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Intestinal Auerbach plexus structures with NADH histochemistry in three strains of spontaneous diabetic rats

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Summary
The enteric nervous system comprises two major systems: the submucosal and the myenteric plexus. The aim of this study was to describe the myenteric plexus from three strains of spontaneous diabetic rats from the histological point of view. Samples of small intestine and of proximal and distal colon were obtained from three spontaneous diabetic strains i.e., fSS, fSMT, B strains and 1-year old Wistar rats. Specimens were stained with NADH (β-nicotinamide adenine dinucleotide, reduced form) histochemical technique and examined with light microscope. Microscopically little modifications in mesh-like structure of intestinal Auerbach’s plexus from fSS were detected in comparison with Wistar rats samples. Intestinal plexus of fSMT and fB rats showed disruption of mesh-like structures, modifications in the slightly colored background (smooth muscle) and augmented vascularization. Small intestine and colon are affected. In short: In our spontaneously diabetic rat models, mesh-like structure of Auerbach’s plexus is strain dependent.

Key words: myenteric plexus, ageing, diabetes, rat intestine.

Estructuras del plexo de Auerbach intestinal con histoquímica del NADH en tres líneas de ratas espontáneamente diabéticas

Resumen
El sistema nervioso entérico comprende dos sistemas mayores: el plexo submucoso y el plexo mientérico. Este estudio describe la estructura histológica del plexo mientérico en tres líneas de ratas espontáneamente diabéticas. Especímenes de intestino delgado, colon proximal y colon distal fueron obtenidos de tres líneas de ratas espontáneamente diabéticas: fSS, fSMT, B y Wistar de 1 año de edad. Los materiales obtenidos fueron procesados con la técnica histoquímica del NADH (β-nicotinamida adenina dinucleotido, forma reducida) y observados en un microscopio óptico. Pequeñas modificaciones histológicas en la estructura reticular del plexo de Auerbach intestinal pueden ser detectados en las ratas fSS cuando son comparadas con las ratas Wistar. La estructura reticular del plexo de Auerbach de las ratas fSMT y fB muestran una desaparición de dicha estructura reticular, disminución de la coloración de base (músculo liso) y un aumento de la vascularización. Tanto el intestino delgado como el colon están afectados. Resumiendo: en nuestros modelos experimentales de ratas diabéticas la estructura reticular del plexo de Auerbach es dependiente de la línea de rata estudiada.

Palabras claves: plexo mientérico, envejecimiento, diabetes, intestino, rata.

The gastrointestinal tract contains the largest number of neurons, outside the central nervous system, constituting with glial cells the enteric nervous system (ENS). ENS is characterized by ganglia and nerve tracts forming different shaped nerve networks within the walls of the gastrointestinal tract. The final motor neurons of the gastrointestinal tract are mostly located within ENS ganglia, the so called the Meissner’s submucosal plexus and the Auerbach’s myenteric plexus. The myenteric plexus is visualized as mesh-like laminar structures with the NADH (β-nicotinamide adenine dinucleotide reduced form) technique developed by Gabella. The mesh formed by ganglia and connecting strands presents a regular, although not quite geometrical...
pattern, which is characteristic of each gut segment and to some extent also of animal species. Most of the early reports about Auerbach’s plexus structure in diabetic rats deals with streptozotocin-treated rats, whereas the present study aims to describe the Auerbach’s plexus by means of the NADH histochemical technique in different spontaneously diabetic rat strains.

Materials and methods

Twenty male rats aged 12 months (n=5 each group), were obtained from different animal husbandries: 1. Obese β line rats (II Mb/Fm b) were raised in the facilities of our School of Medicine (Department of Biology), being the original IIM inbred stock a set of several rat strains bred as parallel lines from a single outbred colony in 1948 (3,4). β rats developed diabetes almost at 8 months of age. 2. eSS (IIME/Fm eSS) derived from the same original IIM inbred colony, developed diabetes at 6 months of age. 3. eSMT strain (II Me/Fm eSMT) raised in the facilities of the School of Medicine (Department of Biology), derived from a crossbred between β and eSS rats, developed diabetes at 3 months of age. 4. Male Wistar rats were provided by the Central Animal House of the School of Biochemistry. The animals fed with commercial balanced food (Cargill, Argentina) and provided with tap water ad libitum, were kept at 22-24°C and 12-h light/12-h dark cycles. The animals were killed at 12 months of age by ether overdose. The abdominal cavity was opened and the small intestine, from duodenal-pyloric junction down to ileo-cecal junction and colon were dissected and removed from ileo-cecal junction up to 2 cm above the anus. Gut was flushed with 4ºC PBS (phosphate buffered saline) to remove content and trimmed off fat and mesentery.

A segment of small intestine was sealed at both ends with linen thread, instilling cold PBS with a syringe at one end. When the specimen started to curl the ligature was tightened. Proximal and distal colon were similarly processed. Accordingly to Freeman et al., proximal (ascending colon) could be distinguished grossly by the presence of oblique mucosal folds; distal (descending) colon, may be distinguished grossly by the presence of longitudinal folds. Gabella's NADH1 reaction was performed with the following modifications: specimens were incubated in 0.5% Triton X-100 for 10min and after a brief washing in PBS, they were incubated for 60-70min in 10mg Nitroblue tetrazolium (Gibco, Japan), 20mg NADH (Gibco, Japan), 20ml PBS and 20ml distilled water. They were rinsed 2-3 times in PBS, and fixed in 3% paraformaldehyde (Merck, USA) in PBS for 24 hours at 4ºC. Samples were obtained cutting the mesenteric border and placed in Petri dishes with the serosa surface down. The mucosa and submucosa were carefully peeled off under dissecting microscope (Leitz) using watchmaker forceps (Brussels’s type # 7), obtaining 1cm wide strip of both circular and longitudinal muscle including the myenteric plexus between the muscle layers attached to the serosa. Samples were rinsed in PBS and mounted with Kerno’s syrup and formaldehyde with the serosa up. The experimental protocol was approved by the Ethical Committee of the School of Medicine, UNR, Argentina.

Results

Small intestine: The mesh-like structure was almost preserved in Wistar rats (figure 1A). Round or oval NADH-positive neurons, lightly stained, NADH-negative cytoplasmic neurons and empty spaces, called "neuronal ghost" by Santer and Baker, were observed. Nerve tracts were almost absent or lightly stained. Smooth muscle fibers from muscularis externa were slightly stained resembling parallel lines and scarce blood vessels were seen. Zones of disrupted mesh-like structures were accompanied with smooth muscle fibers, resembling parallel lines superimposed with clotted stained clusters. In eSS rats (figure 1B) mesh-like structures were preserved. Partial disruption of mesh-like zones could be observed, with isolated or grouped neurons, whereas smooth muscle fibers from muscularis externa were slightly stained, resembling parallel lines superimposed to clotted stained clusters, some empty spaces were seen in ganglia. NADH-positive nerve tracts, increased blood vessels, and some NADH-positive smooth muscles arteries beneath the cellular membrane were seen.

In eSMT rats (figure 1 C, D), the mesh-like structure was disrupted. Neurons were isolated or in small groups, small zones of mesh-like structures could be seen, with empty spaces in ganglia. Arteries were more numerous, with NADH-positive smooth muscles fibers. Smooth muscle fibers from muscularis externa were observed as a background of parallel lines superimposed by tiny NADH-positive particles.
β rats (figure 1E), mesh-like structure was almost totally disrupted, and only a small zone of mesh-like structure was preserved, with isolated or grouped neurons. Some neurons were tightly grouped which cellular erased limits. Other neurons were irregularly shaped with spiny cellular profiles. Muscular blood vessels were NADH-positive. Smooth muscle fibers from muscularis externa in a background of parallel lines superimposed by tiny particles NADH-positive were observed.

Proximal colon: Wistar rats (figure 1F): polygonal and/or elongated ganglia, thicker than the small intestine, were interconnected with other ganglia by nerve tracts. Mesh-like preserved zones were interspersed with complete mesh-like disrupted zones and partially disrupted zones. In ganglia, neuronal spaces were enlarged, with some empty spaces and NADH-negative neurons. Mesh-like disrupted zones, with isolated or grouped neurons, slightly stained smooth muscle fibers from muscularis externa resembling parallel lines were observed.

eSS (figure 1G) and eSMT rats (figure 1I): general structure resembling those of Wistar rats, interspersed with mesh-like disrupted zones and zones of blurred (figure 1H) structures could be observed.
β rats: some small mesh-like preserved zones were interspersed with extended mesh-like disrupted zones (figure 2J) and zones of blurred structures. In disrupted zones isolated and grouped neurons, irregularly shaped, some of them elongated were observed. Arteries were augmented in number with NADH-positive muscle. Smooth muscle fibers from muscularis externa showed clotted stained clusters, and lack of parallel lines.

Distal Colon: Wistar rats (figure 1K): Mesh-like structure was present in almost all the field with ganglia transversally placed. NADH-negative or lightly stained nerve tracts could be observed interconnected among ganglia. NADH-positive neurons, NADH-negative neurons and empty spaces were observed, with neurons of different size. Artery muscle was either NADH-positive or NADH-negative. Smooth muscle fibers from muscularis externa were very slightly stained, resembling parallel lines.

eSS rats: some preserved zones of mesh-like structures with NADH-positive neurons were interspersed with zones of blurred structures (figure 2L) where neurons were slightly stained or absent. NADH-positive nerve tracts (figure 2M), fewer arteries than in Wistar rats, and muscularis externa smooth muscle were observed as parallel lines or clotted material.

eSMT rats (figure 2N): some mesh-like preserved zones interspersed with zones of blurred structures or NADH-positive remaining lines were observed. Some small neurons separated by spaces were present in ganglia. NADH-positive nerve tracts and muscularis externa smooth muscle with clotted stained material or sometimes blurred were observed.
β rats (figure 2O): some animals showed mesh-like preserved zones, interspersed with zones of blurbed structures (*muscularis externa* smooth muscle as parallel lines), while others showed disrupted mesh-like zones, with blurred structure or vestigial NADH-positive lines and NADH-positive nerves.

![Figure 2](image)

**Figure 2.** β rats (J): mesh-like disrupted zones, isolated and grouped neurons are seen. Neurons are irregularly shaped and some of them are elongated (arrowhead). Arteries (a), are augmented in number with NADH-positive muscle. Smooth muscle fibres from *muscularis externa* were blurred stained, losing parallel lines aspect.

**Distal Colon:** In Wistar rats (K): Mesh-like structure is present in almost all the field, ganglia are transversally disposed. Nerve tract (slightly stained) could be observed interconnected between them. Well NADH stained neurons, non NADH stained neurons and empty spaces are seen (arrow). Arteries could be observed (double arrowhead). Smooth muscle fibers from *muscularis externa* are very faintly stained, resembling parallel lines. In eSS rats: (L) zones of blurred structures, where neurons could be observed slightly stained or absent (arrowhead), muscles parallel-line aspect and nerve tracts (t) are slightly stained. These zones are interspersed with mesh-like preserved zones with positive-NADH neurons (M) and positive-NADH nerve tract (arrowhead). Smooth muscle fibers resemble parallel lines. (L) and (M) are microphotographs from the same whole mount slice. In eSMT rats (N): Smooth muscle fibers from *muscularis externa* are clotted stained, without parallel lines aspect. Ganglion is weakly defined (g), neurons are ill-shaped, without nucleus (arrowhead). In β rats (O), total disruption of mesh-like structure is observed. Neurons are isolated or grouped (arrowhead). Artery (a). Bar=50 µm.
Discussion

In our spontaneously diabetic rat models, mesh-like structure of Auerbach's plexus is strain dependent. Thus, in eSS few differences were detected in comparison with Wistar rats, such us partial disruption of mesh structure.

In eSMT and in β rats, disruption of mesh-like structures in myenteric plexus, accompanied by changes in the smooth muscle differed from eSS and Wistar rats. The former were in agreement with Johnson et al.\(^8\) description stating that "the structure of the myenteric plexus consisted of large ganglia interconnected by thick nerve bundles. Numerous fine fibers of the tertiary nerve tract were apparent between the network of interconnected ganglia". Gabella\(^9\) reported also that "the mesh formed by the ganglia and the connecting strands has a regular, although, not quite geometrical pattern which is characteristic of such segment of the alimentary tract and to some extent also of the animal species". Neurons are grouped in a ganglion, whereas there are some isolated neurons within a connecting strand, which was confirmed by our study. Broadly speaking there are two cell types in intramural ganglia: neurons and glial cells, which are associated with a neuropil, formed by neuronal and glial processes.\(^1\) Neurons stained by NADH-diaphorase technique were clearly visible due to purple formalin deposits in the neuronal perikaryum, with unstained nuclei. Neuron size values vary in different parts of the alimentary tract.\(^2\) Stain components are able to reveal differences in neuron type.\(^3\) With NADH technique relatively wide spaces were observed among the neuronal cell bodies composing the ganglia. Heinecke et al.\(^4\) and Young et al.\(^5\) described spaces occupied by unstained neurons, although the nuclei could be observed. The underling overlying smooth muscle was slightly stained. NADH revealed mitochondrial activity,\(^6\) while with Giemsa technique, polyribosomes are blue due to methylene-blue affinity.\(^7\) NADH-diaphorase positive neurons were significantly reduced in eSS and Wistar intestines, as well as in old Sprague Dawley rats\(^8\) and old Wistar rats,\(^9\) while β and eSMT rats showed a neuronal decrease and altered mesh-like structure. The neuron number depends on the technique applied, i.e., NADH,\(^10\) PGP 9.5 (protein gene product) marker,\(^11\) Giemsa.\(^12\) So far, Johnson et al.\(^8\) reported no evidence of atrophic or degenerative neurons. NADH histochemistry in diabetic rats resulted in either increase\(^13\) or diminution\(^14\) of duodenal neurons, in colon\(^15\) and cecum.\(^16\) In streptozotocin-induced diabetes NADH reactive revealed differences ileum innervation in comparison with colon,\(^17\) as observed in our diabetic rats.

Diabetes mellitus affects central and peripheral nervous system: sensory, motor and autonomic nerves.\(^18\) In our experimental animal models, disrupted mesh-like myenteric structures were accompanied with neuronal loss and augmented vascularization. Streptozotocin-induced diabetic rats showed a remarkable correlation between gastrointestinal alterations and axon dystrophy of ileal mesenteric nerves and vasoactive intestinal containing axons.\(^19\) In streptozotocin-induced diabetes microvessels were significantly dilated in diabetic animals.\(^20\) In our spontaneous diabetic models, neuronal diminution was not accompanied by enlargement of remaining neurons, and functional activity was preserved probably due to a compensatory increase in neurotransmitter production of the remaining neuronal elements in the gut as proposed by Dahl et al.\(^21\)

In our experimental animal models, disrupted mesh-like myenteric structures were accompanied with neuronal loss and augmented vascularization, functionally no differences were observed in gross anatomy in colon and stools aspects at the autopsy. Gastrointestinal transit was not impaired by regional loss of myenteric neurons;\(^22\) the rate of neuron loss was organ and region specific\(^23\) and was accompanied with neuronal size modifications.\(^24\) It must be borne in mind that changes in biochemistry and function of sensory neuronal ganglion, apart from myenteric ganglia, were detected in experimental diabetic rats, in spite of relative preservation of neuron numbers observed at 12 months.\(^25\) In diabetic rats, changes in the sensory neuronal number\(^13\) as well as biochemical content\(^13\) have been described. Morphological changes observed in myenteric plexus were indicative of more severe lesions probably due to vascular damage,\(^26\) disruption of mesh-like structures (figure 1D, E; figure 2J), changes in neuron profiles (figure 1E) and augmented blood vessels (figure 1D).

Changes in mesh-like structures in eSMT and β rats might be under genetic or metabolic (triacylglycerolemia) influences, since glycemia (eSS) did not affect mesh-like Auerbach's plexus or sympathetic ganglion cells, and did not produce neuroaxonal dystrophy.\(^27\) Partial preservation of neuronal number at 12 months in Sprague-Dawley diabetic dorsal
root ganglia were reported by Zochodne et al. 28
Summarizing, in our spontaneous diabetes models we can observe: 1) in diabetic eSS rats, that hyperglycemia enhanced the modifications of Auerbach’s plexus affecting circulation and mitochondria. 32) In β and eSMT rats some other factors worsened Auerbach’s plexus structure (hyperacetyl­ triglyceridemia ?). 3) Auerbach’s plexus structure in Wistar rats points out the ageing process.

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