A three-dimensional analysis of jejunal myenteric ganglia: a tool for interpretation of the optical recording of membrane potential

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Abstract

Introduction: Investigating functions of neurons in the enteric nervous system using imaging techniques (e.g. voltage sensitive dyes) requires precise data concerning the three-dimensional structure of enteric ganglia. Recording from small enteric ganglia such as the submucosal plexus allows for single cell resolution recording, while myenteric ganglia are more complex and single cell resolution claim is not straightforward. Therefore, the aim of the present study was to analyze appositions of neuronal somata in myenteric ganglia.
Material and methods: Stretched samples of guinea pig jejunum were fixed and dissected to expose the myenteric plexus. Tissues were stained for Hu protein and confocal microscopic analysis of myenteric ganglia was performed in order to reconstruct three-dimensional allocations of neuronal somata within the myenteric plexuses.
Results: Myenteric neurons form a three-dimensional structure. Analysis of 103 cells showed that 20% were not overlapping with other cells, while 30% and 31% overlapped with 1 and 2 cells, respectively. Looking at the area of overlap, 42% of cells shared less than 10% of their area. On the other hand, 17% of cells had more than 50% of their area shared with others.
Conclusions: These results show that in guinea pig myenteric ganglia neuronal somata form a monolayer, but up to half of the cells may have a significant overlap with other cell somata. Therefore all optical studies claiming single cell resolution recorded from the myenteric plexus should be confirmed by confocal analysis of the ganglia using immunohistochemical identification of cellular structure of analyzed plexuses.

Key words: myenteric plexus, confocal microscopy, three-dimensional structure, neurons, Hu protein.

Introduction

Understanding the behaviour of the enteric nervous system (ENS) requires a combination of knowledge about the structure and the function of the neuronal networks that regulate the majority of gastrointestinal functions. Electrophysiological recordings can reveal important functional information about individual cells but fail to show how this activity is integrated within the complex networks making up the ENS. On the other hand, purely structural studies on neurochemical coding or cell appositions

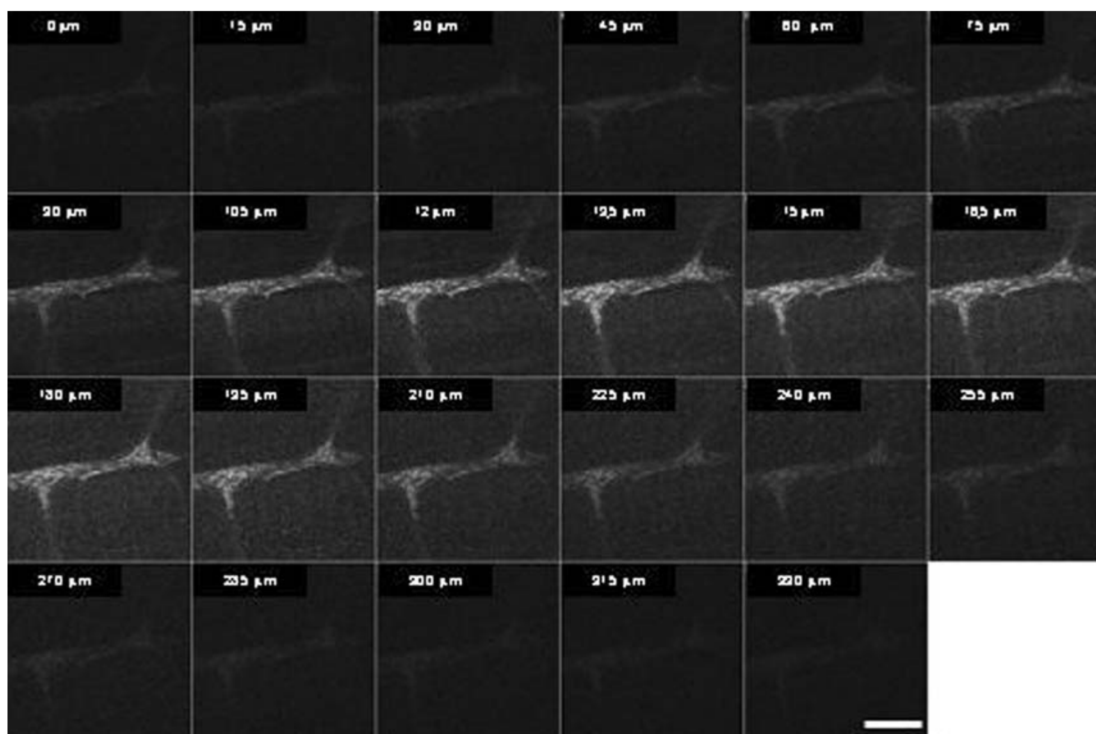

Figure 1. Fluorescence micrographs of staining for Hu-protein in the myenteric plexus of guinea pig ileum. Twenty--three consecutive confocal images of a myenteric ganglion. The depth of the slice was set at 1.5 µm. Scale bar 200 µm

lack information about function but do reveal information about large populations of enteric neurons. The structure and function of myenteric neurons has been the subject of intense research since their discovery in the late 19th century by Auerbach, yet despite this there are still many questions waiting for a precise answer. For instance, little is known about how neuronal signals are propagated between and within enteric ganglia. Recently it has been shown how certain populations of neurons connect with each other [1]. However, this goes only part of the way in showing how the functions of the ENS are orchestrated. The ideal approach would be one in which a large part of the neuronal network was accessible for direct electrophysiological measurement.

Using intracellular recording it has been shown that the morphological classification of neurons according to Dogiel [2] corresponds to their electrophysiological properties. Two types of neurons were identified: S and AH that have Dogiel type I and II morphology, respectively [3]. AH cells have been defined as cells that receive sparse nicotinic cholinergic input and display prolonged hyperpolarization after a single action potential [4-6]. S neurons form a mixed population of monoaxonal motor neurons and interneurons. However, the functional division is not as clear since both types of neurons receive fast and slow synaptic inputs [7]. Therefore, a functional analysis of the spatio-temporal transmission of signals among neurons is the best way to investigate how the information in the ENS is transmitted. The conventional intracellular recording method is not able to give such information. The intracellular recording method has numerous advantages such as a clear record of both incoming and outgoing signals and, when coupled with dye injection, a clear morphological classification. There are, however, certain disadvantages such as biases for

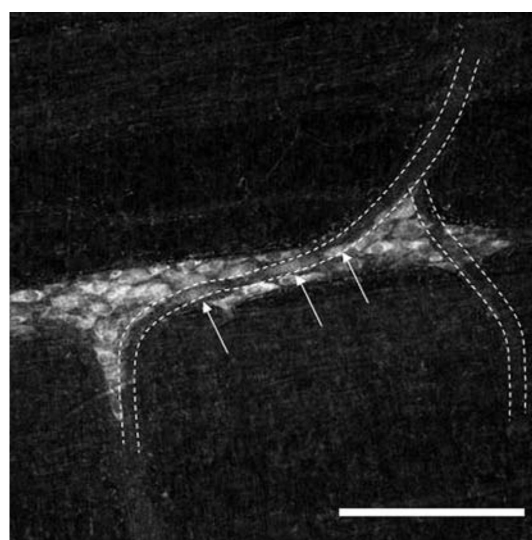

Figure 2. Fluorescence micrographs of staining for Hu--protein in the myenteric plexus of guinea pig ileum. Dotted line shows nerve strands crossing ganglia. Arrows show nerve cells lying over nerve strands. Note that density of cells is higher outside the nerves. Scale bar 200 µm

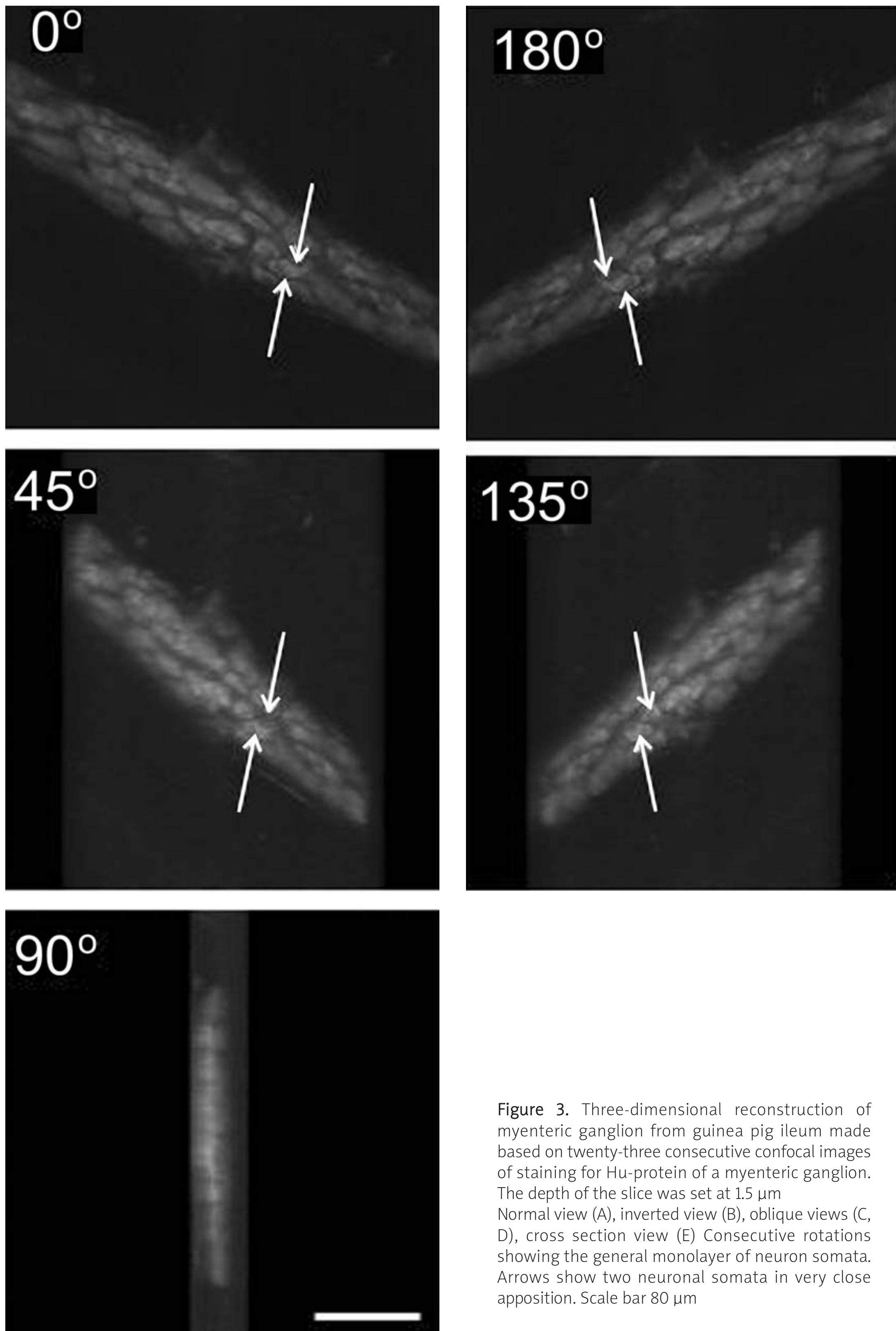

Figure 3. Three-dimensional reconstruction of myenteric ganglion from guinea pig ileum made based on twenty-three consecutive confocal images of staining for Hu-protein of a myenteric ganglion. The depth of the slice was set at 1.5 µm
Normal view (A), inverted view (B), oblique views (C, D), cross section view (E) Consecutive rotations showing the general monolayer of neuron somata. Arrows show two neuronal somata in very close apposition. Scale bar 80 µm

the types of cells impaled, cell trauma due to impalement, and a single cell approach that does not allow for spatial analysis of signal spread and functional analysis of large cell populations. Therefore, a search for an alternative method was underway for many years until finally a reliable optical method of cell membrane potential recording was developed. The first experiments were performed in squid axons showing that changes in optical properties were linearly related to membrane potential [8, 9]. The

studies were further developed toward recording from vertebrate neurons [10]. The function of the ENS has been intensively investigated, showing a number of new features of neuronal function [11-17]. The major assumption made in recent studies is that when using optical recording the system can differentiate signals from adjacent cells [12, 16, 17]. However, there are no dedicated studies investigating positioning of neuronal somata in the guinea pig myenteric plexus, facilitating therefore differentiation of their signal, or presenting that there is an extensive overlapping of neuronal cell bodies that might preclude an analysis of single cell signals. There are two reports addressing the problem of single cell resolution of optical recording [16, 17]. Concerning the submucosal plexus a "virtually two-dimensional" structure was assumed and specially designed spike sorting analysis was applied for recognizing active and non-active cells [16]. However, in the case of experiments applying electrical stimulation the sorting protocol may not be sufficient, especially in the myenteric plexus. Therefore, a precise anatomical analysis is necessary to properly interpret optical signals from neurons. Thus, the aim of the present study was to use confocal microscopy to analyze the three-dimensional structure of myenteric ganglia in the guinea pig ileum and present a numerical expression of neuronal cell soma overlap in the myenteric plexus.

Material and methods

Animals and tissues

The Local Ethical Committee approved all methods used in this study. Three guinea pigs were killed and their gastrointestinal tracts were removed and placed in cold Krebs solution of the following composition (in mM): 117 NaCl, 4.7 KCl, 1.2 $MgCl_2$, 1.2 NaH_2PO_4, 25 $NaHCO_3$, 2.5 $CaCl_2$, and 11 glucose. The jejunum was opened at the mesenteric border, flushed and stretched on a Sylgard-lined Petri dish. Tissues were fixed overnight at 4°C with 4% paraformaldehyde containing 0.2% picric acid in 0.1 M phosphate buffer. After fixing, tissues were washed three times in 0.1 M phosphate buffer. Next, the myenteric ganglion was exposed by blunt removal of mucosa, submucosa and circular muscle. Longitudinal muscle with attached myenteric plexus was pre-incubated for 1 h in 0.1 M phosphate buffered saline solution (PBS) containing 4% horse serum and 0.5% Triton X-100. The tissue was then exposed to primary antiserum diluted in PBS-containing serum and Triton X-100 for 18 h at room temperature. A primary antiserum against Hu protein was used. After three washes in PBS, the preparation was incubated for 2 h in buffer solution containing secondary antibody. Anti-Hu immunostaining has been reported to be an acceptable method for obtaining reliable estimates of total numbers of myenteric neurons in relation to other specific histochemical properties such as histamine binding [18]. The appositions of Hu immunoreactive cell somata were performed using confocal microscopy.

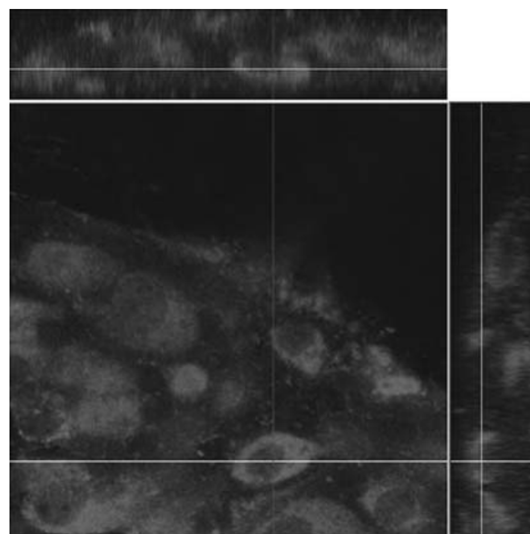

Figure 4. Fluorescence micrographs of staining for Hu--protein in the myenteric plexus of guinea pig ileum (100x). Twenty-three consecutive confocal images of a myenteric ganglion allowed for z axis presentation. The depth of the slice was set at 1.5 µm; x-y (large panel), x-z (upper panel), and y-z (right panel). Scale bar 5 µm

Confocal analysis

Neurons were viewed using a confocal laser-scanning Zeiss LSM 5 PASCAL microscope (Zeiss, Germany) with a HeNe laser of 488 nm and band-pass emission filter of 505 nm. Final magnifications of 40, 63 and 100 were achieved with x40 (Plan-NEOFLUAR, 1.3-Oil, Zeiss), x63 (Plan-APOCHROMAT, 1.4-Oil, Zeiss) and x100 (Plan-APOCHROMAT, 1.4-Oil, Zeiss) lenses, respectively. Volume-rendered 3-D reconstructions of the cells were made using LSM 5 PASCAL Version 3.2 SP2 (Zeiss, Germany) vision image processing. In whole mount preparations a person not involved in the design of the present study chose random ganglia blindly. Optical sections were taken at 0.3-1.5 µm increments. In the present study 12-60 optical sections were made for each ganglion studied and stored on a computer.

Results

Hu-protein staining allowed for confocal imaging of neuronal somata in myenteric ganglia. A general confocal analysis was used to quantify the degree of overlap among neighbouring cells. The thickness of the investigated ganglia ranged from 28 to 31 µm (Figure 1). The average number of cells in one ganglion varied. In one field of view (magnification – 63x) 15-37 neuronal somata could be analyzed for the amount of

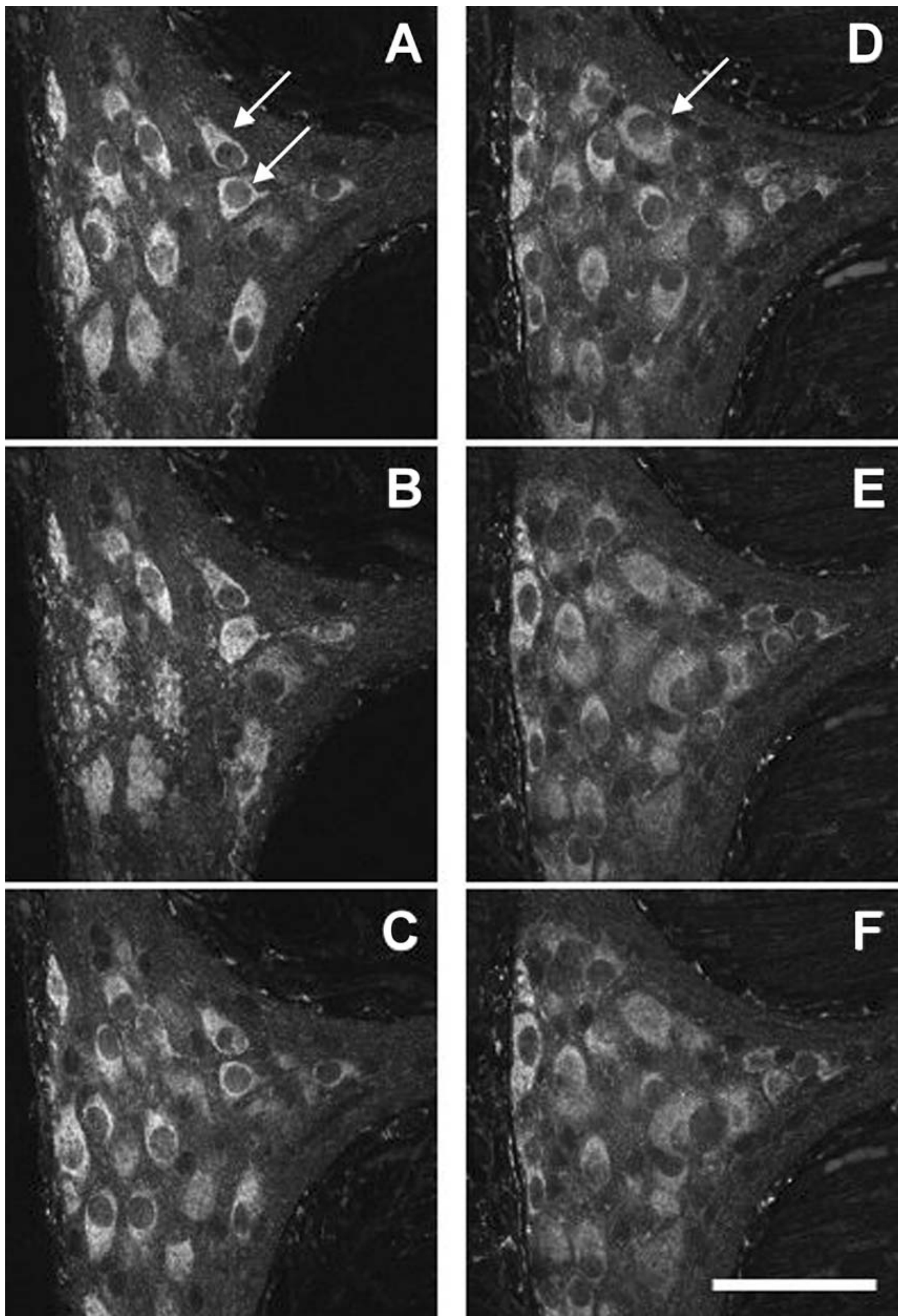

Figure 5. Fluorescence micrographs of staining for Hu-protein in the myenteric plexus of guinea pig ileum (63x). Six consecutive confocal images of a myenteric ganglion. The depth of the slice was set at 1.5 µm. Arrows show overlaying cells. Scale bar 20 µm

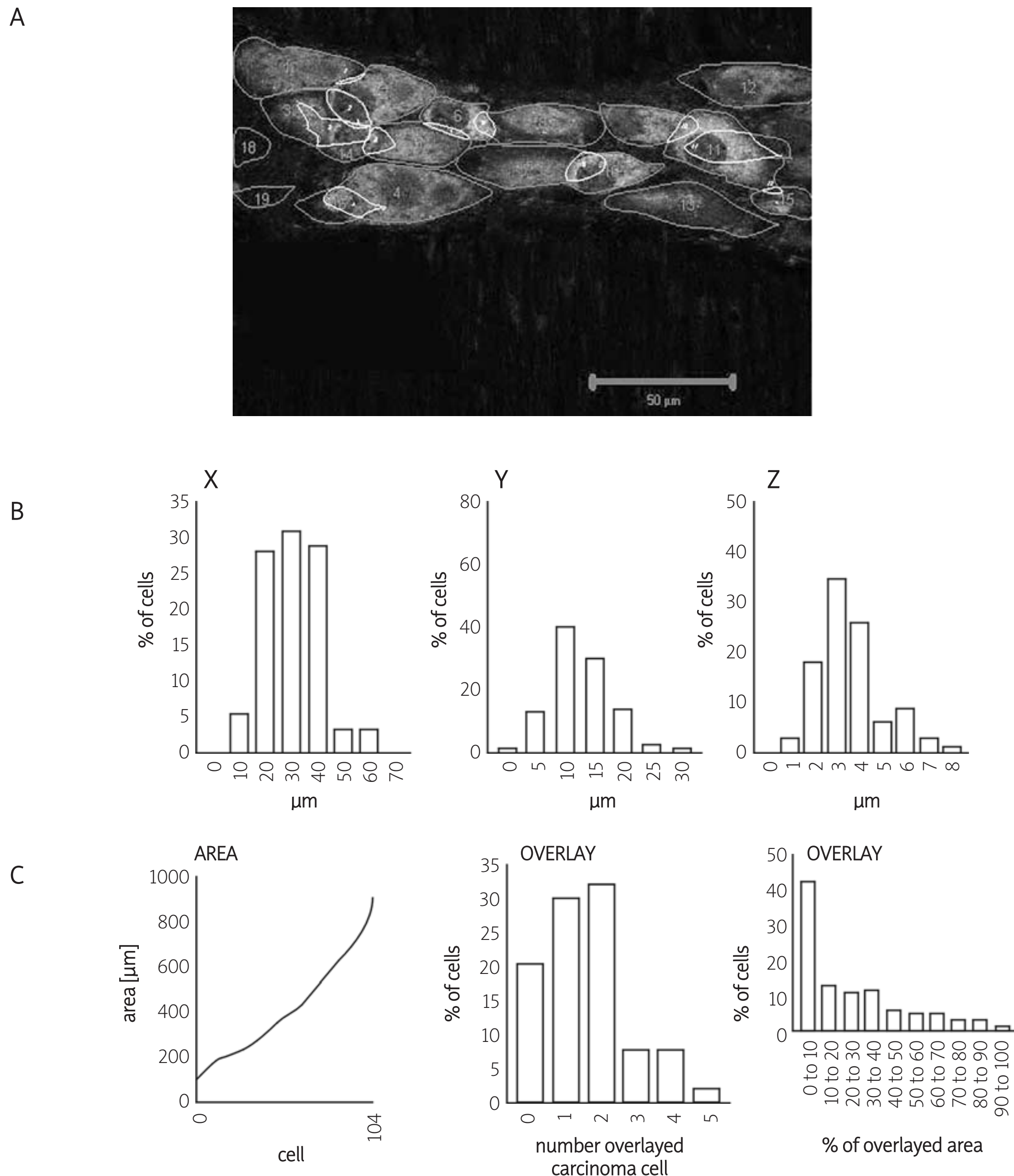

Figure 6. Fluorescence micrograph of staining for Hu-protein in the myenteric plexus of guinea pig ileum (63x) with cell shapes overlaid based on confocal analysis (A). Scale arrow 50 µm. Distribution of neuronal soma sizes (B): circumferential (X), longitudinal (Y) and luminal-serosal (Z). Distribution of neuronal soma areas (C, left panel). Overlay analysis: number of overlay incidences/cell (C, middle panel) and distribution of overlaid area of analyzed cells (C, right panel). Data obtained from 104 cells

overlap with others. The distribution of cells in ganglia was unequal. For example, there were fewer cells over nerve tracts passing through ganglia than in neighbouring areas with only cell bodies. On the other hand, in close proximity to the nerve tracts, cells were packed more tightly (Figure 2). In areas further from nerve fibres, overlap was less frequent and the majority of cells formed a monolayer (Figure 3). An analysis at higher magnification showed that neurons did form a monolayer, but that there were some shifts in the optical plane in which the cells were imaged. In such areas the overlap between neurons was minimal (Figure 4). On the other hand, there were some areas where neurons were very close to each other and formed multi-layered structures with almost total overlapping of adjacent cell bodies (Figure 5). A precise confocal analysis was performed on 103 cells from 4 ganglia. Cell somata have elliptical shapes flattened in the z-axis. The circumferential axis of neuronal somata ranged from 14 to 69 µm, while the longitudinal axis was shorter and ranged from 3 to 34 µm. In the z-axis (luminal-serosal axis) neuronal soma size ranged from 2 to 8 µm^2 (Figure 6). The area of neuronal soma ranged from 100 to 890 µm (Figure 6). In the analyzed population of neurons, 20% did not overlap with any other Hu-positive cell somata. Overlap with one cell

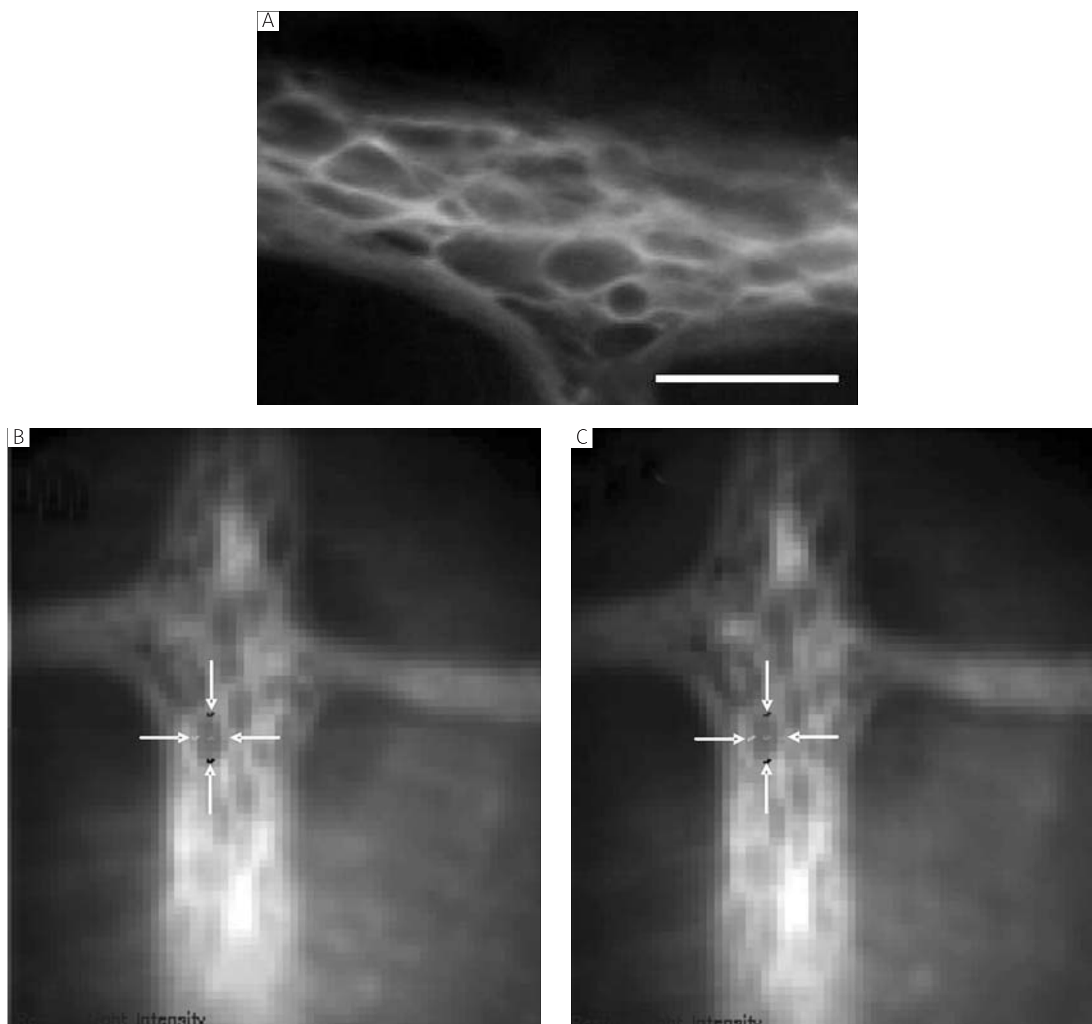

Figure 7. A – fluorescence micrograph of staining using voltage sensitive dye Di-8-ANEPPS (magnification x40 oil immersion objectives (UAPO/340 Olympus, Hamburg, Germany) by using a filter cube equipped with a 545±15 nm excitation interference filter, a 565 nm dichroic mirror and a 590 nm barrier filter (Olympus, Hamburg, Germany). Staining was done using intraganglionic microinjection method. Scale bar 50 µm. B and C – consecutive microphotographs of myenetric ganglion taken by ultrafast camera (RedShirtimaging, Fairfield, CT, USA). The CCD system had 70x70 pixels yielding a spatial resolution of 22 µm with the x40 objective) taken 47 minutes apart. The markers indicated by arrows show that the neuron did not move

was observed in 30%, while 31% made overlays with two neighbouring cells (Figure 6). The majority of the cells showed only marginal overlap. Over 40% of cells showed overlap between 0 and 10% of the vertical soma projection with neighbouring cells. The remaining population of cells showed an equally distributed area of overlap (Figure 6).

Discussion

The topic of the three-dimensional structure of myenteric ganglia that was investigated in the present study has not been previously investigated in detail. A similar analysis was performed in the sympathetic inferior mesenteric ganglion and the sympathetic--parasympathetic pelvic hypogastric ganglion [19]. Further, a detailed three-dimensional study of enteric neurons was performed by the same group [20], but only single neurons and their individual structures were analyzed. They have shown that myenteric neurons have different shaped somata, but on average they are flat with an x-y axis size that is 3- to 4-fold bigger than their z-axis depth, which was shown to range from 8.0 to 11.4 µm. In the present study, the flat disc shape of neuronal somata had similar proportions; however, neurons were on average thinner. The size of neurons in the preparations used for immunohistochemical studies depends on the stretch of the tissue applied during fixing. Unfortunately stretching is necessary to access structures in the gut. However, for these types of experiments the procedure gives reproducible results, so recordings from various plexuses are comparable.

In studies using optical recording of membrane

potential in enteric neurons [11-17] the general assumption has been that the placement of neurons is two-dimensional with minimal overlap between adjacent neuronal somata. The answer to whether this is a true assumption is crucial for a precise analysis of the signals recorded by the optical method. This is most likely true for the submucosal plexus [16, 17], but in the case of the myenteric plexus it seems very unlikely. The present study shows that a certain number of neuronal somata do lay uncovered by others. Therefore, they are readily accessible for optical recording. There are, however, a significant number of cells that overlap with neighbouring cells. To interpret the optical signal from these cells requires a precise structural analysis of the degree of overlap. On the other hand, the view of a ganglion stained with a voltage sensitive dye (Figure 7) suggests that the optical detector receives a considerable signal from only one cell layer [13-15]. Since the optical recording uses relatively simple optics that reduces light loss, the depth of field recorded by the microscope lens depends on the vertical resolution of the signal. The present study provides numerical data that can be used to assess whether applied parameters of the recording machine give a sufficiently shallow focal plane to record the signal from single neuronal somata.

Another problem that arises during recording from the myenteric plexus is muscle movement [17]. Indeed, slight movements happen, but usage of high resolution cameras allow for precise spatial averaging from chosen areas responding to certain neuronal somata for each recording even if they move (Figure 7). In fact an ideal analysis should be based on three-dimensional analysis for each recording, but the speed of the recording is still beyond the requirements for such analysis. There is no available report showing a reliable post-recording anatomical analysis of myenteric ganglia allowing identification of signal origin obtained by the optical method. In the present study it was possible to differentiate cells in various focal planes, showing that it is possible to precisely analyze the distribution of neurons in the myenteric plexus. The light intensity at the margins of average sized neurons changes 3- to 4-fold, which suggests that the signal recorded by the optical method can be interpreted as a signal from a single neuronal soma. Such spatial resolution can be achieved only using a confocal microscope. Fast optical recording is much less sensitive and detectors must receive more light; therefore focal planes are considerably thicker than those of confocal microscopes. The areas of interest – edges of overlapping neuronal somata – are in the range of tenths of micrometres. Such minimal variations are most likely undetectable by the present optical recording machines.

Another thing that has to be mentioned is that structures that were not shown by Hu staining are the sub-cellular structures such as axons and neuropile that fill the space among neuronal somata. This is a problem that has to be taken into consideration when analyzing optical signals. Since there is no single marker for neuronal processes in the myenteric plexus, identification of neuronal strands surrounding neuronal somata has not been done completely yet. An overview of neuronal structures with various coding was shown in a hamster myenteric plexus [21], while precise electron micrographs were shown in a rat [22]. However, analysis suitable for optical recording still needs to be done.

In normal conditions in the gut the tension applied to the myenteric ganglia is lower than that applied when the gut is prepared for neurological experiments. Therefore, surely the structure of ganglia is composed of two or more layers of neuronal soma. However, for experimental conditions the structure achieved in vitro is important, and this was studied in the present study.

Conclusions

The present study illustrates the expected fact that a monolayer of enteric neuronal somata in the myenetric plexus does not exist. However, it is possible to analyze the three-dimensional structure of the myenetric ganglion in detail. Therefore, in the present state of equipment for optical recording the single cell resolution claim, at least concerning the myenetric plexus of the guinea pig jejunum, needs a precise post-recording anatomical analysis. Otherwise it is very likely that a considerable part of the signals received from VSD using optical recording of the cell membrane potential will originate from more than one cell. Moreover, the problem of spontaneous movement and distortion of the ganglion during the experiment has to be evaluated as well as signals coming from nerve strands surrounding neuronal somas.

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