

# Diabetic state affects the innervation of gut in an animal model of human type 1 diabetes

A. Spångéus, O. Suhr and M. El-Salhy

Section for Gastroenterology and Hepatology, Department of Medicine, University Hospital, Umeå, Sweden

**Summary.** Gastrointestinal symptoms in diabetic patients are commonplace, and are believed to be due, at least partly, to neuropathy of the gut. In the present study, therefore, some important neurotransmitters in the myenteric plexus were investigated in non-obese diabetic mice, an animal model of human type 1 diabetes. For this purpose, immunocytochemistry was applied on sections from antrum, duodenum and colon, subsequently quantified by computerized image analysis. Whereas the number of vasoactive intestinal peptide (VIP)-positive neurons was increased in antral myenteric ganglia of diabetic mice, there was a decreased density of nerve fibres in muscularis propria. No difference was seen in the VIP of duodenum and colon. **Acetylcholine**-containing nerve fibres showed an increased volume density in muscularis propria of antrum and duodenum, but a decreased density in colon of diabetic mice, as compared with controls. There was a decreased number of neurons containing nitric oxide synthase (NOS) in myenteric ganglia of antrum and duodenum. No difference was seen in density of NOS-containing nerve fibres in muscularis propria. There was no difference regarding neuropeptide Y (NPY) and **galanin** between diabetic and control mice; nor was there any difference between pre-diabetic NOD mice and controls regarding all bioactive substances investigated. It is concluded that the diabetic state affects the innervation of gut in this animal model. The present findings may be of some relevance to the gastrointestinal symptoms seen in patients with diabetes.

**Key words:** Diabetes, Enteric nerves, Gastrointestinal tract, Immunocytochemistry, NOD mice

## Introduction

Neuropathy is a common complication of diabetes mellitus, occurring in both the central and peripheral

**Offprint requests to:** Magdy El-Salhy, MD PhD, Section for Gastroenterology and Hepatology, Department of Medicine, University Hospital, S-901 85 Umeå, Sweden. Fax: +46-90-143986. e-mail: [magdy.el-salhy@medicin.umu.se](mailto:magdy.el-salhy@medicin.umu.se)

nervous system (Dyck et al., 1993; Locke III, 1995). The gastrointestinal tract is richly innervated with nerves of extrinsic (central nervous system) and of intrinsic origin (enteric nervous system) (Ekblad et al., 1991). These systems are of importance in the regulation of gut functions such as motility, secretion and absorption (Allescher, 1991; Rangachari, 1991; Costa and Brookes, 1994). In diabetes, up to 76% of the patients suffer from clinical symptoms emanating from the gastrointestinal tract, such as nausea, vomiting, heartburn, constipation, diarrhoea and faecal incontinence (Bargen et al., 1936; Kassander, 1958; Feldman and Schiller, 1983; Locke III, 1995). It is possible that neuropathy of the gut is involved in the pathogenesis of these symptoms. Unfortunately innervation of the gut is difficult to study in patients with diabetes, as transmural biopsies are needed. In order to investigate a possible disturbance in the diabetic state, we must therefore use animal models. Several studies made in chemically-induced diabetic rats as well as in genetically diabetic mice (Schmidt et al., 1981; Belai et al., 1985; Ballman and Conlon, 1985; Loesch et al., 1986; DiGiulio et al., 1989; Belai et al., 1991; Wrzos et al., 1997; El-Salhy and Spångéus, 1998a,b; Spångéus and El-Salhy, 1998a) have revealed both ultrastructural and biochemical abnormalities in sympathetic and parasympathetic innervation of the gut, as well as in certain neurotransmitters in the enteric nervous system. However, several important neurotransmitters had not hitherto been studied systematically.

The aim of the present study therefore was to investigate important neurotransmitters, such as VIP, NPY, **galanin**, acetylcholine, adrenaline and NO, in the gastrointestinal myenteric plexus of an animal model of human type 1 diabetes, namely the non-obese diabetic (NOD) mouse (Makino et al., 1980; Kolb, 1987; Tochino, 1987).

## Material and methods

### Animals

Female pre-diabetic and diabetic non-obese diabetic (NOD) mice, aged 22-24 weeks, were investigated. Control animals were non-diabetic age and sex matched

### Diabetic state affects the innervation of gut

BALB/cJ mice, a sister strain of the NOD strain (Fujishima et al., 1989). Each group comprised 7 mice. The animals were housed 5 to each cage in a room with artificial light for 12 h/day and were fed a standard pellet diet (Astra-Ewos AB, Södertälje, Sweden) and water *ad libitum*. They were kept in our vivarium for one week to adapt before being killed. After an overnight fast, the animals were killed in a CO<sub>2</sub> chamber and the antrum, proximal duodenum and distal colon were excised. The mice were characterized and described in detail earlier (El-Salhy et al., 1998). In brief, diabetic NOD mice have glucosuria (detected with Ecur-Test sticks, Boehringer Mannheim), a significantly reduced body weight, significantly lower pancreatic insulin content, significantly lower density of Langerhans' islets as well as insulin cells, and grade 3-4 insulinitis. Pre-diabetic NOD mice are not glucosuric and show a normal pancreatic insulin content. However, they do have significantly reduced body weight, a lower density (though not statistically significant) of Langerhans' islets and of insulin cells, and grade 1-2 insulinitis. No difference was seen between controls and both pre-diabetic and diabetic NOD mice regarding, thickness of the submucosa, or of the circular and longitudinal muscle layer of antrum, duodenum and colon (Spångéus and El-Salhy, 1998a,b; El-Salhy et al., 1998; El-Salhy and Spångéus, 1998c). The local committee on animal ethics at Umeå University approved the investigation.

#### Immunocytochemistry

After overnight fixation in 4% buffered formaldehyde, the tissues were embedded in paraffin wax and cut into 10 µm-thick sections. These were immunostained by the avidin-biotin complex (ABC) method (Dakopatts, Glostrup, Denmark) with microwave antigen retrieval pre-treatment, as described in detail elsewhere (Nyhlin et al., 1997). Briefly, after hydration the sections were placed in a 0.01M citrate buffer (pH 6.0) and then treated in a microwave oven for three 5-min cycles at maximum effect (650W), each cycle in fresh buffer. Sections were then immersed in a 50 ml Tris buffer containing 0.5 ml H<sub>2</sub>O<sub>2</sub> (30%) for 10 min to inhibit endogenous peroxidase, followed by treatment with 1% bovine serum albumin for 10 min to occupy the non-specific binding sites. This was followed by overnight incubation with primary antiserum at room

temperature. Sections were then incubated with the secondary antibody (biotinylated swine anti-rabbit, diluted 1:200) for 30 min at room temperature, followed by incubation for 60 min with avidin-biotin-peroxidase complex (diluted 1:50) at room temperature. Peroxidase was detected with 25 mg 3,3-diamino-benzidine tetrahydrochloride (DAB) in 50 ml Tris buffer containing 10 µl H<sub>2</sub>O<sub>2</sub> (30%). Light counter-staining was effected in Mayer's haematoxylin. The primary antisera used are presented in Table 1.

Specificity controls were performed as described previously (El-Salhy et al., 1993). Briefly, positive controls were obtained by including sections from human antrum, duodenum and colon, as well as paravertebral ganglia from mice. Negative controls included replacement of the primary antibody by non-immune rabbit serum and pre-incubation of the primary antibody with the corresponding peptide. Furthermore, pre-incubation of the primary antibody was performed with structurally related peptides.

#### Computerized image analysis

Immunoreactive nerves were quantified as described earlier (El-Salhy et al., 1997). Briefly, a Quantimet 500MC image processing and analysis system (Leica, Cambridge, England) was connected to an Olympus microscope (type BX50). The software was QWIN (version 1.02, Leica), a computerized image analysis program, together with QUIPS (version 1.02, Leica), an interactive programming system. Quantifications were performed with a x40 objective. At this magnification each pixel of the computer image corresponded to 0.206 µm and each field represented an area of 0.009 mm<sup>2</sup> of the tissue.

The relative volume density of nerve fibres in muscularis propria was quantified in an automated standard analysis sequence according to the classical stereological point-counting method (Weibel and Elias, 1967; Weibel et al., 1969) as adapted for computerized image analysis (El-Salhy et al., 1997; El-Salhy and Spångéus, 1998a). In brief, a 400-point lattice was superimposed on the frame covering muscularis propria. All points not covering muscularis were erased manually by using the computer 'mouse'. Points covering immunostained nerve fibres were marked, and a ratio of points covering nerve fibres and muscle tissue was

Table 1. Primary antisera used

ANTIGEN *	DILUTION	CODE	SOURCE
Porcine vasoactive intestinal peptide, VIP	1:2000	R 7854/01-B5	Euro-diagnostica, Sweden
Synthetic neuropeptide Y, NPY	1:1000	R 840403/B6	Euro-diagnostica
Synthetic galanin 1-29	1:600	R 841606/B2	Euro-diagnostica
Synthetic rat vesicular acetylcholine transporter (VACht)§	1:1000	R 9690/B26-1	Euro-diagnostica
Human neuronal nitric oxide synthase, nNOS	1:400	N 31030	Transduction Laboratories, Denmark
Bovine dopamine-β-hydroxylase	1:50	360PES02	Chemicon International Inc., USA

\*: all the antisera were raised in rabbit.; §: specific for C-terminus.

## Diabetic state affects the innervation of gut

tabulated. In each mouse and for each nerve type, measurements were made in 20 randomly chosen fields from 2-4 different sections, 150  $\mu\text{m}$  apart.

Immunoreactive neurons in myenteric ganglia were measured using a tool kit from the manual menu. Immunolabelled neurons in the ganglion were counted manually, followed by measurements of ganglion area, by drawing around the ganglia using the interactive menu. Ten ganglia per mouse and neurotransmitter were measured.

### Statistical analysis

Comparisons between two groups were performed with the non-parametric, Wilcoxon (Mann-Whitney) test. *P*-values below 0.05 were considered significant.

## Results

### Immunocytochemistry

Nerve fibres immunoreactive (IR) to vasoactive intestinal peptide (VIP)-, neuropeptide Y (NPY)-, galanin-, vesicular acetylcholine transporter (VAcHT)- and nitric oxide synthase (NOS)- were identified in antrum, duodenum and colon of all mouse groups investigated. As dopamine- $\beta$ -hydroxylase was seldom encountered in myenteric plexus of gut from these mice, no further quantification was made. All investigated

nerve types were evenly distributed within muscularis propria. VIP-, NPY-, galanin- and NOS-IR neurons were detected in the myenteric ganglia. As in previous studies (Li and Furness, 1998), no VAcHT-IR neurons could be detected in the myenteric ganglia. Furthermore, the VAcHT-IR nerve fibres tended to be located mostly in the peripheral parts of the ganglia. NOS-IR neurons were larger than VIP-, NPY- and galanin-IR neurons.

The antisera used in the present study immunostained nerve cells in human gut as well as dopamine- $\beta$ -hydroxylase in paravertebral ganglia. No immunostaining was detected when the primary antisera were

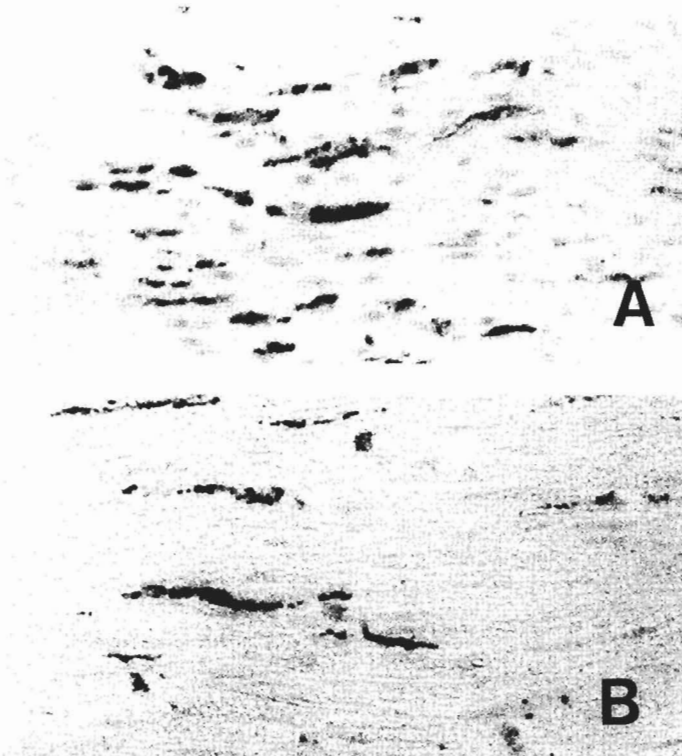


Fig. 1. VIP-immunoreactive nerve fibres in the antral muscularis propria of a control mouse (A) and of a diabetic NOD mouse. (B).  $\times 240$

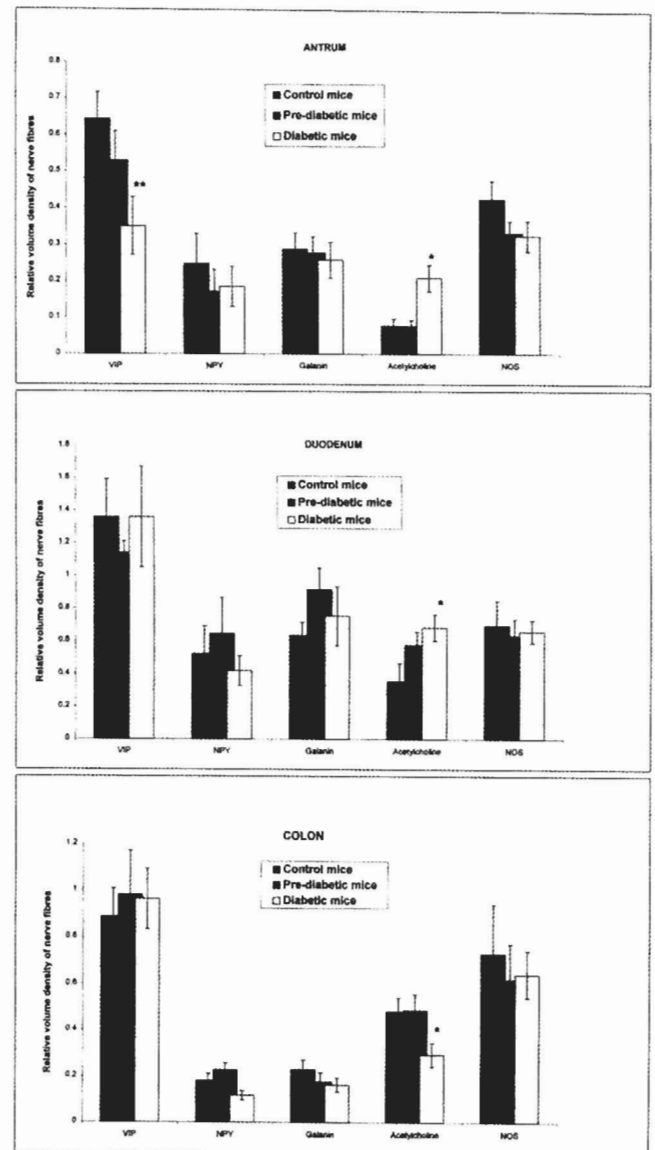


Fig. 2. Relative volume density of immunoreactive nerve fibres in the myenteric plexus of antrum, proximal duodenum and distal colon of controls and non-obese diabetic mice (mean $\pm$ SEM). \*:  $P < 0.05$ , \*\*:  $P < 0.01$ .

pre-incubated with the corresponding bioactive substance or when the antisera were replaced by non-immune rabbit serum. Pre-incubation of primary antibody with structurally related peptides (anti-VIP with secretin, PHI, glucagon and GIP; and anti-NPY with PYY and PP), did not affect the immunostaining.

#### Computerized image analysis

##### Antrum

Diabetic NOD mice showed a significantly decreased relative volume density of VIP-IR nerve fibres, as compared with controls (Fig. 1), whereas the density of VACht-IR nerve fibres was significantly increased. No difference was seen between diabetic NOD mice and controls regarding NPY-, galanin- and NOS-IR nerve fibres. Nor was there any difference between pre-diabetic NOD mice and controls regarding all bioactive substances investigated (Fig. 2). The number of VIP-IR neurons in myenteric ganglia was significantly higher in diabetic mice than in the controls. On the other hand, the number of NOS-IR neurons was decreased (Fig. 3). No difference was found regarding NPY- and galanin-IR neurons in diabetic mice. The number of neurons immunoreactive to all bioactive substances investigated did not differ between pre-diabetic mice and controls (Fig. 4).

##### Duodenum

Diabetic NOD mice showed a significantly increased

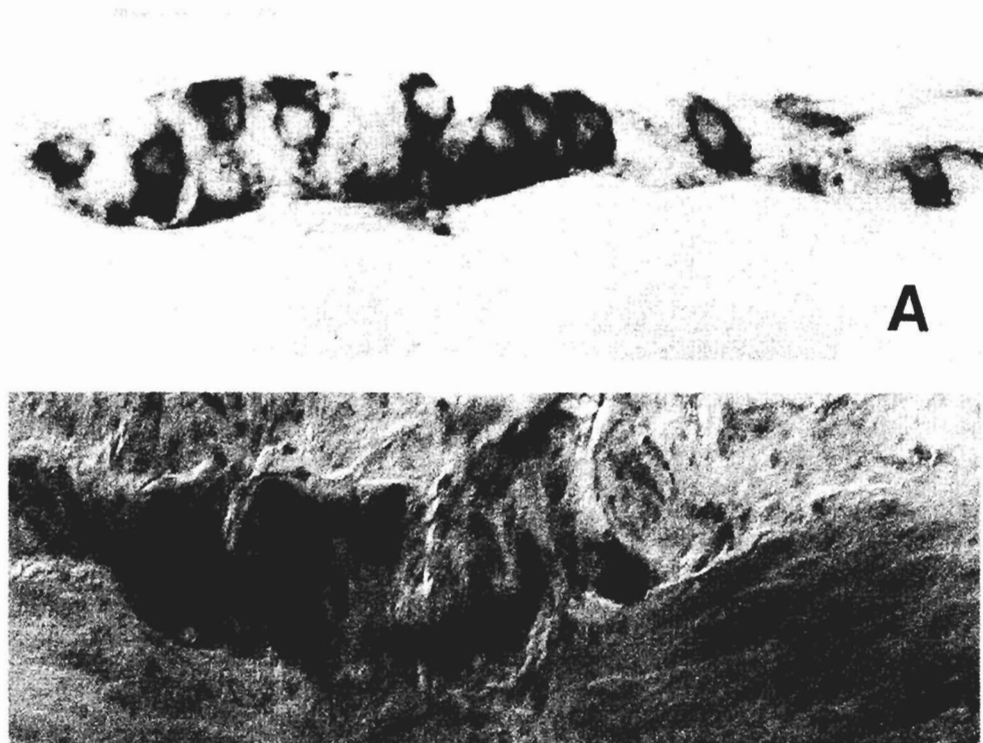
volume density of VACht-IR nerve fibres, as compared with controls. No difference was found regarding volume density of VIP-, NOS-, NPY- or galanin-IR nerve fibres in diabetic mice. Pre-diabetic mice did not differ from controls regarding all bioactive substances investigated (Fig. 2). The number of NOS-IR neurons was significantly decreased in myenteric ganglia of diabetic mice vs controls, whereas no difference was found regarding the number of VIP-, NPY- and galanin-IR neurons. No difference was found in pre-diabetic mice compared with controls regarding all immunoreactive neurons detected (Fig. 4).

##### Colon

In distal colon of diabetic mice, there was a significantly decreased volume density of VACht-IR nerve fibres. No difference was found regarding VIP-, NPY-, galanin- and NOS-IR nerve fibres between diabetic, pre-diabetic and control mice, or regarding VACht-IR nerve fibres between pre-diabetic and control mice (Fig. 2). Nor was there any difference between diabetic, pre-diabetic and control mice regarding all investigated neurons in myenteric ganglia (Fig. 4).

#### Discussion

The present study revealed several abnormalities in the gastrointestinal myenteric plexus of NOD mice, an animal model for human type 1 diabetes. These abnormalities were observed in all parts investigated, namely the antrum, duodenum and colon. The



**Fig. 3.** NOS-immunoreactive neurons in antral myenteric ganglia of a control mouse (A) and of a diabetic NOD mouse (B). x 420

disturbances occurred exclusively in the diabetic mice, indicating that they are related to the diabetic state. Similar results have been reported regarding substance P-IR nerve fibres in colonic myenteric plexus, where these fibres were abnormal in diabetic NOD mice only (El-Salhy and Spångéus, 1998a). However, when a general marker for nerve elements (protein gene product 9.5) was used it was reported that nerve fibre density decreased in the duodenal myenteric plexus of both pre-diabetic and diabetic NOD mice (Spångéus and El-Salhy, 1998a). It is possible that the myenteric plexus is affected in the pre-diabetic state, but that the affected nerve fibres are other than those investigated in the

present study.

Whereas a difference in the thickness of the gut wall has been reported between controls and some animal models of diabetes (Schmidt et al., 1981; Ballman and Conlon, 1985; Belai et al., 1985), no such difference was found in the same NOD mice investigated here (El-Salhy and Spångéus, 1998c; Spångéus and El-Salhy, 1998a,b). Thus, the differences in the myenteric plexus observed are actual changes and not the result of a change in muscle layer volume.

The VAcHT antiserum used in the present study is directed against the vesicular acetylcholine transporter, which occurs mostly in axons (Li and Furness, 1998). This explains why no neurons were identified by this antiserum in the myenteric ganglia. The present findings are in agreement with an earlier study in streptozotocin-induced diabetic rats (Schmidt et al., 1981), where a decreased content of colonic acetylcholine was found. The increased volume density of VAcHT-IR nerve fibres in the antrum and duodenum observed in the present study may have been caused by increased nerve fibre size due to a degenerative process associated with neuropathy (Schmidt et al., 1981; Loesch et al., 1986).

Whereas the antral VIP-IR neurons increased in number in myenteric ganglia, the density of nerve fibres in muscularis propria decreased. This might have been due to an impaired axonal transport of VIP containing axons. A decreased concentration of VIP has been reported previously in stomach of streptozotocin-induced diabetic rats (Ballman and Conlon, 1985). The present study found no difference in proximal duodenum and distal colon. A previous observation has, however, reported increased numbers of VIP-IR neurons in myenteric ganglia of ileum and proximal colon of streptozotocin-induced diabetic rats (Belai et al., 1985). Furthermore, RIA studies on duodenum have shown an increased concentration of VIP in diabetic rats and mice (El-Salhy and Spångéus, 1998b; DiGiulio et al., 1989). These studies have, however, included all the gut wall, which might explain the differences from the present study.

The number of NOS-IR neurons in myenteric ganglia decreased in both antrum and duodenum of diabetic mice. This is in agreement with an earlier observation in streptozotocin-induced diabetic rats, where a smaller proportion of neurons in antral myenteric ganglia expressed NOS (Wrzos et al., 1997), though the same study reported no differences as regards duodenum, ileum and colon.

The present study adds to the growing body of evidence (Schmidt et al., 1981; Ballman and Conlon, 1985; Belai et al., 1985, 1991; Loesch et al., 1986; DiGiulio et al., 1989; Wrzos et al., 1997; El-Salhy and Spångéus, 1998a,b; Spångéus and El-Salhy, 1998a) that the enteric nervous system is affected by the diabetic state in animal models of human diabetes. These findings may be of some relevance for the gastrointestinal symptoms seen in patients with diabetes.

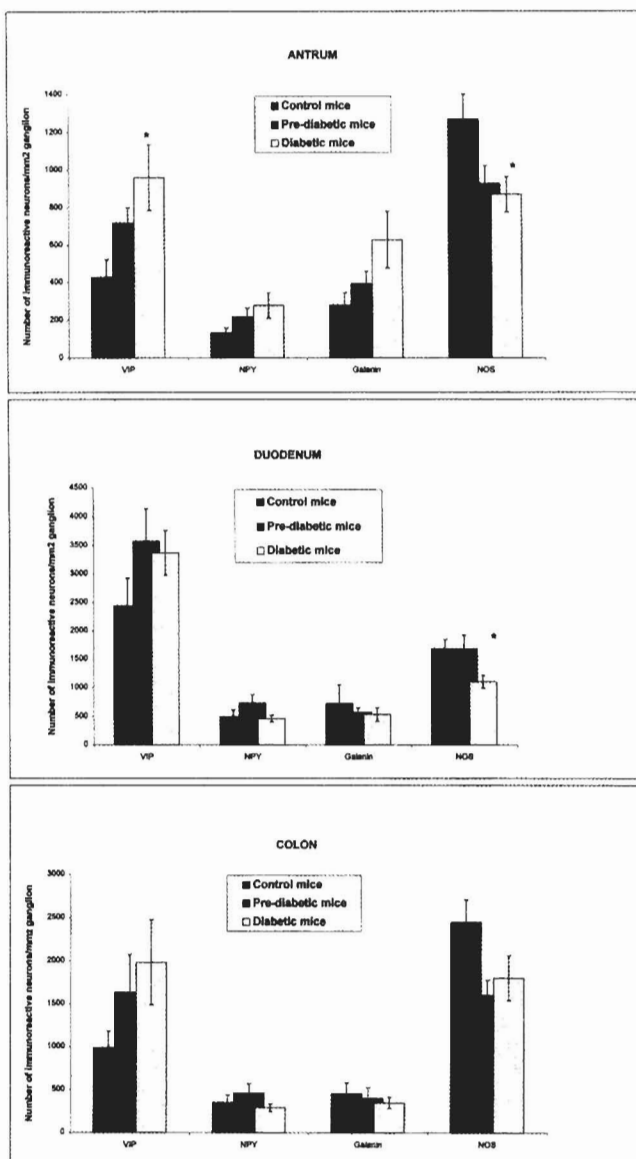


Fig. 4. Immunoreactive neurons per mm<sup>2</sup> of myenteric ganglia in antrum, proximal duodenum and distal colon of controls and non-obese diabetic mice (mean+SEM). Symbols as in Figure 2.



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