Neurovascular Interface in Porcine Small Intestine: Specific for Nitrergic rather than Nonnitrergic Neurons

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Key Words

Blood supply · Enteric nervous system · Nitrergic neuron · Neurohumoral interaction · Vascularization

Abstract

In the 1970s, by using classic histological methods, close topographical relationships between special areas of enteric ganglia and capillaries were shown in the pig. In this study, by application of double and triple immunohistochemistry, we confirmed this neurovascular interface and demonstrated that these zones are mainly confined to nitrergic neurons in the myenteric and the external submucosal plexus. In the upper small intestine of the pig, the respective neurons display type III morphology, i.e. they have long, slender and branched dendrites and a single axon. In another set of experiments, we prepared specimens for electron-microscopical analysis of these zones. Both ganglia and capillaries display continuous basement membranes, the smallest distances between them being 1,000 nm at the myenteric and 300 nm at the external submucosal level. The capillary endothelium was mostly continuous but, at the external submucosal level, scattered fenestrations were observed. This particular neurovascular relationship suggests that nitrergic neurons may require a greater amount of oxygen and/or nutrients. In guinea pig and mouse, previous ischemia/reperfu-

sion experiments showed that nitrergic neurons are selectively damaged. Thus, a preferential blood supply of enteric nitrergic neurons may indicate that these neurons are more vulnerable in ischemia.

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Introduction

In contrast to the deciphering of the structures and functions of the enteric nervous system, its blood supply received less attention during the last decades. In an early publication, silver impregnation was combined with intravascular injection of Indian ink (partly together with gelatine), and a specialized capillary network surround-

Abbreviations used in this paper

BSA	bovine serum albumin
CD31	cluster of differentiation 31
DAB	diaminobenzidine
NADPH	nicotinamide adenine dinucleotide phosphate
NF200	neurofilament 200
nNOS	neuronal nitric oxide synthase
PB	PO ₄ buffer
TBS	tris-buffered saline

Table 1. Antisera

	Host	Dilution	Source
<i>Primary antisera</i>			
Antigen			
CD31	rabbit	1:50	Ab28364; Abcam, Germany
NF200	mouse	1:200	N0142; Sigma, Germany
nNOS	goat	1:200	GTX89962-100; Biozol, Germany
<i>Fluorescence tags for secondary antisera</i>			
Alexa Fluor 555	donkey anti-rabbit	1:1,000	A31572; Life Technologies, Germany
Alexa Fluor 488	donkey anti-mouse	1:1,000	A21202; Life Technologies, Germany
Alexa Fluor 647	donkey anti-goat	1:1,000	A21447; Life Technologies, Germany
<i>Reagents for fine structural immunohistochemistry</i>			
Biotin	sheep anti-mouse	1:400	RPN1001; Amersham Biosciences, Germany
Vectastain Elite ABC kit			PK-6100; Linaris, Germany
3,3'-DAB tetrahydrochloride			CN75.1; Carl Roth, Germany

ing myenteric ganglia was demonstrated in the cat [Iwanow and Radostina, 1937]. By applying principally the same methodological combination, Stach [1977a; 1978a, b; 1979] was able to show that the myenteric and submucosal ganglia of the pig and the cat are composed of morphologically different types of neurons, and that some of them (i.e. uniaxonal neurons with long, slender, branched dendrites) were more intensely surrounded by capillary networks than others.

Ultrastructurally, both enteric ganglia [Baumgarten et al., 1970; Gabella, 1972; Wilson et al., 1981] and intestinal capillaries [Clementi and Palade, 1969; Simionescu et al., 1972] have been thoroughly investigated. Important observations in these and other studies were that the enteric nervous tissue consists of neuronal and glial components which are surrounded by a basement membrane, and that connective tissue and blood vessels do not enter the enteric ganglia and nerve strands in the small and large intestines. Gershon and Bursztajn [1978] demonstrated limited access of macromolecules from the blood stream to myenteric ganglia, and postulated a blood-myenteric plexus barrier. To our knowledge, similar investigations concerning the submucosal plexus were not performed. Furthermore, analysis of specific parts of myenteric or submucosal ganglia was never considered.

In this study, we tried to illustrate both enteric nerve plexus and blood vessels in the porcine small intestine, by means of immunohistochemistry, for cluster of differentiation 31 (CD31, an endothelial marker) and neurofilament 200 (NF200, a structural neuronal marker). In view of an earlier study [Brehmer et al., 1998], we attempted to demonstrate the close proximity of specific myenteric

and submucosal neurons to blood vessels. Furthermore, a fine structural analysis of the capillary segments approaching the enteric ganglia was included. Our aim was to provide morphological and immunohistochemical data as a basis for functional interpretations of the approaching zones between enteric blood vessels and ganglia. This may expand our understanding of gastrointestinal blood flow regulation and neurohumoral interaction [Granger et al., 2015].

Materials and Methods

Tissue Processing

Tissue was obtained from 5 pigs (both sexes) aged between 12 and 15 weeks that had been killed at a registered slaughterhouse in Fürth, Germany. The European Communities Council Directive and animal welfare protocols approved by the local government were followed.

Jejunal segments (each approx. 30 cm in length) were transferred to the laboratory in iced Krebs solution. Thereafter, they were rinsed in Krebs solution at room temperature and transferred into DME/F-12 media (Sigma Chemical Co., St. Louis, Mo., USA) containing 10 mg/ml antibiotic-antimycotic, 50 µg/ml gentamycin, 2.5 µg/ml amphotericin B, 10% fetal bovine serum (all from Sigma), 4 µM nicardipine and 2.1 mg/ml NaHCO₃, bubbled with 95% O₂ and 5% CO₂ at 38.5°C for approximately 2 h.

Immunohistochemistry and Image Acquisition

One half of the segments (approx. 15 cm in length) were immersion-fixed under moderate distension in a solution of 4% formalin in 0.1 M PO₄ buffer (PB; pH 7.4) at room temperature for 2–3 h. They were cut into pieces measuring about 2 cm in length and 1 cm in width which were subjected to whole-mount preparation. This resulted in submucosal whole mounts containing the submucosal plexus and myenteric whole mounts containing

Fig. 1. Capillary network architecture on the level of the myenteric plexus (**a**: red – CD31). A rectangular pattern dominates, but some areas (between the 2 arrowheads) display a more irregular and dense accumulation of capillaries. These surround only parts of the ganglia (**a'**: green – NF200) whereas territories displaying clusters of morphological type II (II) neurons lie outside (**a''**).

the serosa and longitudinal muscle with the adhering myenteric plexus.

Both myenteric and submucosal whole mounts were then pre-incubated for 2 h in 0.05 M tris-buffered saline (TBS; pH 7.4) containing 1% bovine serum albumin (BSA), 0.5% Triton X-100, 0.05% thimerosal and 5% normal donkey serum. After a rinse in TBS for 10 min, they were incubated in a solution containing BSA, Triton X-100 and thimerosal at the same concentrations and the primary antibodies, CD31, NF200 and neuronal nitric oxide synthase (nNOS; table 1) for 72 h (4°C). After an overnight rinse in TBS at 4°C, the secondary antibodies (table 1) were added to the same solution as the primary antibodies (for 4 h at room temperature) followed by a rinse in TBS (overnight at 4°C). Whole mounts were mounted with TBS-glycerol (1:1; pH 8.6). Incubations of whole mounts in solutions lacking primary antisera served as negative controls.

Whole mounts were evaluated using a confocal laser scanning microscope system (Nikon Eclipse E1000-M; Nikon Digital Eclipse C1; Tokyo, Japan) equipped with the following laser configuration: 488 nm argon laser, 543 nm helium-neon-laser, 647 nm diode-laser (from Coherent, Santa Clara, Calif., USA and Melles Griot Inc., Carlsbad, Calif., USA). The 3 laser lines were assigned varying pseudocolors (fig. 1, 2). They were completed utilizing Nikon FreeViewer software (EZ-C1 3.30), Volocity Demo 6.1.1, Adobe Photoshop CS6 and CorelDraw X6.

Ultrastructural Histochemistry and Immunohistochemistry

The other half of the jejunal segments were fixed under moderate distension in a PB-solution containing 0.05% glutaraldehyde in addition to 4% paraformaldehyde. Samples were rinsed in 50% ethanol (4 × 10 min). After washing in PB (4 × 10 min), specimens were incubated in 1% sodiumborohydride in 0.1 M PB for 1 h. Segments were then washed in PB and stored overnight in PB (4°C).

Whole mounts were prepared as described above. To allow visualization and following trimming of type III/nitrergic neurons

for fine structural investigation, 3 methods were tried [Brehmer et al., 1998; Brehmer, 2006]: immunohistochemistry for NF200 and nNOS, each visualized by the diaminobenzidine (DAB) reaction, and histochemistry for the nicotinamide adenine dinucleotide phosphate (NADPH) reaction, which, after formalin fixation, reveals complete colocalization with nNOS immunoreactivity [Brehmer et al., 1998]. Since nNOS/DAB immunohistochemistry resulted in weaker labeling in our specimens, only the NF200/DAB-stained and the NADPH-stained specimens are described and depicted below.

Whole mounts scheduled for NF200/DAB immunohistochemistry were incubated in 40% methanol and 0.3% H₂O₂ dissolved in distilled water (for blocking endogenous peroxidase). Preincubation was carried out in TBS containing 1% BSA, 0.05% thimerosal, 0.05% Triton X and 5% normal donkey serum for 1 h at room temperature. Thereafter, whole mounts were incubated with the primary antibody against NF200 (1:200) in TBS containing 1% BSA, 0.05% thimerosal and 0.05% Triton-X 100 for 72 h (4°C). Specimens were washed for 1 day in TBS (4°C) and were incubated in biotinylated sheep-anti-mouse IgG (1:400; Amersham) dissolved in the same solution as for primary antibodies for 4 h at room temperature. After washing, they were incubated for 3 h at room temperature in avidin-biotin horseradish-peroxidase (Linaris, Germany). Following a rinse in PB, the DAB reaction (Carl Roth, Germany) was carried out.

Whole mounts scheduled for NADPH histochemistry were incubated in a solution containing 0.25 mg/ml nitro blue tetrazolium, 1 mg/ml β-NADPH and 0.5% Triton X-100 in 0.1 M PB (pH 7.4) for about 60 min at 37°C.

All specimens were washed in PB and screened under a stereomicroscope for myenteric and external submucosal ganglia containing significant aggregations of NF200/DAB-stained and NADPH-stained type III neurons, respectively. These whole mounts were trimmed to pieces of about 3 × 3 mm [Brehmer, 2006], dehydrated in graded ethanol solutions and acetone and

Fig. 2. Neurovascular interface at the level of the myenteric (**a**) and the external submucosal plexus (**b**), demonstrated as extended-focus images of the top view in the whole mounts. Left to right: merged images, capillaries (red – CD31), ganglia stained for nNOS (yellow) and NF200 (green). Arrowheads mark territories with clustered dendritic, nNOS-positive neurons approached by capillaries.

flat-embedded in epon [Brehmer et al., 1994]. Appositions between neurons and vessels were located in semithin sections. Thin sections through these areas were cut, stained with lead citrate for 15 min and viewed under a Zeiss 906 electron microscope (Oberkochen, Germany) equipped with the Olympus soft-imaging system (Münster, Germany).

Results

Observations in Whole Mounts Stained for CD31, nNOS and NF200

On the myenteric plexus level, the general capillary pattern was mostly rectangular, paralleling the arrange-

ment of the surrounding muscle layers. Contrasting with this pattern, there were conspicuous small areas with a denser but rather irregular, glomerular arrangement of capillaries (fig. 1). Although pointing to underlying ganglia, these glomeruli covered only parts of them. Most strikingly, nondendritic, multiaxonal type II neurons were located outside of the glomeruli. The glomeruli typically covered accumulations of nitrergic neurons within the ganglia. Based on their NF staining, these were uniaxonal type III neurons with long, slender and branching dendrites distributed radially around their soma (fig. 2a).

In the submucosal whole mounts, there were fewer capillaries between greater vessels when compared to the

Fig. 3. Neurovascular interface at the level of the myenteric plexus, demonstrated in ultrathin sections perpendicular to the whole-mount layer. **a** Overview showing a myenteric ganglion. On one side, the longitudinal muscle layer is present, but on the other side only remnants of the circular muscle layer are present due to the whole-mount preparation. Between the ganglion and small blood vessels (stars), smooth-muscle cells and processes of fibrocytes can be identified. **b** The arrow points at the space between a capillary and a myenteric ganglion. (CM) = Remnants of circular muscle layer; LM = longitudinal muscle layer; N = NADPH-stained neuron.

myenteric plexus level. Nevertheless, small areas with similar capillary nets were obvious. They were exclusively related to parts of the external submucosal ganglia that harbored nitrergic neurons of type III morphology (fig. 2b). In contrast, we did not find accumulations of nitrergic neurons within or dense capillary glomeruli around the internal submucosal ganglia.

In both the myenteric and submucosal specimens, we observed that the more the nitrergic type III neurons were clustered, the more striking was the capillary net around them, and vice versa, i.e. the less they were clustered, the less capillaries were seen. In the latter case, the arrangement of a capillary near a single nitrergic neuron looked rather like accidental proximity.

Fine Structural Observations

Ultrathin cross sections through regions of myenteric and external submucosal ganglia harboring clusters of NF200-stained and NADPH-stained type III neurons, respectively, were studied.

Around the myenteric ganglia (fig. 3), small vessels including capillaries were found, both on the luminal and abluminal side. Sometimes, processes of fibrocytes or single smooth-muscle cells were located in between. The smallest distances between the basal laminae of the capillary and the myenteric ganglion measured were about 1,000 nm. Capillaries revealed both a continuous endothelium and basement membrane.

Around the external submucosal ganglia, capillaries were also found on both sides (luminally and abluminally). In contrast to the myenteric level, the capillaries were located closer to the ganglia (fig. 4a–c), with the smallest distances observed being about 300 nm. Both the ganglionic

and the capillary surfaces were covered by a basement membrane. The capillary endothelium was usually continuous, but scattered fenestrations were also found (fig. 4d).

In both the myenteric and external submucosal neurons approached by capillaries, the neuronal surfaces were covered directly by the basement membrane without intervening glial cells.

Discussion

In this study, we confirmed the close topographical relationship between particular enteric neurons and capillary nets (neurovascular interface), which was first described by combining silver impregnation with intravascular filling [Stach 1977a; 1978a, b; 1979]. As an alternative approach, we took advantage of immunohistochemical colabeling of neurons and endothelium. This enabled us to chemically characterize the respective neurons and analyze the fine structure of the approaching vessels.

Enteric Neurons Approached by Capillaries

Earlier studies by Stach [1977a; 1978a, b; 1979] showed that long-dendritic, uniaxonal neurons were preferentially surrounded by capillaries. Only subsequently were these neurons termed as type III neurons in the pig small intestine [Stach, 1982]. The reasons for this belated denotation were analyzed previously [Brehmer et al., 1999].

Later studies documented that these type III neurons are nitrergic [Timmermans et al., 1994; Brehmer and Stach 1997; Brehmer et al., 1998; Timmermans et al., 2001]. Both the questions of the equivalence of silver impregnation and NF immunohistochemistry and of the

Fig. 4. Neurovascular interface at the level of the external submucosal plexus, demonstrated in ultrathin sections perpendicular to the whole-mount layer. Arrows point at the space between the ganglia (among nerve fibers). **a** Capillary with continuous endothelium on one side of a ganglion. **b** Capillaries on both sides of a ganglion (the left capillary contains erythrocytes and the right one contains a leukocyte). **c** Extremely narrow gap between a ganglion and a capillary. **d** Occasionally, fenestrations of the endothelium could be identified (arrowheads). N = NF200-marked neurons.

morphochemical identification of nNOS-reactive type III neurons have been dealt with previously [e.g. in Brehmer, 2006]. Thus, it was tempting to test the hypothesis that the approached neurons were nitrergic and that, by applying NF costaining, they display type III morphology. The neurons not preferentially approached by capillaries, among them the morphologically striking, nondendritic type II neurons, were shown to be nonnitrergic but cholinergic [Brehmer et al., 2004; Wolf et al., 2007]. It can be assumed that additional neuroactive substances are colocalized with nNOS in the respective myenteric and submucosal type III neurons [Timmermans et al., 2001; Kapp et al., 2006; Wojtkiewicz et al., 2012; Petto et al., 2015]. The further deciphering of the chemical code of porcine type III neurons including quantitative data will be performed in a future project. The desirable quantification of the neurovascular interface will be difficult to realize since smaller accumulations of nitrergic neurons are approached by less conspicuous capillary nets. It will thus be difficult to define the cut-off criteria.

Capillaries Approaching Enteric Neurons

Early electron-microscopical investigations revealed fenestrations in the mucosal and submucosal but not in the intramuscular capillaries [Clementi and Palade, 1969; Simionescu et al., 1972; Gershon and Bursztajn, 1978]. We also found differences between the myenteric and submucosal capillaries that we investigated. The shortest distances between ganglionic surface and capillary was considerably smaller on the external submucosal than on the myenteric level (300 vs. 1,000 nm). Occasional endothelial fenestrations were seen in external submucosal rather than in myenteric capillaries. This difference in endothelial architecture and in distance between capillaries and ganglia will be discussed below.

Functional Interpretation of the Neurovascular Interface

The most likely interpretation of the differential capillary supply of various enteric neuron types (most strikingly: type III vs. type II) may be that different types of neurons may require different amounts of oxygen and/or nutrients. In guinea pig and mouse, Rivera et al. [2009; 2011b] found selective damage to nitrergic neurons following experimental ischemia and reperfusion. Although these results and our morphochemical data were raised in different species, it may be concluded that enteric nitrergic neurons require a preferred blood supply (in the pig, e.g. type III neurons), and are therefore more vulnerable to limitation of the blood flow [Rivera et al., 2011a] than

other neurons (in the pig, e.g. type II neurons). The difference in endothelial architecture and in distance between capillaries and ganglia may be due to the fact that the nitrergic type III neurons are more aggregated in the external submucosal ganglia [Stach, 1977b] than in the myenteric ganglia.

Endothelial fenestrations are characteristic for capillaries of endocrine glands. Given their occasional presence found in capillaries approaching external submucosal ganglia, it may be that, probably in addition to the above function, products released from respective neurons enter the blood stream or influence capillary function. A first step to test this hypothesis should be to check the further chemical coding of the respective nitrergic neurons, followed by functional experiments. In contrast, the neurovascular interface described here may represent a gate from the blood stream to neurons, beyond oxygen and nutrients, for toxic agents suspected to cause, for example, Parkinson's disease [Braak et al., 2006; Pan-Montojo et al., 2010].

Studies on the topographical relationship between enteric ganglia and blood vessels in human intestines are ongoing. The distribution pattern of putative neurovascular interface (if it exists in humans) onto the different enteric plexus may differ markedly from those in the pig since, for instance, the neuronal composition of pig and human external submucosal ganglia is quite different [Beuscher et al., 2014].

Acknowledgements

The excellent technical assistance of Karin Löschner, Stefanie Link, Anita Hecht, Andrea Hilpert and Hedwig Symowski is gratefully acknowledged.

Disclosure Statement

The authors declare that there is no conflict of interests.

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