Gastrointestinal Motility Changes and Myenteric Plexus Alterations in Spontaneously Diabetic Biobreeding Rats

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Background/Aims

Type 1 diabetes is often accompanied by gastrointestinal motility disturbances. Vagal neuropathy, hyperglycemia, and alterations in the myenteric plexus have been proposed as underlying mechanism. We therefore studied the relationship between vagal function, gastrointestinal motiliy and characteristics of the enteric nervous system in the biobreeding (BB) rat known as model for spontaneous type 1 diabetes.

Methods

Gastric emptying breath test, small intestinal electromyography, relative risk-interval variability, histology and immunohistochemistry on antral and jejunal segments were performed at 1, 8 and 16 weeks after diabetes onset and on age-matched controls.

Results

We observed no consistent changes in relative risk-interval variability and gastric emptying rate. There was however, a loss of phases 3 with longer duration of diabetes on small intestinal electromyography. We found a progressive decrease of nitrergic neurons in the myenteric plexus of antrum and jejunum, while numbers of cholinergic nerve were not altered. In addition, a transient inflammatory infiltrate in jejunal wall was found in spontaneous diabetic BB rats at 8 weeks of diabetes.

Conclusions

In diabetic BB rats, altered small intestinal motor control associated with a loss of myenteric nitric oxide synthase expression occurs, which does not depend on hyperglycemia or vagal dysfunction, and which is preceded by transient intestinal inflammation.
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Key Words

Diabetes mellitus; Electromyography; Enteric nervous system; Gastric empyting; Rats, inbred BB

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Introduction

Diabetes mellitus in humans is frequently associated with gastrointestinal motility disorders such as delayed gastric emptying, constipation and diarrhea.[1,2] In animal models of diabetes, gastrointestinal motility disorders have also been described.[3-5] The abnormalities of motor control in diabetes are thought to originate at least in part from visceral autonomous neuropathy,[2] more specifically from changes in vagal innervations.[3] On the other hand, abnormalities of the intrinsic enteric nervous system have also been reported, and they may also contribute to abnormal motor control. Most of these studies were carried out in animals where diabetes was induced by streptozotocin,[4,6-15] which is not devoid of some direct neurotoxic properties.[16] Spontaneously diabetic biobreeding (BB) rats[17] are considered as a useful model for human type1 diabetes as they develop a pancreatic infiltration with destruction of the insulin producing beta cells around 2-3 months of age. More recent studies also reported changes in neurotransmitter content of the myenteric plexus in spontaneously diabetic BB rats.[18,19] Assessment of smooth muscle contractility in vitro in response to electrical field stimulation and ligand application confirmed altered neural motor control.[7,18-20] In the jejunum of spontaneously diabetic BB rats, decreased nitrergic motor control and decreased neuronal nitric oxide synthase (nNOS) protein expression occur, while purinergic transmission is unaffected.[19] However, the time-course of these alterations has not been studied in detail, and it is unclear to which extent gastrointestinal motility in vivo is affected, as very few studies have been devoted to motility measurements in these models. Furthermore, conflicting reports exist on the influence of blood glucose levels on gastric emptying: both hypoglycemia[21] and hyperglycemia[22] have been reported to accelerate gastric emptying and conversely, hyperglycemia was also reported to induce delayed gastric emptying.[23]

In this study we examined in vivo upper gastrointestinal motility and changes in neurotransmitter content of the myenteric plexus in spontaneously diabetic BB rats. We correlated these changes to measurements of relative risk (RR)-interval variability[24-26] to assess diabetic neuropathy of the vagal nerve. Gastric emptying of semi-solids and small intestinal electromyography (EMG) were used to monitor motility changes. Immunohistochemistry was used to assess changes in neurotransmitter content in the myenteric plexus. We measured RR-interval variability to determine whether vagal neuropathy was present. Since an in-flammatory infiltration in the colonic wall in BB rats has been reported[27,28] we also performed myeloperoxidase (MPO) activity measurements and histologic examinations to detect such an infiltrate in the upper gastrointestinal tract.

Materials and Methods

Biobreeding Rats

In the BB rat breeding colony of our institution, approximately 2/3 of the rats develop diabetes around the third month of life (93 ± 3 days). We screened for development of diabetes by glycosuria testing (Clinistix®; Bayer, Boca Raton, FL, USA) twice a week. Diabetic rats received insulin according to 2 regimens, maintaining either chronic hyperglycemia or near-normoglycemia. After development of glycosuria, confirmation of the diagnosis was made by the measurement of hyperglycemia of > 200 mg/dL (Glucometer Elite®; Bayer). After diagnosing diabetes, long-acting insulin (Ultratard®; Novo Nordisk, Bagsvørd, Denmark) was administered subcutaneously once daily to maintain blood glucose levels between 300 and 400 mg/dL, and to prevent ketonuria (Ketostix®; Bayer). Non-diabetic littermates were used as sex- and age-matched controls. Although chronic glycemia in diabetic rats was significantly higher than in controls, fasting glycemias at the time of acute experiments were often in the normal range. To determine more clearly the effect of chronic hyperglycemia on the EMG findings, a subgroup of rats were treated by subcutaneously implanting small insulin coated rods (LinPlant, Linshin Canada Inc., Toronto, Ontario, Canada). These rods, which release a continuous amount of insulin, result in chronic near-normoglycemia. All procedures were approved by the Animal Care and Use Committee of the University of Leuven.

Gastric Emptying

After 1, 8 and 16 weeks of diabetes, animals underwent gastric emptying studies using a ^{14}C-octanoic acid breath test, which has been described elsewhere.[29] Briefly, after an overnight fast, rats ate a semi-solid test meal doped with ^{14}C-octanoic acid, which is absorbed into the small intestine, but not the stomach. Octanoic acid is preferentially metabolized in the liver to form $^{14}CO_2$, which is then exhaled. Immediately after ingestion of the test meal, animals were placed in an airtight container with continuous airflow. The $^{14}CO_2$ in the outgoing air was measured and fitted to an excretion curve. $T_{1/2}$ was calculated and compared between diabetic and control rats.

Small Intestinal Electromyography

One or two days after the breath test, animals were prepared for small intestinal EMG testing. After laparotomy, 4 bipolar electrodes (Nichrome wire, 80 μm diameter, insulated except at the tip) were attached at regular intervals to the jejunum[30,31] and tunneled subcutaneously. The electrodes, protected by a metal wire coil fastened to the skin, were led outside the cage. After the operation, all rats moved freely in individual cages and had unrestricted access to food and water. One week later, all rats had fully recovered. After an overnight fast, small intestinal EMG signals were recorded and integrated every 10 seconds for at least one hour, using a similar method as previously reported.[31-33] The signals were digitized (WINDAQ; DATAQ Instruments Inc., Akron, OH, USA and Microsoft Corp., Redmond, WA, USA) and analyzed semi-automatically.

Activity fronts were defined as periods of at least one minute, in which the integrated signal was raised above a set threshold.[31] Progression over the electrodes was checked visually, as were the phases 1 (absence of activity for at least 30 seconds, within 3 minutes after the end of an activity front). Phase 3 was characterized by (1) an aborally migrating activity front and (2) which was followed by a phase 1. After EMG recordings, animals were sacrificed, the exact locations of electrodes were determined and the jejunum was removed.

Relative Risk-interval Variability

The RR-interval, the time between 2 successive R-spikes in the QRS complex, is determined by heart rate. Heart rate normally displays a respiration-related variation, which is decreased in autonomic nervous system dysfunction.[23,24] Thus, loss of RR-interval variability can be used to look for autonomous neuropathy. In this study, 3 subcutaneous thoracic electrodes were implanted, tunneled subcutaneously and led outside the cage. After a week, electrocardiogram signals were recorded and RR-interval variability was determined. Using spectral power analysis and an algorithm developed in our institution,[25] we determined the low frequency/high frequency ratio (LF/HF ratio) of RR-intervals. The low frequencies (0.195 to 0.740 Hz) are predominantly dependent on sympathetic input; the high frequencies (between 0.78 and 2.50 Hz) are more under parasympathetic (vagal) control. In autonomous diabetic neuropathy, especially the HF component is reduced, resulting in a higher LF/HF ratio.[23,24]

Immunohistochemistry

After gastric emptying test, small intestinal EMG and electrocardiogram recording, animals were sacrificed and the small intestine and stomach were removed out for histochemical and immunohistochemical studies of the myenteric plexus. Prior to the labeling experiments, the absence of non-specific cross-reactions between antisera, and the correspondence between nNOS-immunoreactivity and nicotinamide adenine dinucleotide phosphatase (NADPH) diaphorase reactivity were confirmed. Triple labeling immunohistochemistry (neuron specific enolase [NSE], choline acetyltransferase [ChAT] and nNOS) evaluated the neurotransmitter content of the myenteric neurons: longitudinal muscle-myenteric plexus preparations (LMMPP) of jejunum and the antrum were placed 2-3 hours in blocking buffer, 48 hours in primary antibody buffer and 2-3 hours in fluorescence labeled secondary antibodies buffer (Table 1). After mounting

Table 1. List of Primary and Secondary Antisera Used in Staining of the Myenteric Plexus

Primary antisera					
Antigen	Antigen function	Host	Type	Dilution	Source
NSE	Pan-neuronal marker	Rabbit	Polyclonal	1:1000-1:3000	Polysciences, Warrington, USA
ChAT	Excitatory NT production	Goat	Polyclonal	1:100	Chemicon Int. Inc., Temecula, USA
nNOS	Inhibitory NT production	Mouse	Monoclonal	1:150	Transduction Lab., Lexington, USA
Secondary antisera					
Antigen	Label	Host	Type	Dilution	Source
Rabbit IgG	AMCA	Donkey	Polyclonal	1:250-1:500	Jackson ImmunoRes. Lab., West Grove, USA
Goat IgG	FITC	Donkey	Polyclonal	1:50	Jackson ImmunoRes. Lab., West Grove, USA
Mouse IgG	Texas red	Donkey	Polyclonal	1:500	Jackson ImmunoRes. Lab., West Grove, USA

NSE, neuron specific enolase; ChAT, choline acetyl transferase; NT, neurotransmitter; nNOS, neuronal nitric oxide synthase; AMCA, aminomethylcoumarin; FITC, fluorescein isothiocyanate.
The used anti-nNOS does not react with inducible NOS.

the LMMPP on glass slides, immunoreactive (IR) cell bodies were counted in 100 jejunal or antral myenteric ganglia for both diabetic and control groups. Nerve cells were identified by NSE immunoreactivity.

Histology and Assessment of Inflammation

A segment of jejunum was placed in 4% formaldehyde for at least 24 hours, then embedded in paraffin. H&E staining was performed on full thickness slides. Signs of inflammation were checked light optically by an experienced pathologist.

MPO activity was measured as previously described.[34] Briefly, the mucosa was separated from the underlying layers by blunted dissection in 0.9% NaCl and immediately snap-frozen in liquid nitrogen. After homogenization of the tissue in Hexadecyl-trimethylammonium bromide buffer, the homogenate was centrifuged (12,500 g, 4°C, 30 minutes) and supernatant MPO activity was measured spectrophotometrically ($\lambda = 460$ nm) after addition of H_2O_2 and O-dianisidine hydrochloride. Results were expressed in units/mg tissue.

Statistical Methods

Results are shown as mean $\pm$ SEM. ANOVA (analysis of variance), Student's t, Chi-square and Fisher exact test were performed using Microsoft Excel 97 or GraphPad InStat 3.00. Bonferoni's correction was used for multiple testing.

Results

Animal Cohorts and Control of Diabetes

The mean glycemia of rats with insulin rod implants was 88 $\pm$ 15 mg/dL, which was comparable to control rats (94 $\pm$ 6

Table 2. Age and Food Intake at Gastric Emptying Testing in Control and Diabetic Biobreeding Rats at Various Time Points

	Control	Diabetes	Student's t test
Age at test (days)			
Week 1	99 $\pm$ 4	112 $\pm$ 13	NS
Week 8	142 $\pm$ 3	158 $\pm$ 12	NS
Week 16	213 $\pm$ 6	207 $\pm$ 6	NS
Food ingested			
(mg/100 g body weight)			
Week 1	195 $\pm$ 9	172 $\pm$ 8	NS
Week 8	189 $\pm$ 5	200 $\pm$ 4	NS
Week 16	189 $\pm$ 9	166 $\pm$ 13	NS
ANOVA	NS	NS	

mg/dL, NS); and significantly lower than insulin-injected rats (319 $\pm$ 16, $P < 0.001$). All other characteristics (age at onset of diabetes, duration of diabetes and location of electrodes) did not differ between insulin rod-implanted and insulin-injected rats. It should be noted that although chronic glycemia in diabetic rats was significantly higher than in controls, fasting glycemias at the time of acute experiments in this study were within the same range (97$\pm$15 vs. 73$\pm$3 mg/dL, NS).

Gastric Emptying

Gastric emptying breath tests were performed in 27 diabetic insulin-injected animals and 35 non-diabetic controls at 1, 8 and 16 weeks. Both groups were age and sex-matched and the amount of food ingested did not differ at any of the 3 time points (Table 2). No major differences in gastric emptying rate were observed (Fig. 1). Gastric emptying rate did not differ between diabetic and control rats at 1 ($T_{1/2}$ 50.6 $\pm$ 3.8 vs. 49.6 $\pm$ 2.8 minutes, NS), 8 ($T_{1/2}$ 43.6 $\pm$ 2.3 vs. 34.1 $\pm$ 2.7 minutes, NS) and 16 weeks ($T_{1/2}$ 55.5 $\pm$ 15.4 vs. 57.9 $\pm$ 9.5 minutes, NS). Gastric emptying in control rats at 8 weeks was significantly faster than in diabetic rats at 8 weeks and than control rats at other time points, but this was no longer significant after Bonferroni correction for multiple testing.

Small Intestinal Electromyography

Small intestinal EMG was performed in 22 diabetic animals and 18 non-diabetic controls. The location of the electrodes did not differ between the 2 groups. The first electrode was located 13.0 $\pm$ 0.3 cm distal to the pylorus, the second was located at 4.4
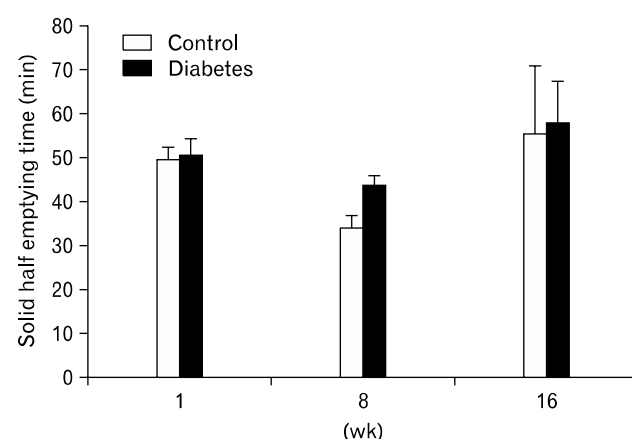

Figure 1. Gastric half emptying times for semi-solids in diabetic and sex- and age-matched control rats at 1, 8 and 16 weeks after onset of diabetes. No significant differences occurred between both groups at any time point.

± 0.3 cm more distally, and the third and fourth electrodes followed at distances of 4.4 ± 0.2 and 4.2 ± 0.2 cm, respectively.

The number and duration of activity fronts and phases 3 did not differ significantly between both groups at individual time points (Table 3). However, after 1 and 8 weeks of diabetes, the proportion of propagated activity fronts was significantly lower than in control rats (43/61 vs. 61/71 and 48/85 vs. 39/53, respectively, both $P < 0.05$), but this was no longer significant after 16 weeks (30/42 vs. 15/22, respectively, NS). Over time, the number of phases 3 decreased and the interval between phases 3 lengthened significantly in the diabetic group, but not in the control group (Fig. 2). The number of phases 3 per hour did not differ significantly between diabetic and control rats at 1 (4.9 ± 1.7 vs. 3.8 ± 1.3, NS), 8 (2.2 ± 0.6 vs. 3.5 ± 0.8, NS) and 16 weeks (1.8 ± 1.0 vs. 2.5 ± 1.0, NS). Similarly, duration of activity fronts shortened significantly over time in control rats, but not in diabetic rats (Table 3).

Compared to rats receiving daily subcutaneous insulin injections, the subgroup of 8 rats in which glycemia was tightly controlled with insulin rod implants, did not differ in number (7.5 ± 2.2 per hour, NS), duration (3.5 ± 0.3 minutes, NS), proportion of activity fronts that were propagated (26/39, NS) and number of phases 3 (3 ± 2 per hour, NS).

Relative Risk-interval Variability

RR-interval measurements were performed in 6 insulin- treated diabetic and 5 control animals, at 1 and 16 weeks. LF/HF ratio did not differ significantly between diabetic and control animals after 1 and 16 weeks (0.53 ± 0.26 vs. 0.58 ± 0.08 and 0.33 ± 0.16 vs. 1.04 ± 0.13, respectively, both NS). In the diabetic

group, there was no alteration over time in the LF/HF ratio (0.53 ± 0.26, 0.51 ± 0.13 and 0.33 ± 0.16, NS). The overall LF/HF ratio in diabetic animals was 0.47 ± 0.11 and 0.73 ± 0.11 (NS) in control animals.

Jejunal Inflammation

After one week of diabetes, light optical imaging and leukocyte antibodies revealed only occasional, submucosally localized, inflammatory cells. After 8 weeks of diabetes, thickening of the muscle layers, and prominent transmural inflammatory infiltrate were apparent. Inflammatory cells could be seen adjacent to ganglion cells in both of submucoal and myenteric plexuses (Fig. 3). After 16 weeks, the inflammation had subsided again, with only the occasional presence of submucosal inflammatory cells. Thickening of the muscle layers was no longer present. MPO activity was increased after 8 weeks of diabetes compared to controls (6.4 ± 2.1 vs. 1.3 ± 0.2 U/mg, $P < 0.05$). No significant difference was found at 16 weeks (1.6 ± 0.4 vs. 1.3 ± 0.3 U/mg, NS).
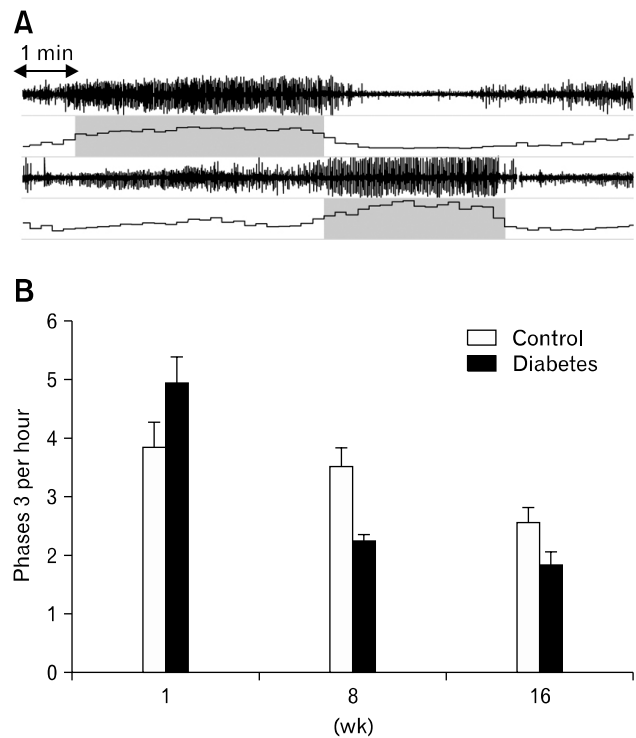

Figure 2. (A) Example of semi-automatic analysis of small intestinal electromyography of 2 consecutive electrodes (E1 and E2). Activity fronts (▨) are defined as periods of at least 1 minute, in which the integrated signal is raised above the set threshold. (B) Number of phases 3 per hour in diabetic and sex- and age-matched control rats at 1, 8 and 16 weeks after onset of diabetes. Over time, the number of phases 3 decreased significantly in the diabetic group.

Table 3. Small Intestinal Electromyography Results

	Control	Diabetes	Student's t test
Number of activity fronts per hour			
Week 1	9.8 ± 1.5	9.9 ± 1.3	NS
Week 8	10.7 ± 2.6	9.2 ± 0.9	NS
Week 16	9.3 ± 1.1	7.5 ± 2.2	NS
ANOVA	NS	NS	
Duration of activity fronts (min)			
Week 1	3.5 ± 0.3	3.4 ± 0.1	NS
Week 8	2.8 ± 0.2	3.2 ± 0.2	NS
Week 16	2.6 ± 0.3	3.4 ± 0.4	NS
ANOVA	$P < 0.05$	NS	

Immunohistochemistry

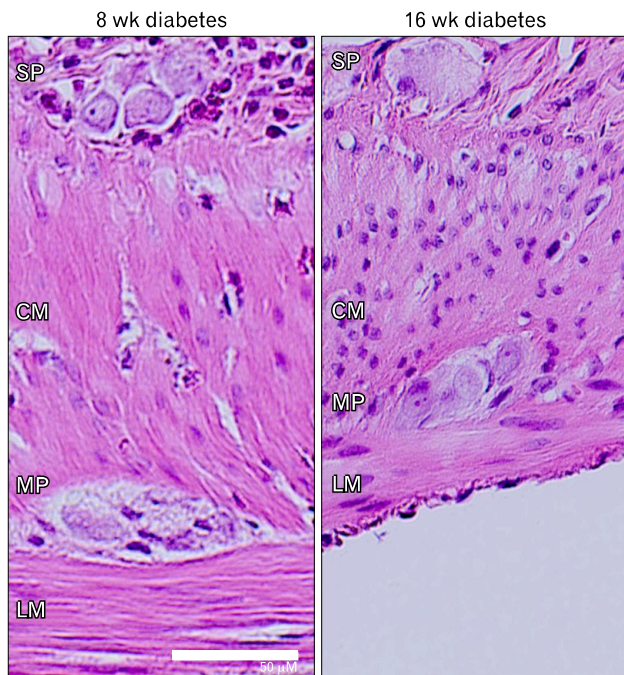In the myenteric plexus of the gastric antrum, the number of

Figure 3. Representative image of 8 and 16 weeks diabetic rat by H&E staining. Eight weeks after onset of diabetes, infiltration of polymorphonuclear leukocyte cells into enteric nervous system (SP, submucosal plexus; MP, myenteric plexus) was present with thickening of the muscular layers (CM, circular muscle; LM, longitudinal muscle). However, 16 weeks after onset of diabetes, no inflammatory infiltrate was seen. Scale bar = 50 μm.

NSE-IR neurons per ganglion did not differ significantly between diabetic rats and matched controls after 1, 8 and 16 weeks of onset of diabetes. At 16 weeks, the number of ChAT-IR neurons per ganglion was higher in diabetic animals, but this was no longer significant after correction for multiple testing (Table 4). Over time, antral nNOS-IR, but not NSE- and ChAT-IR, decreased significantly in both groups ($P < 0.005$ for diabetes, $P < 0.001$ in controls). Counts of nNOS myenteric neurons were lower in diabetic than in control animals at every time point (Fig. 4). In the myenteric plexus of the jejunum, the number of NSE-IR and ChAT-IR did not differ between diabetic and control animal. Over time, jejunal NSE- and ChAT-IR did not vary significantly in either group (Table 4). However, nNOS-IR was significantly decreased at week 8 and 16 (Fig. 4). The number of nitrergic neurons per ganglion in the antrum was significantly lower in diabetic animals compared to controls at 1 (6.1 ± 0.4 vs. 8.1 ± 0.4, $P < 0.001$), 8 (4.1 ± 0.3 vs. 5.2 ± 0.4, $P < 0.05$) and 16 weeks (2.9 ± 0.2 vs. 5.5 ± 0.3, $P < 0.001$) between both groups. The number jejunal nitrergic neurons was significantly lower in diabetic animals compared to controls at 8 (3.0 ± 0.3 vs. 3.7 ± 0.3, $P = 0.05$) and 16 (2.3 ± 0.2 vs. 3.4 ± 0.3, $P < 0.01$) but not at 1 week (6.6 ± 0.4 vs. 7.3 ± 0.4, NS). As in the antrum, jejunal nNOS-IR decreased significantly over time in both diabetic and control animals ($P < 0.001$). The number of nitrergic neurons was not significantly correlated to gastric emptying times, activity fronts or phases 3, except for a significant correlation between the number of phases 3 and nitrergic neuron counts at week 16 in diabetic rats (R = 0.490, $P < 0.05$).

Table 4. Immunohistochemistry

NSE (cells per ganglion)	Antrum			Jejunum		
	Control	Diabetes	Student's *t* test	Control	Diabetes	Student's *t* test
Week 1	34.0 ± 0.7	35.1 ± 0.9	NS	28.9 ± 0.6	31.4 ± 0.8	NS
Week 8	33.4 ± 0.6	35.7 ± 1.0	NS	30.5 ± 0.9	30.2 ± 0.7	NS
Week 16	34.6 ± 0.8	37.1 ± 0.9	NS	31.4 ± 0.8	32.3 ± 0.8	NS
ANOVA	NS	NS		NS	NS	

ChAT (cells per ganglion)	Antrum			Jejunum		
	Control	Diabetes	Student's *t* test	Control	Diabetes	Student's *t* test
Week 1	30.1 ± 0.7	31.8 ± 0.9	NS	26.3 ± 0.6	31.5 ± 2.9	NS
Week 8	30.5 ± 0.6	32.9 ± 1.0	NS	27.6 ± 1.0	27.6 ± 0.7	NS
Week 16	30.6 ± 0.8	34.2 ± 0.9	NS	28.9 ± 0.7	30.0 ± 0.8	NS
ANOVA	NS	NS		NS	NS	

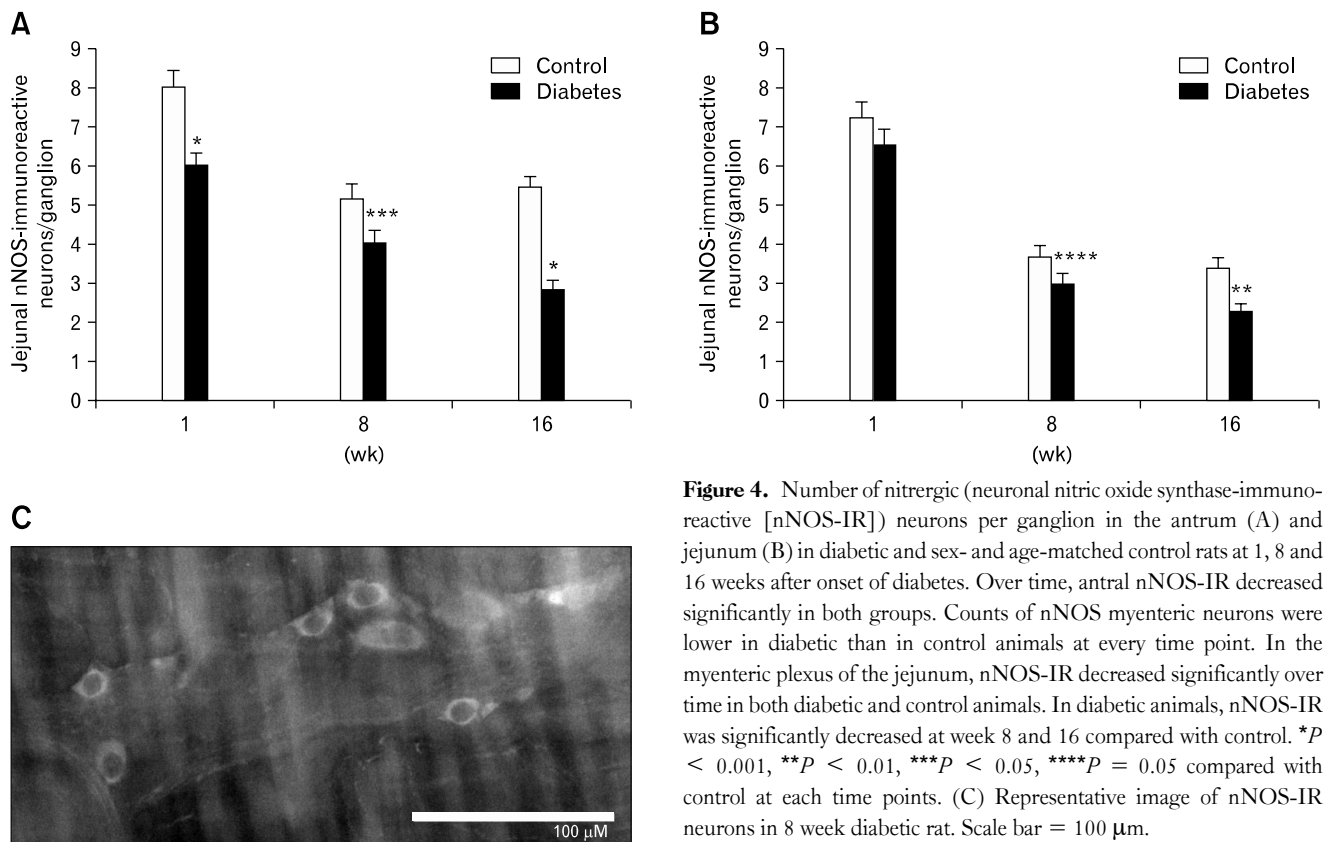NSE, neuron specific enolase; ChAT, choline acetyl transferase.

Figure 4. Number of nitrergic (neuronal nitric oxide synthase-immunoreactive [nNOS-IR]) neurons per ganglion in the antrum (A) and jejunum (B) in diabetic and sex- and age-matched control rats at 1, 8 and 16 weeks after onset of diabetes. Over time, antral nNOS-IR decreased significantly in both groups. Counts of nNOS myenteric neurons were lower in diabetic than in control animals at every time point. In the myenteric plexus of the jejunum, nNOS-IR decreased significantly over time in both diabetic and control animals. In diabetic animals, nNOS-IR was significantly decreased at week 8 and 16 compared with control. *$P < 0.001$, **$P < 0.01$, ***$P < 0.05$, ****$P = 0.05$ compared with control at each time points. (C) Representative image of nNOS-IR neurons in 8 week diabetic rat. Scale bar = 100 μm.

Discussion

In this study, we examined in vivo gastric and jejunal motility in spontaneously diabetic BB rats, and we explored the possible correlations with autonomic neuropathy or with changes in myenteric innervation. We did not observe any consistent signs of vagal neuropathy or delayed gastric emptying, but we did observe changes in small intestinal motor control. At the level of the myenteric plexus, the total number of neurons and the number of Chat-IR neurons per ganglion did not differ between controls and diabetic animals. In the antrum and the jejunum, the number of nNOS-IR neurons was significantly decreased in diabetic animals compared to controls. At 8 weeks, diabetic animals were characterized by the presence of transmural inflammatory infiltrate.

On studying the semi-liquid gastric emptying by means of a breath test, we found no changes by the duration of diabetes. No correlation was found between acute glycemia levels and the rate of gastric emptying. We did observe changes in nNOS immunoreactivity, but these were not correlated with the rate of gastric emptying. The absence of consistently delayed gastric empty-

ing is at variance with several studies reporting delayed gastric emptying in diabetic animals.[8,9,23] However, most of these studies were done in the streptozotocin or non-obese diabetic (NOD) models, and they used liquid meals or non-nutrient solids to measure emptying rate.[8,9,23] As the control of liquid emptying depends strongly on nitrergic control of pyloric motor function,[9,35,36] it is conceivable that liquid emptying is differentially affected by diabetic neuropathy. One of the main functions of nitrergic nerves in the stomach, proximal to the pylorus, is to mediate gastric accommodation to ingestion of a meal.[37] In human, impaired accommodation is not associated with changes in gastric emptying.[38,39] Hence, assessment of proximal stomach motor function, and especially meal-induced accommodation, could be considered in future studies in the diabetic BB rat model. As gastroparesis in man is mostly observed in type 1 diabetics with a long-standing disease history, we cannot rule out the occurrence of gastric emptying disturbances with longer follow-up, beyond 16 weeks diabetes in the BB rat model.

In the small intestine, we found EMG alterations, with a progressively decreasing number of phases 3 and a progressive lengthening in interval of phases 3, and a decreased proportion of

adequately propagated activity fronts in diabetic rats. In strepto-zotocin induced diabetes, a lengthening of the cycle interval and a decrease of phases 3 have been reported as early as 4 days after the onset of diabetes.[12,13] These alterations in small bowel motility control are likely to be related to decreased nitrergic neuronal activity in the enteric nervous system. Indeed, we previously reported that NOS-inhibition by a 3-day oral administration of L-NG-nitroarginine methyl ester (L-NAME) induced changes on small intestinal EMG similar to those observed in diabetes: a decrease of phases 3 leading to lengthened migrating myoelectric complex cycle and decreased proportion of normally propagated activity fronts.[31] We observed similar changes in small bowel motor control and nNOS expression in a rat model of 2,4,6-trini-trobenzene sulfonic acid (TNBS)-induced jejunitis, again supporting a direct relationship between altered motor control and loss of nNOS expression and function.[31]

In our immunhistochemcal studies of myenteric plexus neurons, we found no consistent changes in NSE-IR and ChAT-IR neurons, but diabetic animals had a decreased number of nNOS-IR myenteric neurons in the antrum and jejunum. The finding of decreased nitrergic neuronal numbers confirms previous observations in spontaneous and induced animal models of Type 1 diabetes.[9,10,18] These changes seem relevant from a pathophysiological perspective, as previous in vitro studies reported decreased nitrergic relaxation of the stomach and small intestine in diabetic animals.[9,14,18] Similar loss of nitrergic motor control has been reported in the small intestine in aging animals.[40] We confirmed altered inhibitory motor control in the small bowel, which can be attributed to loss of nitrergic motor function in the present study and in a previous study on TNBS-induced small bowel dysmotility.[31]

It is unclear whether the decreased nNOS-IR in the present study reflects neuronal loss or altered nNOS expression. Guo et al[16] reported increased apoptosis in the myenteric plexus of strep-tozotocin induced diabetic rats, suggestive of loss of myenteric neurons in this diabetes model. In the present study, we did not detect a reduction in the number of NSE-IR neurons in diabetic animals, potentially arguing against neuronal loss. However, the number of nNOS-IR neurons is small in comparison to the total neuronal count, and it is conceivable that the decrease of nNOS population may not significantly affect the total number of neurons per ganglion.

The mechanism underlying loss of nNOS-IR neurons in the diabetic BB rat remains to be established. Vagal neuropathy has been proposed as an important contributor to diabetes-associated changes in gastrointestinal motility control, and especially in delayed gastric emptying.[3,8,41] However, RR-interval variation studies failed to demonstrate cardiac autonomic neuropathy in the cohort of BB rats in the present study. The finding seems to be at variance with previous studies,[23,42,43] but it is likely that this is related to the shorter follow-up in our study, as other studies reported altered RR-interval variability after 4 to 6 months. Moreover, although nNOS mRNA expression in the gastric myenteric plexus is under vagal control, neuronal NOS expression in the rat jejunum is independent of the vagus nerve.[40] Hence, vagal neuropathy is unlikely a mechanism to explain the changes in neural motor control that we observed at 8 and 16 weeks. Hyperglycemia also does not seem to determine these changes, as animals with less or more stringent glycemia control showed similar disturbances.

We did find evidence for a transient inflammatory infiltration at 8 weeks of diabetes, and we hypothesize that this contributed to the changes in neural motor control in the present study. Inflammation of the stomach, small intestine and colon has been reported in BB rat in the pre- or early diabetic stage.[27,28] In the colon, this was accompanied by damage to the myenteric and sub-mucosal plexus and impairment of colonic motor function.[27] The cause of this inflammatory infiltrate is unclear, and it might reflect impaired intestinal barrier function in the diabetic BB rat[44] or it could be a manifestation of the auto-immune inflammatory processes that are highly prevalent in this animal model.[45] The finding of transient inflammation followed by a decrease of myenteric nNOS-IR and a loss of inhibitory small intestinal motor control is reminiscent of our findings in TNBS-induced jejunitis.[31] Transient inflammation may lead to a down-regulation of nNOS through iNOS induction producing large quantities of NO[46,47] or through apoptotic cell death. On the other hand, in the present study, the changes in nNOS-IR persisted after resolution of the inflammation. Since several auto-antibodies implicated in diabetes can also be found on neurons,[48] one could also hypothesize that the intestinal infiltration is of auto-immune origin and contributes to permanent damage to nitrergic neurons. A case report in a diabetic woman, in whom severe gastrointestinal motor disturbances were accompanied by a marked reduction in expression of nNOS and inhibitory peptides on a jejunal biopsy, suggests that similar events may occur in human.[49]

In conclusion, BB rats display a progressive decrease in nNOS-IR myenteric neurons in the antrum and jejunum. This is not associated with signs of vagal neuropathy or with consistent change in the gastric emptying of semi-solids, but coincides with

transient inflammation of the gastrointestinal tract. Small in-testinal EMG shows abnormalities in inhibitory motor control reminiscent of the effects previously observed after chronic ad-ministration of the NOS-inhibitor L-NAME and after transient TNBS-induced inflammation. These observations suggest that diabetic BB rats have altered intestinal motor control, attributable to loss of nitrergic control and potentially related to transient in-flammatory infiltrates.

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