donkey anti human IgG Cy3 (Dianova) in phosphate buffered saline/sodium azide (0.1%) with 4% horse serum and Triton X-100 (0.5%) at a dilution of 1:500).

After incubation in the blocking solution, human tissues were incubated for 40h at room temperature in a primary antibody solution with biotin-conjugated mouse HuC/HuD antibody (A-21272, Thermo Fisher Scientific) in phosphate buffered saline/sodium azide (0.1%) with 4% horse serum and 0.5% Triton X-100. After washing (3×10 min in phosphate buffered saline), the tissues were incubated for 90–120min in a solution of Cy3 streptavidin (016-160-084, Dianova) in phosphate buffered saline/sodium azide (0.1%) with 4% horse serum and Triton X-100 (0.5%) at a dilution of 1:500.

All preparations were finally washed in phosphate buffered saline (3×10 min) and mounted and coverslipped in Citifluor AF1 (Science Services) on poly-l-lysine-coated slides. During mounting, we took care to restore the tissue to its stretched size.

The slides were analyzed with a fluorescence microscope (BX61 WI, Olympus, Hamburg) equipped with a Cy3 filterblock (U-M41007, Olympus), a monochrome CCD camera (Fluoview II, Olympus), and controlled by the cell^p software (Olympus).

For tissues from mouse and guinea pig, we counted all neurons in 2–4 fields of view per animal and region at magnifications of 10x.

At this magnification one field of view corresponds to an area of $0.0236\,cm^2$. With the area stretch factor, we calculated the density of neurons and with the total area of each region the total number of neurons in the respective region. Results are given as average per region and animal and as average ± standard deviation per region over all animals. In the submucous plexus of human tissue samples, we used the same strategy and counted all neurons in 1–3 fields of view at a magnification of 10×. When ganglia in one field of view were at different focus planes, we acquired a z-stack and used the function for Extended Focal Imaging of the cell^p software. Because the ganglia in the human myenteric plexus were larger than in animal tissues, we used the microscopy software and a motorized xy-table (LStep 12/2, Märzhäuser, Germany) to stitch mosaic pictures from 3×3 pictures at a magnification of 10×. We counted then all neurons in 1–3 of these mosaic pictures. Results are given as average per region and patient and average ± standard deviation per region over all patients. Standard deviations for the total number of neurons over all regions were calculated from the sum of the variances of the individual regions.

2.5 | Statistics

Statistical analysis was done with SigmaPlot 12.5 (Systat Software Inc.). Comparisons were done as analysis of variance between different intestinal regions as indicated in the results.

The data that support the findings of this study are available from the corresponding author, MS, upon reasonable request.

3 | RESULTS

3.1 | Mouse tissue

Summaries of all values for tissue dimensions, neuronal densities, and total neuron numbers are given in Tables 1 and 2 and Figure 1.

Staining and counting of neurons were done in three animals. For technical reasons we were not able to perform staining in the jejunum of one mouse. This jejunum was replaced with the jejunum of a fourth mouse. The n-number for the staining experiments is therefore $n = 3$. For statistical tests (analysis of variance), we divided the gastrointestinal tract into three segments: the upper GIT consisted of the esophagus, forestomach, and glandular stomach. Small bowel included duodenum, jejunum, and ileum while large bowel included the cecum, the proximal colon, and the distal colon. Analysis of variance was performed only within the three segments and not between them. Staining quality for each plexus was high in all regions examined and individual neurons could be easily differentiated.

Ganglia in the myenteric plexus of the esophagus were small and had no clear orientation with respect to longitudinal or circular muscle. We did not find a ganglionated submucous plexus. Neuronal density in the myenteric plexus (4956 ± 1227 $1/cm^2$) was similar to the forestomach ($p = 0.2$) but significantly lower than

in the glandular stomach ($p < 0.001$). The total number of enteric neurons in this region was calculated to be 3038 ± 1656 neurons. In the forestomach and glandular stomach, myenteric ganglia were larger and were also irregularly distributed. However, the neuronal density was significantly lower in the forestomach in comparison to the glandular stomach ($14{,}416 \pm 5858$ vs. $41{,}457 \pm 8133$ $1/cm^2$, $p < 0.001$). Similar to the esophagus, we could not detect a ganglionated submucous plexus. With the surface areas of forestomach and glandular stomach from Table 1, we found $30{,}099 \pm 11{,}717$ neurons in the forestomach and $65{,}558 \pm 8006$ neurons in the glandular stomach.

In all parts of the small intestine, the ganglia of the myenteric plexus had an elongated shape and were clearly oriented in the direction of the circular muscle. We could not detect significant differences between the neuronal densities in the myenteric plexus of the three segments of the small intestine (duodenum: $26{,}996 \pm 3331$ $1/cm^2$, jejunum: $30{,}149 \pm 2556$ $1/cm^2$, ileum: $45{,}771 \pm 12{,}672$ $1/cm^2$). The duodenum had a total number of $68{,}918 \pm 8654$ neurons, the corresponding values for jejunum and ileum were $660{,}707 \pm 123{,}013$ neurons and $282{,}935 \pm 63{,}997$ neurons. The ganglia of the submucous plexus showed neither a particular shape nor a clear directionality. Again, neuronal densities were not significantly different between the three segments (duodenum: $23{,}628 \pm 1345$ $1/cm^2$, jejunum: $26{,}086 \pm 3103$ $1/cm^2$, ileum: $13{,}052 \pm 1995$ $1/cm^2$). The total number of neurons in the submucous plexus was calculated to be $61{,}556 \pm 15{,}142$ neurons in the duodenum, $564{,}603 \pm 50{,}781$ neurons in the jejunum, and $81{,}030 \pm 1133$ neurons in the ileum.

Ganglia in the myenteric plexus of the cecum were elongated and oriented in the direction of the circular muscle. Ganglia in the submucous plexus had variable shapes and no particular anatomical orientation. The density of neurons was $14{,}709 \pm 2226$ and 7104 ± 1017 $1/cm^2$, respectively. The myenteric plexus of the proximal colon was very dense. Ganglia were oriented in the direction of the circular muscle and often showed no clear borders. The neuronal density ($95{,}048 \pm 8287$ $1/cm^2$) was higher than in the cecum or distal colon ($p < 0.001$ for cecum and distal colon). In the submucous plexus of the proximal colon, we found a neuronal density of $26{,}309 \pm 13{,}376$ $1/cm^2$, which was significantly higher than in the cecum and the distal colon ($p = 0.046$ and $p = 0.037$, respectively). Ganglia in the distal colon appeared similar to those in the proximal colon with better defined borders between individual ganglia. The neuronal density was $41{,}602 \pm 7346$ $1/cm^2$. Ganglia of the submucous plexus were scattered and relatively small with a neuronal density of 3936 ± 1483 $1/cm^2$. In the myenteric plexus of cecum, proximal and distal colon, we calculated total numbers of $74{,}051 \pm 14{,}523$ neurons, $278{,}435 \pm 80{,}246$ neurons, and $246{,}893 \pm 61{,}418$ neurons. Values for the submucous plexus were $35{,}756 \pm 6771$, $75{,}850 \pm 36{,}680$, and $22{,}812 \pm 6850$ neurons, respectively. Summating the values for all regions, we calculated $1{,}710{,}632 \pm 35{,}069$ nerve cells in the myenteric and $841{,}609 \pm 78{,}300$ neurons in the submucous plexus. This added up to a total of $2{,}552{,}241 \pm 70{,}174$ neurons in the enteric nervous system of the mouse. When we averaged the neuronal densities

TABLE 1 Dimensions of the regions of the gastrointestinal tract for mice, guinea pig, and humans

	Mouse		Guinea pig		Human	
	Length Circumference [cm]	Area [cm^2]	Length Circumference [cm]	Area [cm^2]	Length Circumference [cm]	Area [cm^2]
Esophagus	1.77±0.40 0.33±0.06	0.60±0.21	5.2±2.0 1.00±0.2	5.47±3.0	n.d.	n.d.
Forestomach/ proximal stomach	n.a.	2.12±0.60	5.4±1.0 8.27±1.2	45.33±14.4	n.d.	n.d.
glandular stomach	n.a.	1.65±0.53	n.a.	n.a.	n.a.	n.a.
Antrum	n.d	n.d.	1.9±0.4 5.70±0.6	11.14±3.3	n.d.	n.d.
Duodenum	2.60±0.53 1.00±0.10	2.61±0.65	10.5±2.8 1.47±0.1	15.30±3.5	24 6.6±1.7	157.6±39.8
Jejunum	20.50±2.29 1.07±0.12	21.90±3.64	44.7±5.0 1.43±0.1	64.33±11.6	20 7.6±1.4	1520.0±277.8
Ileum	7.00±1.00 0.90±0.00	6.30±0.90	67.0±7.5 1.60±0.3	117.25±13.2	30 5.6±0.1	1670.0±34.6
Cecum	2.77±0.31 1.82±0.13	5.01±0.22	15.3±2.1 8.33±0.8	127.17±15.1	7 5.0	35.0
Proximal colon/ Ascending colon	2.67±0.58 1.10±0.17	2.90±0.62	21.7±3.8 2.25±0.3	42.25±7.4	23 8.0±0.0	184.0±0.0
Transverse colon	[a]	[a]	[a]	[a]	58 10.0±1.8	581.9±102.2
Descending colon	[a]	[a]	[a]	[a]	33 6.5±1.8	249.2±7.0
Distal colon/ Sigmoid colon	4.87±0.64 1.22±0.08	5.90±0.52	53.7±10.2 1.60±0.1	80.50±15.3	49 7.0±0.6	341.4±27.9
Rectum	[a]	[a]	2.8±0.3 1.13±0.1	3.20±0.3	20 6.8	136.0

Note: Per species, length and circumference (with standard deviation) are given in on column and the surface area in a second column. Area was calculated as average from the individual animals and therefore deviates from the product of length and circumference. Areas for mouse stomach were measured directly with a microscope. Lengths for human gastrointestinal tract were taken from literature ([18,19]) while circumference data correspond to values from actual samples. Abbreviations: n.a., not applicable; N.d., not determined.

[a]In mouse and guinea pig, the colon was divided only into a proximal and a distal part.

over all regions, we found a mean density of 35,011±25,017 1/cm^2 ($n = 9$ regions) for the myenteric and 16,685±9098 1/cm^2 ($n = 6$ regions) for the submucous plexus.

3.2 | Guinea pig tissue

Summaries of all values for tissue dimensions, neuronal densities, and total neuron numbers are given in Tables 1 and 2 and Figure 2.

For these experiments we used tissues of three animals. For technical reasons we had to substitute the stomach in one guinea pig and the rectum in another guinea pig. N-numbers are therefore $n = 3$ for all reported numbers. The gastrointestinal tract was divided in three regions: The upper GIT consisted of the esophagus, the proximal stomach (fundus/corpus), and the distal stomach (antrum). Small bowel consisted of duodenum, jejunum, and ileum and the large bowel of the cecum, the proximal and the distal colon and

the rectum. Statistical comparisons (ANOVA) were only done within these three regions and not between the regions. Staining quality was comparable to the staining in mouse tissue. Due to the thicker muscle layers and connective tissue, the background staining (auto-fluorescence) was more visible. However, ganglia and neurons could always be clearly distinguished from the background.

No ganglia could be detected in the submucous layer of the esophagus and the stomach. This agrees with a recent study in rats that found only a very small amount of neurons in the sub-mucosal plexus of the stomach.[16] The myenteric plexus of the esophagus contained small ganglia with an irregular shape. The neuronal density was 7316±2629 1/cm^2, giving a total number of neurons in the esophagus of 42,582±27,060. Ganglia in the myenteric plexus of proximal and distal stomach were larger and had an irregular shape. The proximal stomach had a neuronal density of 10,953±2916 1/cm^2 and a total number of 468,578±23,198 neurons. The distal stomach showed a neuronal density of
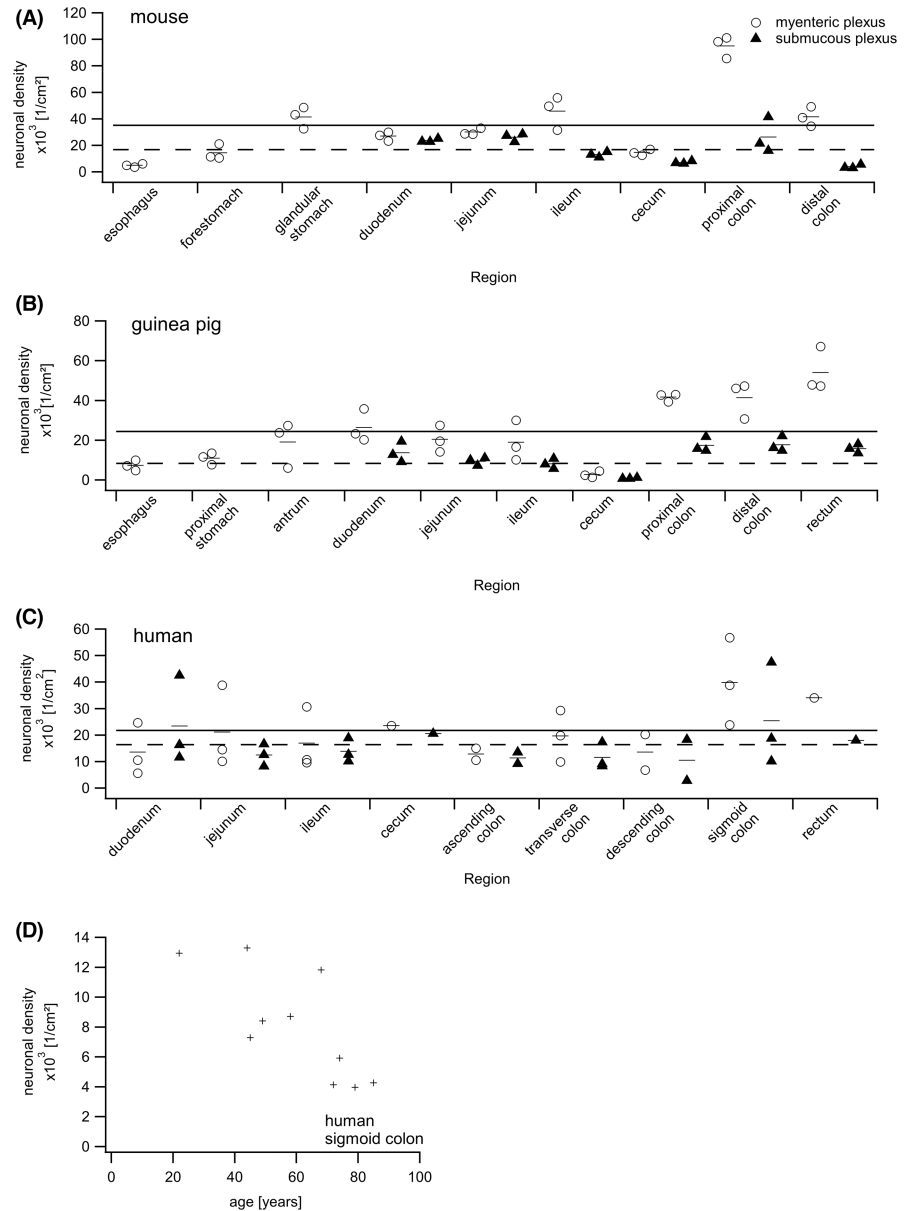
TABLE 2 Neuronal densities and total number of neurons in the gastrointestinal tract of mice, guinea pig, and humans

	Mouse		Guinea pig		Human	
	myenteric	submucous	Myenteric	submucous	myenteric	submucous
Esophagus	4956±1227 (3) 3038±1656	n.a.	7316±2629 (3) 42,582±27,060	n.a.	n.d.	n.a.
Forestomach/proximal stomach	14,416±5858 (3) 30,099±11,717	n.a.	10,953±2916 (3) 468,578±23,198	n.a.	n.d.	n.a.
Glandular stomach	41,457±8133 (3) 65,558±8006	n.a.	n.a.	n.a.	n.a.	n.a.
Antrum	n.d.	n.a.	19,085±11,436 (3) 223,665±174,812	n.a.	n.d.	n.a.
Duodenum	26,996±3331 (3) 68,918±8654	23,628±1345 (3) 61,556±15,142	26,466±8256 (3) 387,500±40,485	13,734±5214 (3) 198,463±31,004	13,613±9861 (3) 2,347,640±2,227,420	23,463±16,646 (3) 4,067,247±3,809,054
Jejunum	30,149±2556 (3) 660,707±123,013	26,087±3103 (3) 564,603±50,781	20,444±6691 (3) 1,365,547±665,094	9434±1924 (3) 621,670±225,921	21,186±15,453 (3) 29,406,509±15,420,403	12,477±4206 (3) 19,005,291±8,063,279
Ileum	45,771±12,672 (3) 282,935±63,997	13,052±1995 (3) 81,030±1133	18,959±10,179 (3) 2,289,639±1,466,945	8107±2529 (3) 964,910±393,207	16,993±11,858 (3) 28,652,284±20,627,118	13,900±4489 (3) 23,187,380±7,312,677
Cecum	14,709±2226 (3) 74,051±14,523	7104±1017 (3) 35,756±6771	2755±1578 (3) 349,224±188,650	836±270 (3) 105,442±30,692	16,993 (1) 825,517	13,900 (1) 719,005
Proximal colon/Ascending colon	95,048±8287 (3) 278,435±80,246	26,309±13,376 (3) 75,850±36,680	41,711±2052 (3) 1,766,521±358,340	17,406±3755 (3) 722,366±98,014	12,816±3211 (2) 2,358,121±590,737	11,365±3083 (2) 2,091,152±567,236
Transverse colon	a	a	a	a	19,680±9714 (3) 10,811,723±3,869,951	11,583±4999 (3) 6,480,137±1,909,671
Descending colon	a	a	a	a	9041±9493 (2) 3,345,760±2,270,254	10,533±10,945 (2) 2,585,869±2,653,078
Distal colon/Sigmoid colon	41,602±7346 (3) 246,893±61,418	3936±1483 (3) 22,812±6850	41,379±9242 (3) 3,423,305±1,289,086	17,697±3923 (3) 1,445,377±498,357	39,811±16,482 (3) 13,816,279±6,715,261	25,432±19,508 (3) 9,043,559±7,607,037
Rectum	a	a	54,086±11,306 (3) 171,832±29,834	15,735±2249 (3) 50,803±12,544	34,037 (1) 4,629,005	18,012 (1) 2,449,578
Subtotals myenteric and submucous	35,011±25,017 1,710,632±35,069	16,685±9098 841,609±78,300	24,315±16,627 10,488,393±2,469,031	11,850±6122 4,109,030±767,664	21,698±9492 96,192,838±27,088,789	16,367±5655 69,629,218±14,208,274
Total	2,552,241±70,174		14,597,423±3,110,560		165,822,056±30,588,847	

Note: In each cell, the neuronal density in neurons per cm^2 is given in the first line and total number of neurons in the second line. Number of specimens is given in parentheses. Abbreviations: n.a., not applicable; N.d. not determined.

[a] In mouse and guinea pig, the colon was divided only into a proximal and a distal part.

FIGURE 1 Neuronal densities in unstretched preparations of the myenteric (○) and submucosal plexus (▲) in the gastrointestinal tract of (A) mouse, (B) guinea pig, and (C) human. Solid and dashed lines in the graphs represent the average neuronal density for myenteric and submucosal plexus, respectively. Note the different scaling for the y-axis in the graphs. (D) The neuronal density in stretched preparations of the human sigmoid colon is negatively correlated to the age (Spearman rank order correlation, $R = -0.81$, $p = 0.003$).

19,085 ± 11,436 1/cm² with a total number of 223,665 ± 174,812 neurons. We found no statistical differences in the neuronal densities among the three regions of the upper GIT. Similarly, there were no significant differences in the neuronal densities in the myenteric plexus between the regions of the small intestine (duodenum: 26,466 ± 8256 1/cm², jejunum: 20,444 ± 6691 1/cm² and ileum: 18,959 ± 10,179 1/cm²). Total numbers of myenteric neurons were 387,500 ± 40,485 in the duodenum, 1,365,547 ± 665,094 in the jejunum, and 2,289,639 ± 1,466,945 in the ileum. Myenteric ganglia in all parts of the small bowel were large. However, while the ganglia in the duodenum had an irregular shape, the ganglia in the jejunum and ileum were elongated and oriented in the direction of the circular muscle.

Ganglia in the submucous plexus of the small bowel were smaller than those in the myenteric plexus and had irregular shapes. Similar to our results in the myenteric plexus, neuronal densities in the submucous plexus were not different (duodenum: 13,734 ± 5214 1/

cm², jejunum: 9434 ± 1924 1/cm², ileum: 8107 ± 2529 1/cm²) and yielded a total number of 198,463 ± 31,004, 621,670 ± 225,921, and 964,910 ± 393,207 in the duodenum, jejunum, and ileum, respectively.

Ganglia in the myenteric plexus of the cecum were small and showed no clear anatomical orientation. Submucosal ganglia also were small with irregular shapes. Neuronal density in the myenteric plexus was 2755 ± 1578 1/cm² and significantly different from the other parts of the large bowel (cecum vs. proximal colon: $p = 0.001$, cecum vs. distal colon: $p < 0.001$, cecum vs. rectum: $p < 0.001$, ANOVA with post hoc Holm-Sidak). The neuronal density in the submucous plexus was 836 ± 270 1/cm² and differed between the regions of the large bowel (cecum vs. proximal colon: $p < 0.001$, cecum vs. distal colon: $p < 0.001$, cecum vs. rectum: $p = 0.001$, ANOVA with Holm-Sidak). We calculated the total number of neurons to be 349,224 ± 188,650 in the myenteric plexus and 105,442 ± 30,692 in the submucous plexus.
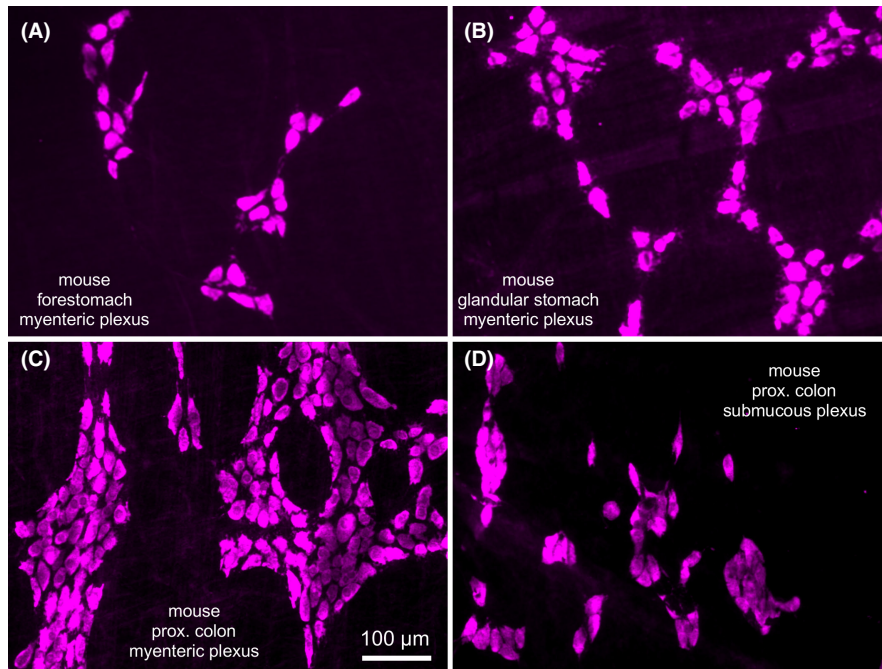
FIGURE 2 Immunohistochemistry with a panneuronal antibody against HuC/D in stretched samples of the enteric nervous system of the mouse. The neuronal density in the myenteric plexus of the forestomach (A) is lower than in the glandular stomach (B). Ganglia in the myenteric plexus of the proximal colon (C) are larger than in the submucous plexus (D). Scale bar in (C) applies to all figures.
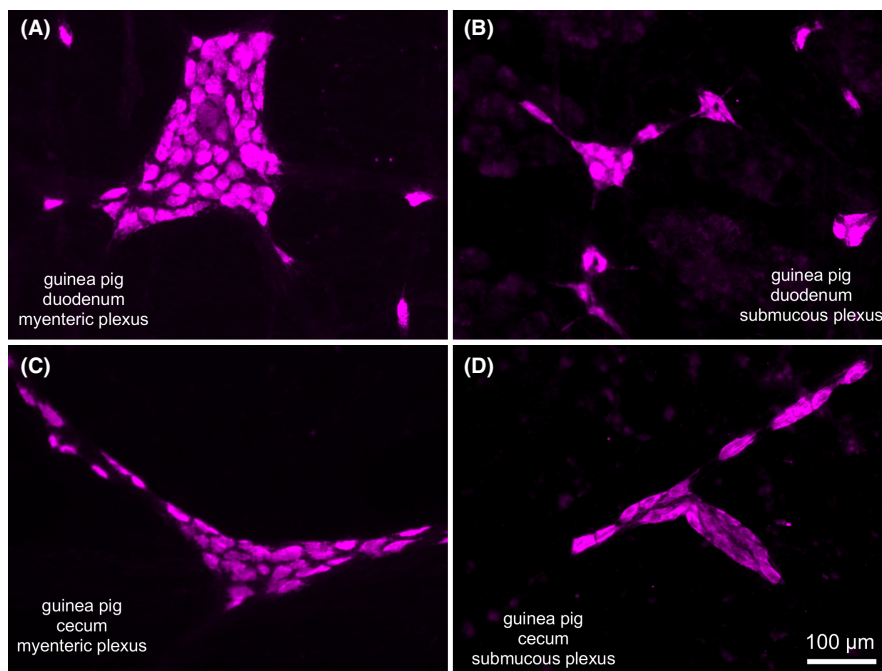

FIGURE 3 Immunohistochemistry with a panneuronal antibody against HuC/D in stretched samples of the enteric nervous system of the guinea pig. Neuronal density and ganglion size in the myenteric plexus are higher in duodenum (A) and cecum (C) than in the submucous plexus (B, D). Scale bar in (D) applies to all figures.

The myenteric plexus in the proximal colon contained large ganglia, which often merged into each other. In contrast to this, the ganglia in the distal colon and the rectum were smaller and could be distinguished more easily. Neuronal densities in these three regions were found to be $41,711 \pm 2052$, $41,379 \pm 9242$, and $54,086 \pm 11,306$ $1/cm^2$ and were not statistically different from each other (proximal colon vs. distal colon: $p = 0.9$, proximal colon vs. rectum: $p = 0.2$, distal colon vs. rectum: $p = 0.2$, ANOVA with Holm-Sidak). We calculated the total numbers of neurons in the myenteric plexus as $1,766,521 \pm 358,340$ for the proximal colon, $3,423,305 \pm 1,289,086$ for the distal colon, and $171,832 \pm 29,834$ for the rectum. Ganglia in the submucous plexus of the proximal colon and the rectum appeared to be small while the submucosal ganglia in the distal colon were larger. Neuronal density was $17,406 \pm 3755$, $17,697 \pm 3923$, and $15,735 \pm 2249$ $1/cm^2$ for the proximal colon, the distal colon, and the rectum, respectively. Like in the myenteric plexus we found no significant differences in the neuronal densities between these regions (proximal colon vs. distal colon: $p = 0.9$, proximal colon vs. rectum: $p = 0.8$, distal colon vs. rectum: 0.8). The total number of neurons could be calculated as $722,366 \pm 98,014$ (proximal colon), $1,445,377 \pm 498,357$ (distal colon) and $50,803 \pm 12,544$ (rectum). When we added the number of neurons for all regions, we found $10,488,393 \pm 2,469,031$ neurons for the myenteric plexus and $4,109,030 \pm 767,664$ neurons for

FIGURE 4 Immunohistochemistry with a panneuronal antibody against HuC/D in stretched samples of the human enteric nervous system. Neuronal density varies strongly between myenteric and submucous plexus in the duodenum (A, B) and the sigmoid colon (C, D) but also between the regions. Note different scale bars in all figures.
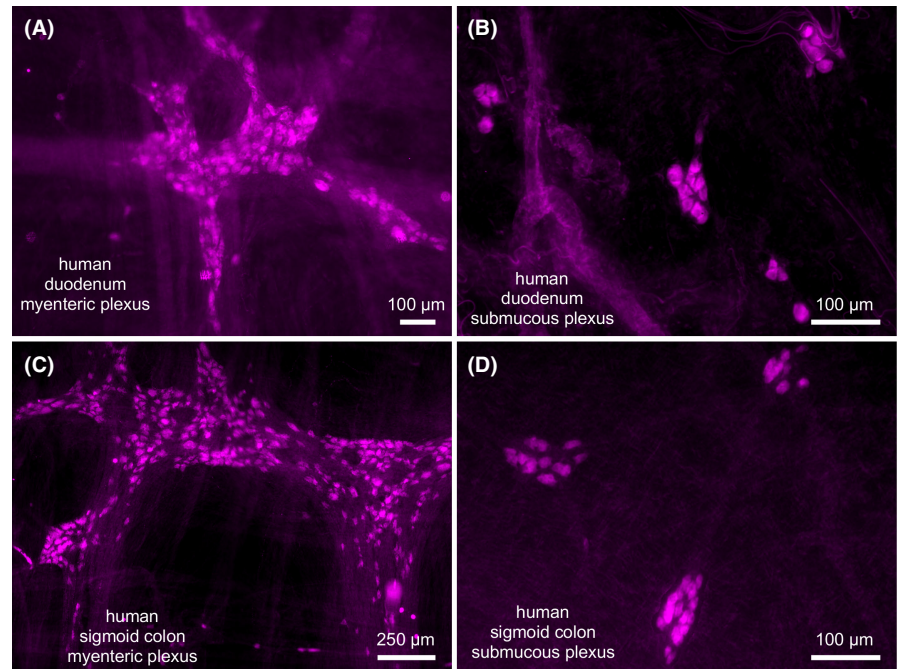
the submucous plexus. Thus, the total number of neurons in the enteric nervous system of the guinea pig was $14{,}597{,}423 \pm 3{,}110{,}560$. Averages of the neuronal densities over all regions showed a significantly larger value ($24{,}315 \pm 16{,}627$ $1/cm^2$, $n = 10$ regions) for the myenteric than for the submucous plexus ($11{,}850 \pm 6122$ $1/cm^2$, $n = 7$ regions) (paired t-test, $p = 0.008$).

3.3 | Human tissue

Summaries of all values for tissue dimensions, neuronal densities, and total neuron numbers are given in Tables 1 and 2 and Figure 3.

Due to thicker muscle layers and more connective tissue, immunohistochemistry in human tissue showed a higher background intensity than in mouse or guinea pig tissue. Staining quality was however always good enough for a reliable quantification of neuron numbers in myenteric and submucosal plexus. Because we were not able to determine the total length of the bowel region in the respective patients, we assumed a length of 24 cm for the duodenum, 200 cm for the jejunum 300 cm for the ileum, 7 cm for the cecum, 23 cm for the ascending colon, 58 cm for the transverse colon, 33 cm for the descending colon, 49 cm for the sigmoid colon, and 20 cm for the rectum. These values were based on two studies that measured intestinal lengths either intraoperatively (small bowel) or with computed tomography colonography (large bowel).[18,19]

Ganglia in the myenteric and the submucous plexus of all examined regions were of variable shapes and showed no obvious anatomical orientation. The neuronal densities in the myenteric plexus of duodenum, jejunum, and ileum were $13{,}613.4 \pm 9860.9$, $21{,}186.1 \pm 15{,}453.4$, and $16{,}993.3 \pm 11{,}858.1$ $1/cm^2$, respectively with no statistically significant differences between these regions

($p = 0.7$). With the diameter of our samples and the abovementioned lengths for the different regions, we calculated surface areas and total number of myenteric neurons for each region (see Table 1). The duodenum had $2{,}347{,}640 \pm 2{,}227{,}420$ neurons while the jejunum had $29{,}406{,}509 \pm 15{,}420{,}403$ neurons, and the ileum had $28{,}652{,}284 \pm 20{,}627{,}118$ neurons. The corresponding neuronal densities and total neuron numbers in the submucous plexus were $23{,}462.8 \pm 16{,}646.3$ and $4{,}067{,}247 \pm 3{,}809{,}054$ neurons (duodenum), $12{,}476.6 \pm 4206.3$ $1/cm^2$ and $19{,}005{,}292 \pm 8{,}063{,}279$ neurons (jejunum), and $13{,}900 \pm 4489$ $1/cm^2$ and $23{,}187{,}380 \pm 7{,}312{,}677$ neurons (ileum). Again, we could not find statistically significant differences between the neuronal densities in the submucous plexus of the small bowel. Only one sample from a cecum was available for staining. The density of neurons in the myenteric plexus was $23{,}586.2$ and $20{,}543.0$ $1/cm^2$ in the submucous plexus. We calculated the total number of neurons in the myenteric plexus to be $825{,}517$ and $719{,}005$ in the submucous plexus. This single sample was not included in the statistical comparisons (ANOVA) for the large bowel. Neuronal densities in the myenteric plexus of the ascending colon, transverse colon, descending colon, and sigmoid colon were $12{,}815.9 \pm 3210.5$, $19{,}679.7 \pm 9713.7$, 9041.4 ± 9493.0, and $39{,}810.5 \pm 16{,}481.6$ $1/cm^2$, respectively. The total number of myenteric neurons was $2{,}358{,}121 \pm 590{,}737$, $10{,}811{,}723 \pm 3{,}869{,}951$, $3{,}345{,}760 \pm 2{,}270{,}254$, and $13{,}816{,}279 \pm 6{,}715{,}261$ for the respective regions of the large bowel. The neuronal densities in both plexus were comparable between the regions. The densities were $11{,}365.0 \pm 3082.8$ $1/cm^2$ for the ascending colon, $11{,}582.5 \pm 4999.2$ $1/cm^2$ for the transverse colon, $10{,}532.5 \pm 10{,}944.5$ $1/cm^2$ for the descending colon, and $25{,}431.8 \pm 19{,}507.6$ $1/cm^2$ for the sigmoid colon. Using the areas listed in Table 1, we calculated total neuron numbers in the submucous plexus as $2{,}091{,}152 \pm 567{,}236$, $6{,}480{,}137 \pm 1{,}909{,}671$, $2{,}585{,}869 \pm 2{,}653{,}078$, and $9{,}043{,}559 \pm 7{,}607{,}037$ for ascending,

transverse, descending, and sigmoid colon, respectively. For one rectal preparation we found a neuronal density of 34,036.8 $1/cm^2$ in the myenteric plexus and 18,011.6 $1/cm^2$ in the submucous plexus. The total number of neurons in the myenteric or submucous plexus was 4,629,005 or 2,449,578, respectively. This single sample was not included in the analysis of variance.

Age might have an effect on the density and/or total number of enteric neurons.[13,14,20,21] In contrast to the samples from mouse or guinea pig, the samples from human tissue came from patients with different ages. We therefore evaluated the neuronal density in 10 myenteric plexus preparations from the human sigmoid colon. For these experiments we calculated the density only in fully stretched preparations. Age of the patients was between 22 and 85 years (four female, six male), and we found a negative correlation between age and neuronal density (Spearman rank order correlation, $R = -0.81$, $p = 0.003$; Figure 4).

With the results for the individual regions, we finally calculated the number of neurons in the human ENS (without esophagus and stomach) to be $96,192,838 \pm 27,088,789$ myenteric neurons and $69,629,218 \pm 14,208,274$ submucous neuron. Thus, we found in total $165,822,056 \pm 30,588,847$ neurons in the human ENS. For the stomach we had only access to preparations of maximally stretched tissue from four patients without information about the original dimensions of the samples. In these specimens we found a neuronal density of 1665 ± 219 $1/cm^2$. With an assumed stretch factor of 4, we would calculate a neuronal density of 6660 $1/cm^2$. With a surface of 80 cm^2 for the esophagus and 200 cm^2 for the stomach[22] and this neuronal density, these two organs would harbor approximately 1.9 million additional neurons. Thus, we would assume a total of 168 million neurons in the human ENS. Average densities over all studied regions were not significantly different between the myenteric ($21,698 \pm 9492$ $1/cm^2$) and the submucous plexus ($16,367 \pm 5655$ $1/cm^2$; paired t-test, $p = 0.072$).

We also compared the densities over all regions between the different species. However, no significant differences were detected for the densities in the myenteric or submucous plexus of mouse, guinea pig, and human (ANOVA, $p = 0.3$ for myenteric plexus, $p = 0.4$ for submucous plexus).

4 | DISCUSSION

To the best of our knowledge, our study provides the first comprehensive nerve cell counts in the ENS of the three different species mostly used in gastrointestinal research. We believe that our technical approach yielded reliable counts of neuronal densities and total number of neurons in the ENS. We consider the use of identical methods in one study as a significant progress to compare nerve cell density in the ENS of mouse, guinea pig, and human. The values for the individual regions showed a good repeatability between individual animals. A recent study showed total neuron numbers in the rat stomach that are very close to the values that we obtained in the guinea pig.[16] As both species are also similar

in size and body weight, this further validates our experimental approach and demonstrates a good comparability between these species. The variations observed for human samples are attributed to the age range, a phenomenon previously reported.[13,14,20,21] It has to be noted, however, that the number of human specimens in our study is limited for some intestinal regions. For a robust statistical analysis, much higher n-numbers in clearly defined age groups would be needed.

There are publications that evaluate neuronal density in different species and intestinal regions.[8–10,12,14–16,23,24] However, none of them provided an in-depth nerve cell counts along all regions of the gastrointestinal tract. This is also supported by the conclusion, that the variations in the methods used made it impossible to compare data on neuronal densities in the human ENS from various studies.[25] A study by Gabella compared neuronal densities and numbers in the small intestine of mice, guinea pigs, and sheep.[8] Neuronal densities were calculated for preparations of distended intestine and cannot be compared directly with the numbers in the present study. However, they show a similar relation: Neuronal density in the myenteric plexus was highest in the mouse, lower in guinea pig, and lowest in sheep (10,600: 8600: 2500 $1/cm^2$). The present study also found the highest density in mouse tissue (e.g., duodenum: 45,771 $1/cm^2$), an intermediate value in guinea pig tissue (18,959 $1/cm^2$), and the lowest value in human tissue (16,993 $1/cm^2$). It is tempting to conclude from these relations that smaller animals have higher neuronal densities in the ENS. This is supported by tracing studies, which have shown longer projections for, e.g., interneurons in the myenteric plexus of the human colon in comparison to similar preparations in the guinea pig.[26–28] As a consequence, interneurons signal over longer distances and hence lower neuronal densities are needed. On the other hand, animals differ not only in size but also in their diet, day–night rhythm, or life expectancy and each of these factors might influence the ENS as well. Another aspect that needs to be taken into consideration is the plasticity of the ENS. The ENS is not only able to adapt ("learn") but also seems to possess a remarkable degree of neurogenesis with a rapid turnover of neurons and the ability to regenerate after an insult.[5,29–32]

So far, numbers representing the size of the ENS were mainly based on estimations or extrapolations. For the human ENS, for example, values between 10 and 600 million have been claimed. However, these figures seemed to be based on plausibility rather than actual counts.[2,11,33] The present study found 168 million neurons in the human ENS, including estimated 1.6 million neurons in the esophagus and stomach.

One of the strongest factors that influences the total number of neurons in the ENS is, however, the length of the gastrointestinal tract. While it is straightforward to evaluate the length of the GIT in mouse or guinea pig, it is much more challenging to directly measure these values in the human GIT. We had to use data from the literature. Determinations of this depend heavily on the method employed: We found values for small bowel between 261 cm (range 205–371 cm)[34] and 998.5 cm (range 630–1510 cm)[35] and for colon between 109 cm (range 91–125 cm)[34] and 189 cm (range 120–299 cm).[18] One study

compared repeated intraoperative measurements of small bowel length and found considerable differences between the first and the second measurement (580 vs. 485 cm).[36] These results show that the lengths of the intestinal segments vary strongly due to different measuring techniques and between individuals. We finally decided to use values from Teitelbaum et al. for small intestine and from Khashab et al. for large intestine.[18,19] The measurements in the small intestine were performed in a single center in a well-defined group of 240 individuals without known small intestinal problems. Length measurements were done intraoperatively with special care to apply only minimal longitudinal stretch and manipulation to prevent shortening of the intestine.[19] Measurements in the large intestine were done with a roentgenographic method (computed tomography colonography, CTC) in a large group of 505 asymptomatic adults. With CTC it is possible to construct a three-dimensional model of the complete large intestine in-vivo where all segments can be measured with high precision.[18]

The number of neurons in the ENS is often compared to the number of neurons in the spinal cord.[11,24,37] Comparing our data with literature data for the spinal cord shows that these numbers seem to match quite closely for the species examined: Mice of the same strain as we used (C57Bl6) have 2.3–2.8 million neurons in the spinal cord while we found 2.5 million neurons in the ENS.[38] We did not find data for the spinal cord of guinea pigs and can compare here only to numbers for rats, which have 8 million neurons in the spinal cord, similar to our finding of 14.6 million neurons in the guinea pig ENS.[39] Finally, we found 168 million neurons in the human ENS, which is relatively close to 222 million neurons in the human spinal cord.[40] These values indicate that the number of neurons in the ENS and spinal cord are comparable, but the suggestion that the ENS contains more neurons than the spinal cord is not really justified.

In summary, we provide the first in-depth nerve cell counts in mouse, guinea pig, and human ENS using identical techniques, thus allowing direct comparison of neuronal densities and total neuron numbers. Neuronal densities were comparable between the three species and total neuron numbers were related to body size and intestinal length.

AUTHOR CONTRIBUTIONS

Conceived and designed the experiments: KM and MS. Performed the experiments: SD, BK. Analyzed the data: KM. Contributed materials: ED and FZ. Wrote the manuscript: KM and MS. All authors contributed to editing and revising the manuscript. All authors read and approved the final manuscript.

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CONFLICT OF INTEREST

The authors have no competing interests.

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