Published in final edited form as:

Auton Neurosci. 2011 February 24; 160(1-2): 69–81. doi:10.1016/j.autneu.2010.10.006.

Regional differences in neostigmine-induced contraction and relaxation of stomach from diabetic guinea pig

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Abstract

Delayed gastric emptying and autonomic neuropathy have been documented in patients with diabetes mellitus. Some medications used to treat delayed gastric emptying enhance release of acetylcholine from autonomic neurons to strengthen gastric contractions. Autonomic coordination among gastric regions may be altered in diabetes resulting in poor outcomes in response to prokinetic drugs. Fundus, antrum, and pylorus from STZ or control guinea pigs were treated with neostigmine to mimic release of acetylcholine from autonomic neurons by prokinetic agents. In diabetic animals, neostigmine-induced contractions were weaker in fundus and pylorus but similar in antrum. The muscarinic receptor antagonist 4-DAMP or the nicotinic receptor antagonist hexamethonium reduced neostigmine-induced contractions. Activation of presynaptic muscarinic receptors on nitrergic neurons was impaired in fundus and antrum from diabetic animals. Nerve-stimulated contractions and relaxations, number of nNOS myenteric neurons, and tissue choline content were reduced in fundus from diabetic animals. Despite reduced number of myenteric neurons, tissue choline content was increased in antrum from diabetic animals. Since cholinergic motility of each gastric region was affected differently by diabetes, prokinetic drugs that nondiscriminately enhance acetylcholine release from autonomic neurons may not effectively normalize delayed gastric emptying in patients with diabetes and more selective medications may be warranted.

Introduction

Gastric emptying, delivery of contents to the small intestine at the appropriate rate for optimal digestion and absorption of ingested nutrients, is highly regulated by parasympathetic reflexes, enteric neural reflexes, and gastrointestinal (GI) hormones. After a

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swallow, gastric accommodation of the meal involves fundic relaxation by activation of the vagal inhibitory pathway (Cannon et al., 1911). Stored fundic contents are gradually delivered to the caudad stomach where peristaltic contractions, modified by the vagal excitatory pathway (Langley, 1898), reduce food particle size and combine gastric secretions with the ingested meal. As antral peristaltic contractions force gastric contents towards the pylorus, small amounts spurt into the duodenum when the sphincter is open, but contents are repelled back into the gastric body when the sphincter is closed (Cannon, 1911). Gastrointestinal hormones released from mucosal endocrine cells in response to luminal nutrients modulate gastric emptying rate (Burks et al., 1985) to optimize carbohydrate, protein and fat digestion and absorption.

Some patients with diabetes mellitus (DM) have delayed gastric emptying (Kloetzer et al., 2010; Maggs et al., 2008; Shakil et al., 2008) which slows carbohydrate delivery to the small intestine and delays intestinal glucose absorption thereby interfering with postprandial blood glucose control by insulin (Khoo et al., 2009). In turn, abnormal blood glucose can alter gastric emptying rate in patients with DM (Khoo et al., 2009; Maggs et al., 2008). According to Ma *et al.* (Ma et al., 2009), delayed gastric emptying can result from a distinct malfunction of a single region of gut or from impaired coordination of motor activity among gastric regions. In patients with DM, separate studies have shown impaired fundic accommodation (Kumar et al., 2008; Samsom et al., 1995; Samsom et al., 1998; Troncon et al., 1998) and weak or disordered antral contractions (Rosztoczy et al., 2004; Samsom et al., 1996). Therefore, both impaired relaxation and contraction of gastric regions may contribute to a slower than normal rate of gastric emptying in patients with DM.

Regions of the stomach may be affected differently by the low concentration of effective insulin and high plasma glucose that characterize DM, weakening the autonomic coordination required for efficient gastric emptying. Using gastric regions from the same type 2 diabetic db/db mice, bethanechol-induced contractions were decreased in fundus, unchanged in the antrum, and increased in pylorus (James et al., 2004). One goal of this study was to determine if the normal variation in cholinergic contractions of fundus, antrum, and pylorus characterized for control animals was uniformly altered in type 1 diabetic animals.

Prokinetic medications used to treat patients with gastroparesis include those that enhance endogenous Ach release from autonomic neurons (Hasler, 2007; Park et al., 2006; Patrick et al., 2008; Rayner et al., 2005; Sanger et al., 2008). One goal of this study was to use neostigmine, a reversible inhibitor of acetylcholinesterase (AChE) to mimic the action of prokinetic agents. In the presence of neostigmine, Ach breakdown is slowed and activation of cholinergic receptors is prolonged. Since endogenous Ach released by prokinetic medications can activate both muscarinic and nicotinic receptor subtypes, selective antagonists were used to evaluate the individual contributions of Ach at the smooth muscle neuroeffector junction and in the neuronal synapse to neostigmine-induced contractions of gastric tissues from control and diabetic animals.

Using animal models of DM, the causes of gastrointestinal motility abnormalities have been further defined and include autonomic neuropathy of the vagus nerve (Kniel et al., 1986; Regalia et al., 2002; Robertson et al., 1980) and enteric neurons (Chandrasekharan et al., 2007). Since acetylcholine (ACh) is the primary excitatory neurotransmitter of autonomic neurons, it is not surprising that the activity and expression of enzymes involved in the synthesis and disposition of ACh were altered in diabetic animals. In STZ guinea pigs, choline acetyltransferase (ChAT) immunoreactive neuronal processes in the ileal tertiary plexus, representing excitatory enteric motor neurons innervating longitudinal muscle, were increased in density as compared to control animals suggesting increased expression of the

synthetic enzyme (LePard, 2005). To investigate if neuronal Ach content was altered in diabetes, the concentrations of choline, a precursor in Ach synthesis, in whole pieces of fundus, corpus, and antrum were determined.

Activation of cholinergic receptors on enteric neurons relaxed gut smooth muscle by increasing generation of nitric oxide from L-arginine by the enzyme neuronal nitric oxide synthase (nNOS). In this study, Ach-induced relaxation of tissues from gastric regions was evaluated after selective antagonism of either muscarinic or nicotinic receptors. In addition, since expression of nNOS was decreased in animal models of DM (Cellek, 2004; Takahashi, 2003), the number of nNOS immunoreactive neurons in the myenteric plexus of fundus, corpus, and antrum from control and diabetic animals was determined.

In animal models of DM, gastric motor dysfunction may result from either a low concentration of effective plasma insulin, abnormal plasma c-peptide concentration, or elevated plasma glucose. This study uses a streptozotocin (STZ)-induced model of type 1 DM in guinea pigs to capitalize on the wealth of information on gastric enteric neurochemical coding and motor circuits published for guinea pig (Michel et al., 2000; Schemann et al., 1995; Vanden Berghe et al., 1999). Similar to rodent models, insulin and c-peptide were very low in STZ guinea pigs (Gorray et al., 1986; Howell et al., 1971; Junod et al., 1969; Lundquist et al., 1975). However, hyperglycemia and ketosis were more mild in STZ guinea pigs (Elliott et al., 1977; Gorray et al., 1986; Hootman et al., 1998; Johnson, Jr., 1978; Peterssen et al., 1970; Schlosser et al., 1984a; Schlosser et al., 1987) as compared to STZ rats (Hawkins et al., 1986; Junod et al., 1969). Hence, to focus on the contribution of insulin deficiency to autonomic neuropathy and motor dysfunction, the effect of neostigmine on gastric motility was determined in STZ-treated guinea pigs.

Materials and Methods

Induction of diabetes by streptozotocin

Diabetes was induced in male Hartley guinea pigs (n=26, 200–300 g, Charles River Laboratories, Inc, Portage, MI) by a single intraperitoneal (ip) injection of STZ (50 mg/mL, 280 mg/kg, Sigma, St. Louis, MO) dissolved in citrate buffer (5M, pH 4.5) (Schlosser et al., 1984b). Control animals (n=19) were given a single ip injection of citrate buffer. Animals were euthanized by CO₂ narcosis 5–6 weeks after injection. Stomach was removed and placed in warmed, oxygenated Krebs solution of the following composition (mM): NaCl, 117; KCl, 4.7; CaCl₂, 2.5; MgCl₂, 1.2; NaH₂PO₄, 1.2; NaHCO₃, 25; glucose, 11. Kidneys were removed, blotted and weighed to determine the ratio of final body weight to kidney weight.

Preparations for motility recordings

Using razor blades, the pyloric sphincter was cut from antrum and duodenum then mounted in the tissue bath using metal stirrups. Stomach was cleaned of contents, trimmed of mesentery, cut along the greater curvature, pinned, and stretched mucosa side down in a sylgard-lined petri dish. Whole thickness strips of fundus (including portions of the corpus, typically 3 per animal) and antrum (caudal from the incisura angularis, typically 2 per animal) were cut with razor blades along either the longitudinal or circular muscle axis. Two sets of stitches (4-0 suture) were made at each end of the preparation, then loops were tied. Using the loops, tissues were mounted to hooks, lowered into a 10 mL organ bath (8 channel Myobath, World Precision Instrument, Sarasota, FL), maintained at 37°C by a heater/circulating bath (DC10, Haake Thermo Electron Corporation, Karlsruhe, Germany), and continuously bubbled with 95% O₂/5% CO₂. Contractile responses were recorded using isometric force transducers, then digitized (PowerLab 8SP, ADInstruments, Colorado

Springs, CO), and saved to a computer hard drive. Voltage for nerve stimulation was applied across platinum wire electrodes mounted to the tissue holder (6, volts, DS8000 stimulator, World Precision Instrument, Sarasota, FL).

At the start of each experiment, resting tension was adjusted to approximately 1.0 g and tissues were washed 6×10 min with normal Krebs solution to establish baseline. For antrum and fundus, at least one basal contraction was required to peak ≥ 0.2 g or tissue was replaced. All pylori were used. At the end of the experiment, the wet weights of all tissues were obtained and some gastric preparations were frozen at -20°C for analysis of choline.

Treatment with neostigmine or bethanechol

Experiments were performed to determine the effect of bethanechol (Sigma, St. Louis, MO), a muscarinic receptor agonist, and neostigmine (Sigma, St. Louis, MO), an antagonist of AchE, on motility. Tissues were treated with increasing doses of drug for 5 minutes with 3×10 minute washes between each dose.

Mechanism of neostigmine-induced contraction

In separate experiments, tissues were pretreated for 2 min with either vehicle, the muscarinic receptor antagonist 4-diphenylacetoxy-N-methylpiperidine (4-DAMP, Sigma) or the nicotinic neural receptor antagonist hexamethonium (C6, Sigma) then treated with 10 μM neostigmine for 5 min.

Analysis of smooth muscle contractions

Contractions were analyzed using computer software (Chart 5 version 5.5.4, ADInstruments, Colorado Springs, CO). Computer analysis identified prominent peaks over 2 min for calculation of frequency. Basal tone was calculated by average force over 2 min. Basal tone and frequency of contraction were determined in absence and presence of 4-DAMP or hexamethonium. Computer analysis calculated area under the curve for a maximum of 5 min. Area under the curve ($\text{g}\cdot\text{s}$) was determined for all contractions in the absence (no drug) or presence of bethanechol or neostigmine as well as for contractions elicited by bethanechol or neostigmine after pretreatment with 4-DAMP or C6. In addition, the area of bethanechol- or neostigmine-induced contraction after drug pretreatment (4-DAMP, C6) was compared to the area of bethanechol- or neostigmine-induced contraction after vehicle pretreatment and data were expressed as either percent inhibition or percent change with positive and negative values representing an increase and decrease in AUC, respectively. For the neostigmine dose-response curve, data were also expressed as area of contraction per gram of tissue wet weight. However, given that whole thickness strips of tissue and intact pylorus contain mass that does not contribute to force of contraction (Hasler, 2010), most data were expressed as area of contraction. For neostigmine dose-response curves, curve fitting was performed using 4 parameter logistic standard curve fitting (Sigma Plot 10) with r^2 ranging from 0.74 to 0.97 and EC_{50} derived from the curve fit data.

Choline tissue content

Tissues used in motility experiments and additional separate pieces of stomach were frozen at -20°C . After thawing on ice, each piece was weighed then ground in normal Krebs (final volume 2 ml) using a manual grinding tube (Fisher Scientific). The slurry was transferred into a microcentrifuge tube, then centrifuged for 4 minutes at $14,000\text{ min}^{-1}$. Supernatant from each microcentrifuge tube was aspirated, avoiding the pellet, and placed in a clean centrifuge tube then frozen at -20°C until analysis for choline.

Tissue supernatant was assayed for choline content by enzymatic assay using a chemiluminometer (Model 20 photometer, Mt. View, CA). A reaction mixture was made

consisting of 7.5 ml normal Krebs, 100 μ L of 250 U/mL choline oxidase, 50 μ L of 2 mg/mL horseradish peroxidase, 100 μ L of 1 μ M luminol (Sigma), and 80 μ L of 1000 U/mL cholinesterase. Tissue supernatant (100 μ L) was vortexed with 150 μ L of reaction mixture for 60 seconds. After a 5 second delay, the light value was read by the chemiluminometer. The choline concentration in each 100 μ L sample was determined using standard curves to choline (10–1000nM). The correlation coefficients (r^2) for each standard curve were ≥ 0.98 .

Neuronal nitric oxide synthase (nNOS)

The number of myenteric neurons immunoreactive (ir) for nNOS were counted in sections of fundus, corpus, and antrum from a group of control and diabetic animals not used in tissue bath experiments. Tissues were fixed overnight at 4°C in modified Zamboni's fixative (15% saturated picric acid and 2% formaldehyde in 0.1 M PBS, pH 7.40) then washed 3 $\times$ 10 min in dimethyl sulphoxide followed by PBS (0.1 M, pH 7.40). Tissues were incubated overnight in a dark, humidified chamber with primary antisera to nNOS (rabbit, 1:500, N53130, BD Transduction Labs, Lexington, KY) and HuC/HuD (HuC/D, mouse, 1:200, A21275, Molecular Probes, Eugene, OR), a panneuronal marker that stains neuronal cell bodies but not processes. Both primary antibodies were applied to tissues at the same time at the final concentration. Tissues were rinsed 3 $\times$ 10 min in PBS then incubated for 2 hrs at room temperature in a dark, humidified chamber with secondary antisera for nNOS (sheep anti-rabbit Cy3, 1:100, C2306, Sigma Chemical Company, St. Louis, MA). HuC/D was directly tagged with Alexa Fluor 488. Finally, tissues were washed 3 $\times$ 10 min in PBS and mounted with buffered glycerol. Fluorescence was viewed using appropriate filters on a microscope with ultraviolet light source (Leica DMIL) and images were taken using attached digital camera (MagnaFire, Optronics).

Myenteric ganglia were selected for analysis based only on quality of HuC/D staining, not the presence or absence of nNOS-ir neurons or size of ganglia. The numbers of HuC/D-ir neurons (2976–4992 per group) and nNOS-ir neurons (990–1410 per group) were counted at 40 $\times$ for 5–39 images per animal (100–139 ganglia) by a blinded investigator (KD). Every nNOS-ir neuron was also positive for HuC/D. Data were expressed as number of HuC/D-ir neurons or nNOS-ir neurons per individual ganglion and percent nNOS-ir neurons per total HuC/D-ir neurons per ganglion.

Statistics

All data were expressed as mean $\pm$ SEM. The n represents the number of preparations or ganglia. Data were statistically analyzed using SPSS (version 14.0, Chicago, IL). Outliers of control and diabetic groups were determined using a boxplot graph and removed prior to comparisons using the Student's t-test or factorial analysis of variance (ANOVA) with pairwise comparisons (Percy, 1996). Depending on the analysis, independent factors for ANOVA included 1) control or diabetic, 2) dose of neostigmine or hexamethonium, 3) drug (vehicle, 4-DAMP, hexamethonium), 4) region (fundus, antrum, pylorus), 5) longitudinal or circular muscle axis, or 6) neostigmine exposure (single, multiple). Main effects and interactions were determined and Sidak correction was used for post-hoc analysis. Prevalence of nerve-stimulated contractions and relaxations was determined using Chi Square analysis. For cell count data, regional differences within control or diabetic group were determined using the nonparametric Kruskal-Wallis test with post-hoc Mann-Whitney U test. The effect of control and diabetic was determined for each region using Mann-Whitney U test. Bonferroni correction was used to adjust the overall p value for multiple comparisons to $p < 0.05$. Statistically significant differences between control and diabetic animals were denoted using * $p < 0.05$, whereas statistically significant differences within control or diabetic group were denoted as † $p < 0.05$ or ‡ $p < 0.05$.

Results

Animal characteristics

Before injection of STZ, both groups of animals (control $n=13$, diabetic $n=20$) had similar body weights [g: control, 272 ± 5 ; diabetic, 265 ± 4]. At the time of the experiment 5–6 weeks after STZ injection, diabetic animals weighed less than control animals [g: control, 487 ± 15 ; diabetic, $360\pm18^*$; $*p<0.05$]. Diabetic animals had a higher kidney weight to body weight ratio [g/gx 10^{-3} : control, 4.22 ± 0.06 ; diabetic, $5.83\pm0.16^*$; $*p<0.05$]. In diabetic animals, the stomachs were commonly distended suggesting delayed gastric emptying (Watkins et al., 2000). Motility preparations from control and diabetic animals were similar weight [Main effect: control/diabetic; $F(1,178)=0.274$] [mg: control ($n=78$), 62.5 ± 3.8 ; diabetic ($n=106$), 58.9 ± 3.9] and pylori were heavier than preparations from fundus or antrum [Main effect: region; $F(2,178)=94.622$; $p<0.05$] [mg: fundus ($n=94$), 45.8 ± 2.1 ; antrum ($n=59$), 52.6 ± 2.4 ; pylorus ($n=31$), $119.8\pm8.2^+$; $^+p<0.05$]. There was a trend for antral preparations from diabetic animals to be lighter than those from control animals [Interaction: control/diabetic*region; $F(2,178)=2.979$; $p=0.053$] [mg: control ($n=19$), 62.8 ± 4.3 ; diabetic ($n=40$), 47.8 ± 2.5].

Basal motility

Gastric preparations from control and diabetic animals had similar frequencies of basal contraction prior to any drug treatment. Frequency of contraction was highest in antrum [Hz: control ($n=11$), 0.08 ± 0.00 ; diabetic ($n=18$), 0.07 ± 0.01], followed by fundus [Hz: control ($n=19$), 0.04 ± 0.01 ; diabetic ($n=23$), 0.05 ± 0.01], then pylorus [Hz: control ($n=6$), 0.01 ± 0.00 ; diabetic ($n=9$), 0.01 ± 0.00].

In the fundus and antrum from control or diabetic animals, neither 4-DAMP (1 μM) nor hexamethonium (100, 300 μM) altered the frequencies of contractions (data not shown). Using pylorus from control or diabetic animals, contractions were absent after pretreatment with 4-DAMP or the high dose of hexamethonium. At the lower dose of hexamethonium, contractions persisted in control, but not diabetic pylorus [Hz: control ($n=6$), 0.03 ± 0.02 ; diabetic ($n=9$), $0\pm0^*$; $*p<0.05$]. In all gastric preparations from control or diabetic animals, no change in tone was observed after treatment with 4-DAMP or hexamethonium (data not shown).

Nerve-stimulated contractions and relaxations of gastric preparations

Frequency of nerve-stimulated contractions and relaxations varied with stomach region (Fig. 1A). All pylori from control and diabetic animals contracted, but did not relax, in response to nerve stimulation. Some fundic and antral preparations contracted then relaxed, while others only contracted or only relaxed. Within control animals, nerve-stimulated contractions were more prevalent in fundus than antrum, but within diabetic animals, the opposite was observed ($^+p<0.05$). Hence, nerve-stimulated contractions were more prevalent in diabetic antrum but less prevalent in diabetic fundus as compared to control regions ($*p<0.05$). Nerve-stimulated relaxations were more prevalent in both control and diabetic fundus as compared to antrum ($^+p<0.05$).

Strength of nerve-stimulated contractions varied with gastric region from control and diabetic animals (Fig. 1B) [Interaction: control/diabetic*region; $F(1,91)=15.893$, $p<0.05$] but not with circular or longitudinal axis of preparation [Interaction: control/diabetic*region*circular/longitudinal; $F(1,91)=0.00$, $p=0.991$]. Within control animals, nerve-stimulated contractions were stronger in fundus than antrum ($^+p<0.05$). In contrast, within diabetic animals, antral nerve-stimulated contractions were stronger as compared to fundus ($^+p<0.05$). Hence, nerve-stimulated contractions of diabetic fundus, but not antrum,

were weaker as compared to control animals (* $p < 0.05$). Similarly, nerve-stimulated contractions of pylorus were weaker in diabetic as compared to control animals [area (g*s): control (n=3), 13 ± 2 ; diabetic (n=9), $8 \pm 1^*$; * $p < 0.05$].

Strength of nerve-stimulated relaxations did not vary with gastric region from control and diabetic animals [Interaction: control/diabetic*region; $F(1,91)=2.010$, $p=0.160$] or with circular or longitudinal axis of preparation [Interaction: control/diabetic*region*circular/longitudinal; $F(1,91)=0.987$, $p=0.323$]. Overall, nerve-stimulated relaxations were weaker in diabetic as compared to control tissues [Main effect: control/diabetic; $F(1,91)=4.818$, $p < 0.05$] and stronger in fundus as compared to antrum [Main effect: region; $F(1,91)=3.971$, $p < 0.05$]. Regardless of region, relaxations of preparations cut on the longitudinal muscle axis, but not the circular muscle axis, were weaker in gastric tissues from diabetic as compared to control animals (Fig. 1B, * $p < 0.05$) [Interaction: control/diabetic*LM/CM; $F(1,91)=4.511$, $p < 0.05$].

Muscarinic contractions (Fig. 2)

Cholinergic contractions were evaluated in response to direct activation of muscarinic receptors by bethanechol (10 μ M). In general, bethanechol-induced contractions were strongest in pylorus but similar in fundus and antrum ($p < 0.05$) [Main effect: region; $F(2,77)=23.192$, $p < 0.05$]. There was a strong trend for bethanechol-induced contractions to be weaker in diabetic as compared to control animals [Main effect: control/diabetic; $F(1,77)=3.846$, $p=0.053$]. In diabetic animals, bethanechol-induced contractions were 7% stronger in fundus, 34% weaker in antrum, and 21% weaker in pylorus as compared to regions from control animals. To confirm that bethanechol contracted smooth muscle by activating muscarinic receptors, the ability of the selective muscarinic receptor antagonist 4-DAMP (1 μ M) to reduce the area of bethanechol-induced contractions was also determined. 4-DAMP was less effective in inhibiting bethanechol-induced contractions of fundus (83–88%) as compared to antrum (96–99%) or pylorus (99–100%) [Main effect: gastric region, $F(2,77)=9.944$, $p < 0.05$] but was equally effective in control and diabetic animals [Main effect: control/diabetic; $F(1,77)=0.587$, $p=0.45$].

Effect of longitudinal and circular muscle axis on neostigmine-induced gastric contractions (Fig. 3)

Whole thickness strips of fundus and antrum from control and diabetic animals cut on either the circular or longitudinal muscle axis were exposed to increasing concentrations of neostigmine (0.1–50 μ M). Within diabetic but not control antrum, there was a trend for preparations cut on the longitudinal muscle axis to contract more strongly to all neostigmine doses than those cut on the circular muscle axis (Fig. 3B) [Interaction: control/diabetic*region*longitudinal/circular; $F(1,419)=3.370$, $p=0.067$] [area (g*s): control: circular (n=21), 113 ± 26 ; longitudinal (n=28), 91 ± 19 ; diabetic: circular (n=77), 101 ± 12 ; longitudinal (n=52), 137 ± 19] whereas control and diabetic fundic preparations cut on the circular or longitudinal axis contracted similarly to neostigmine doses (Fig. 3A) [area (g*s): control: circular (n=69), 113 ± 16 ; longitudinal (n=87), 118 ± 10 ; diabetic: circular (n=64), 52 ± 4 ; longitudinal (n=80), 68 ± 6]. In general, neostigmine-induced contractions of preparations cut on the circular, but not longitudinal, muscle axis were weaker in diabetic as compared to control animals [Interaction: control/diabetic*longitudinal/circular; $F(1,419)=4.475$, $p < 0.05$] [area (g*s): circular: control: (n=90), 113 ± 14 ; diabetic: (n=141), $78 \pm 7^*$; * $p < 0.05$] [area (g*s): longitudinal control: (n=115), 112 ± 9 ; diabetic: (n=132), 94 ± 9 ; NS]. However, since axis of the preparation did not alter neostigmine dose-response curves of antral and fundic preparations from control and diabetic animals (Fig. 3) [Interaction: control/diabetic*region*longitudinal/circular*dose; $F(6,419)=0.316$, $p=0.928$], the axis of the preparation was ignored in subsequent analyses.

Neostigmine-induced contractions (Fig. 4 and Fig. 5)

Increasing concentrations of neostigmine dose-dependently increased the area of neostigmine-induced contractions of fundus, antrum, and pylorus from control and diabetic animals (Fig. 4) [Interaction: control/diabetic*region*dose; $F(13,511)=1.933$, $p<0.05$]. The doses of neostigmine resulting in half-maximal contractions (EC_{50}) were similar between control and diabetic animals with pylorus most sensitive to neostigmine [control, 0.29 ± 0.19 μ M; diabetic, 1.33 ± 0.45 μ M] and fundus least sensitive to neostigmine [control, 1.2 ± 1.1 μ M; diabetic, 3.3 ± 2.5 μ M]. The minimum effective dose producing measurable contractions was lowest in control pylorus (1 μ M) and highest in control fundus and diabetic antrum (10 μ M).

In diabetic but not control animals, neostigmine-induced contractions of fundus were weaker than antrum (Fig. 5). Within control animals, antrum and fundus contracted similarly to all doses of neostigmine, but contractions of pylorus were stronger than antrum and fundus at 1.0 μ M and 10 μ M neostigmine ($\dagger p<0.05$). Within diabetic animals, contractions of diabetic fundus were weaker than diabetic antrum at 10 and 30 μ M neostigmine ($\ddagger p<0.05$), and weaker than diabetic pylorus at 10, 30, and 50 μ M neostigmine ($\dagger p<0.05$).

At some doses, neostigmine-induced contractions of gastric regions were significantly weaker in diabetic as compared to control animals (Fig. 4) [Interaction: control/diabetic*region*dose; $F(13,511)=1.933$, $p<0.05$]. For diabetic fundus, the dose-response curve to neostigmine was shifted to the right with neostigmine-induced contractions at 10 and 30 μ M weaker than control ($*p<0.05$) and a strong trend for weaker contractions observed at 1 μ M ($p=0.054$). The maximum contraction was weaker in diabetic as compared to control fundus. For diabetic pylorus, the dose-response curve to neostigmine was shifted to the right with 0.3, 0.5, and 1 μ M neostigmine contractions weaker in diabetic than control ($*p<0.05$) and a trend for weaker contractions observed at 10 μ M neostigmine ($p=0.088$). The maximum contraction was similar in control and diabetic pylorus. In contrast, antral contractions to all neostigmine doses were similar in preparations from control and diabetic animals.

In some tissues, once the maximum contraction to neostigmine was elicited, increasing the neostigmine dose resulted in a decrease in the area of contraction (Fig. 4). Within control tissues, area of contractions at 10 μ M neostigmine were significantly weaker than at 50 μ M neostigmine ($\dagger p<0.05$) for fundus and pylorus, but not antrum. Within diabetic tissues, only in the antrum was there a trend for a decrease in area at 50 μ M as compared to 10 μ M neostigmine ($p=0.094$).

Similarly, when data for force of contractions was expressed as area per gram tissue weight, neostigmine-induced contractions of gastric regions were altered in diabetic as compared to control animals [Interaction: control/diabetic*region*dose; $F(13,511)=1.842$, $p<0.05$]. Contraction of diabetic fundus was weaker than control fundus at 10 μ M neostigmine [area/g tissue: control ($n=27$), 5.34 ± 1.03 ; diabetic ($n=24$), $2.09\pm0.31^*$; $*p<0.05$] and at 30 μ M neostigmine [area/g tissue: control ($n=21$), 5.68 ± 1.16 ; diabetic ($n=19$), $2.55\pm0.43^*$; $*p<0.05$]. Contraction of diabetic pylorus was weaker than control pylorus at 0.5 μ M neostigmine [area/g tissue: control ($n=4$), 5.06 ± 3.43 ; diabetic ($n=5$), $0.39\pm0.14^*$; $*p<0.05$], at 1 μ M neostigmine [area/g tissue: control ($n=7$), 6.02 ± 4.04 ; diabetic ($n=7$), $1.16\pm0.73^*$; $*p<0.05$], and at 10 μ M neostigmine [area/g tissue: control ($n=7$), 7.39 ± 4.54 ; diabetic ($n=8$), $3.35\pm1.32^*$; $*p<0.05$]. In contrast, contraction of diabetic antrum was stronger than control antrum at 10 μ M neostigmine [area/g tissue: control ($n=8$), 2.63 ± 0.86 ; diabetic ($n=22$), $5.92\pm1.02^*$; $*p<0.05$] probably since there was a strong trend for diabetic antral preparations to be lighter than control antral preparations (see Animal characteristics).

Contraction of tissues to a single dose of neostigmine (Table 1)

In order to determine the individual roles of muscarinic and nicotinic receptors in mediating neostigmine-induced contractions, separate experiments were performed. In these mechanistic experiments, tissues were first pretreated with vehicle followed by 10 μ M neostigmine. Contractions elicited by this single dose of neostigmine were significantly different than contractions obtained at the same dose in the dose-response experiments [Interaction: control/diabetic*region*neostigmine exposure; $F(2,167)=5.025$, $p<0.05$]. A single dose (SD) of 10 μ M neostigmine produced weaker contractions than 10 μ M neostigmine in the dose-response experiments (MDR) in control fundus, but stronger contractions in control antrum, control pylorus, and diabetic pylorus ($\dagger p<0.05$) with a trend observed for stronger contractions in diabetic fundus ($p=0.07$). In contrast, for diabetic antrum, contractions to the same doses of neostigmine were similar regardless of experimental protocol. In contrast to the dose-response experiments ($*p<0.05$), similar strength contractions were observed after a single dose of 10 μ M neostigmine in diabetic as compared to control fundus ($p=0.331$).

Role of muscarinic receptors in neostigmine-induced contraction

With muscarinic receptors blocked by 4-DAMP (1 μ M), Ach activated nicotinic neural receptors on noncholinergic excitatory and inhibitory enteric motor neurons to alter force production. 4-DAMP was equally effective in inhibiting neostigmine-induced contractions of all gastric regions from control and diabetic animals [Interaction: control/diabetic*region; $F(2,77)=0.027$, $p=0.973$] [%inhibition: fundus: control ($n=19$), 94 ± 2 ; diabetic ($n=22$), 88 ± 3 ; antrum: control ($n=11$), 97.1 ± 0.4 , diabetic ($n=17$), 91 ± 2 ; pylorus: control ($n=6$), 98 ± 1 ; diabetic ($n=8$), 94 ± 6]. There was a strong trend for 4-DAMP to be less effective in inhibiting neostigmine-induced contractions of tissues from diabetic as compared to control animals [Main effect: control/diabetic; $F(1,77)=3.980$, $p=0.050$].

The areas of contractions evoked by neostigmine (Neo) after 4-DAMP (4D) pretreatment were different in regions of stomach from control and diabetic animals (Fig. 6) [Interaction: control/diabetic*region; $F(2,79)=5.256$, $p<0.05$]. These contractions in the presence of 4D Neo were weaker in diabetic pylorus as compared to control pylorus ($*p<0.05$), stronger in diabetic antrum as compared to control antrum ($*p<0.05$), and similar in control and diabetic fundus. Within diabetic but not control animals, contractions of pylorus in the presence of 4-DAMP Neo were significantly weaker than fundus or antrum ($\ddagger p<0.05$).

Role of nicotinic receptors in neostigmine-induced contraction

With nicotinic receptors blocked by hexamethonium (100 or 300 μ M), Ach activated muscarinic receptors located postjunctionally on smooth muscle and presynaptically on excitatory and inhibitory enteric neurons to alter force production. Hexamethonium (100 or 300 μ M) was equally effective in reducing the area of neostigmine-induced contractions in all gastric regions from control and diabetic animals [Interaction: control/diabetic*region*dose; $F(2,151)=0.720$, $p=0.489$] [%inhibition by 300 μ M C6: fundus: control ($n=18$), 82 ± 4 ; diabetic ($n=20$), 73 ± 4 ; antrum: control ($n=10$), 91 ± 1 , diabetic ($n=17$), 82 ± 3 ; pylorus: control ($n=6$), 95 ± 1 ; diabetic ($n=9$), 83 ± 9]. In contrast, regional differences were observed in the ability of 100 μ M hexamethonium to alter neostigmine-induced contractions [Interaction: region*dose; $F(2,151)=10.588$, $p<0.05$]. At 100 μ M, hexamethonium moderately reduced the neostigmine-induced contraction of control and diabetic pylorus, weakly reduced the neostigmine-induced contraction of control and diabetic antrum, and had no effect in control and diabetic fundus [%inhibition by 100 μ M C6: fundus: control ($n=19$), $-15\pm8\ddagger$; diabetic ($n=21$), $-2\pm5\ddagger$; antrum: control ($n=11$), 17 ± 7 , diabetic ($n=17$), 22 ± 7 ; pylorus: control ($n=6$), $54\pm10\ddagger$; diabetic ($n=9$), $41\pm8\ddagger$; $\ddagger p<0.05$ as compared to fundus and antrum, $\ddagger p<0.05$ as compared to antrum]. At the higher dose of

hexamethonium (300 μ M), these regional differences were not observed. The areas of contractions evoked by neostigmine after hexamethonium (300 μ M) pretreatment were stronger in tissues from diabetic as compared to control animals regardless of region [Main effect: control/diabetic: $F(1,78)=7.821$, $p<0.05$] [area (g*s): control (n=35), 28 ± 5 ; diabetic (n=49), $46\pm5^*$; $*p<0.05$].

Contributions of muscarinic and nicotinic receptors to neostigmine-induced contractions

Either 4-DAMP or hexamethonium (300 μ M) was equally effective in inhibiting the neostigmine-induced contractions of gastric regions from control or diabetic animals [Interaction: control/diabetic*region*drug; $F(2,151)=0.075$, $p=0.927$]. Percent inhibition of neostigmine-induced contractions was weaker in diabetic as compared to control animals [Main effect: control/diabetic; $F(1,151)=10.926$, $p<0.05$], weakest in fundus as compared to pylorus or antrum [Main effect: region; $F(2,151)=5.736$, $p<0.05$], and stronger by 4-DAMP than hexamethonium [Main effect: drug; $F(1,151)=15.783$, $p<0.05$]. The greater effectiveness of 4-DAMP was also evident in that the areas of contractions evoked by neostigmine after 4-DAMP pretreatment were smaller than those evoked by neostigmine after hexamethonium (300 μ M) pretreatment in tissues from both control and diabetic animals [Interaction: control/diabetic*drug; $F(1,157)=6.587$, $p<0.05$].

Comparison of contractions evoked by neostigmine after 4-DAMP or hexamethonium (300 μ M) pretreatment to basal contractions (Fig. 6). Different strengths of contractions were evoked in fundus or pylorus from control or diabetic animals after exposure to 4-DAMP or hexamethonium followed by neostigmine (Neo) as compared to basal contractions [Fundus: Interaction: control/diabetic*drug; $F(2,129)=3.907$, $p<0.05$] [Pylorus: Interaction: control/diabetic*drug; $F(2,37)=4.253$, $p<0.05$]. Areas of contractions evoked by neostigmine after 4-DAMP pretreatment (4D Neo) were weaker than areas of basal contractions in absence of drug (No Drug) for both control and diabetic fundus ($\dagger p<0.05$), but not control or diabetic pylorus. Areas of contractions evoked by neostigmine after hexamethonium pretreatment (C6 Neo) were weaker than areas of basal contractions in absence of drug for control fundus ($\dagger p<0.05$) but stronger for diabetic pylorus ($\dagger p<0.05$).

In contrast, using antrum from control or diabetic animals, areas of contraction were similar after exposure to 4-DAMP or hexamethonium followed by neostigmine as compared to basal contractions [Interaction: control/diabetic*drug; $F(2,82)=0.081$, $p=0.922$]. Overall, area of contraction was stronger in preparations from diabetic as compared to control animals ($p<0.05$) [Main effect: control/diabetic; $F(1,82)=4.606$, $p<0.05$] and contractions evoked by neostigmine after 4-DAMP pretreatment (4D Neo) were weaker, but contractions evoked by neostigmine after hexamethonium pretreatment (C6 Neo) were stronger, as compared to basal contractions (No Drug) ($p<0.05$) [Main effect: drug; $F(2,82)=13.752$, $p<0.05$].

Tissue Choline (Table 2)

Tissue choline was altered in gastric regions from control and diabetic animals [Interaction: control/diabetic*region; $F(2,118)=7.753$, $p<0.05$]. The amounts of choline in tissues were increased in antrum, reduced in fundus, but similar in pylorus. Within control animals, choline was lower in the antrum and pylorus as compared to the fundus ($\dagger p<0.05$), but within diabetic animals, choline was higher in antrum and fundus as compared to pylorus ($\dagger p<0.05$).

Neuronal Nitric Oxide Synthase (Table 3)

There was a wide range of HuC/D-ir neurons per individual ganglion [control fundus, 6–69; diabetic fundus, 5–63; control corpus, 5–80; diabetic corpus, 8–83; control antrum, 5–117;

diabetic antrum, 9–110]. Within control or diabetic animals, antrum contained a higher number of HuC/D-ir neurons per ganglion than fundus ($\dagger p < 0.05$) or corpus ($\ddagger p < 0.05$). In addition, within diabetic animals, corpus contained a higher number of HuC/D-ir neurons per ganglion than fundus ($\dagger p < 0.05$). The numbers of HuC/D-ir myenteric neurons per ganglion were significantly decreased in diabetic fundus and antrum as compared to control fundus and antrum ($*p < 0.05$).

There was a narrower range of nNOS-ir neurons per individual ganglion [control fundus, 1–26; diabetic fundus, 0–19; control corpus, 0–25; diabetic corpus, 0–35; control antrum, 1–25; diabetic antrum, 0–36]. Within control animals, there were more nNOS-ir neurons in ganglion from antrum as compared to corpus ($\ddagger p < 0.05$). The number of nNOS-ir myenteric neurons per ganglion was reduced in fundus, but not corpus or antrum, from diabetic as compared to control animals ($*p < 0.05$).

Due to the wide range of HuC/D-ir neurons per ganglion, when expressed as a percent, there was a wide range of nNOS-ir neurons per total HuC/D-ir neurons in the ganglion [control fundus, 7–85%; diabetic fundus, 0–100%; control corpus, 0–86%; diabetic corpus, 0–89%; control antrum, 6–100%; diabetic antrum, 0–54%]. Within control or diabetic animals, the percent of total myenteric neurons positive for nNOS was decreased in antrum as compared to fundus ($\dagger p < 0.05$) or corpus ($\ddagger p < 0.05$). Gastric regions from control and diabetic animals were similar.

Discussion

Neostigmine, an AchE inhibitor that increases endogenous Ach levels, was used to mimic the mechanism of action of prokinetic drugs. In each gastric region, cholinergic contractions were altered in diabetic as compared to control animals, but the mechanism varied in each region (Fig. 7). Smooth muscle muscarinic receptor activation by cholinergic agonists was reduced in gastric regions from diabetic animals. Impaired activation of presynaptic muscarinic receptors by endogenous Ach resulted in weaker relaxations of diabetic fundus and antrum. Noncholinergic excitatory pathways were suppressed in diabetic pylorus. These inconsistent alterations in cholinergic muscarinic and nicotinic receptor control of gastric regional contractions in diabetic guinea pigs, ascribed primarily to low insulin, may contribute to the impaired ability of the autonomic nervous system to effectively modulate gastric emptying rate.

Basal motility

In fundus and antrum from both control and diabetic animals, basal contractions were infrequent and insensitive to both 4-DAMP and hexamethonium suggesting little involvement of Ach through muscarinic or nicotinic receptors in maintaining basal myogenic motility. In these tissues, spontaneous basal release of endogenous Ach was efficiently broken down by AchE as observed in murine antral circular smooth muscle (Forrest et al., 2006).

Low gastric cholinergic tone at rest may reflect nominal vagal cholinergic nerve activity. Vagal efferent nerve activity was required for basal gastric cholinergic tone in dogs (Azpiroz et al., 1987) and guinea pigs (Berthoud et al., 2002). Since motility in our studies was recorded *in vitro* using whole thickness tissue, low basal frequency of contractions with little cholinergic tone reflected scant active vagal input to fundic and antral preparations at rest, and a difference between control and diabetic tissues was not observed (Fig. 4). Similarly, basal force production (as inferred from graphs of agonist dose-response) was similar in fundus, antrum, and pylorus from control and type 2 diabetic mice (James et al., 2004) and in antrum from control and STZ rats (Maruyama et al., 1999). The basal frequency of

contraction was so low in both control and diabetic pylori that the contributions of muscarinic and nicotinic receptors to basal frequency and tone were not assessed.

Nerve-stimulated contractions and relaxations

In guinea pig antrum, nerve-stimulated contractions were observed in 59% of antral smooth muscle strips with relaxations in 41% of antral smooth muscle strips (James et al., 2003). These percentages are similar to those observed in control antrum (Fig. 1A). In diabetic antrum, a higher percentage, but not strength, of nerve-stimulated contractions may reflect the increased tissue content of choline as compared to control antrum (Table 2). Nerve-stimulated contractions of fundus were less prevalent and significantly weaker in diabetic as compared to control animals (Fig. 1B) which may reflect not only weaker activation of smooth muscle muscarinic receptors in diabetic fundus (Fig. 2), but also decreased vagal input to enteric neurons suggested by reduced tissue content of choline (Table 2) and ineffectiveness of low dose hexamethonium to reduce neostigmine-induced contractions. In addition, nerve-stimulated contractions were also weaker in diabetic pylorus as compared to control. Since nerve-stimulated contractions of pylorus were reported to be totally cholinergic (James et al., 2003), weaker nerve-stimulated contractions most likely reflected reduced activation of muscarinic receptors by Ach in diabetic pylorus as was observed for bethanechol (Fig. 2).

Separate studies reported that nerve-stimulated relaxations of fundic longitudinal muscle strips (Jenkinson et al., 1995), but not antral circular muscle strips (Gangula et al., 2007), were significantly weaker in diabetic as compared to control rats. In this study, when determining the impact of diabetes on nerve-stimulated relaxations of fundic and antral preparations from the same stomach, data were similar in control and diabetic animals. Instead, relaxations of preparations cut on the longitudinal but not circular muscle axis were weaker in diabetic as compared to control animals (Fig. 1B) and regardless of gastric region or axis of preparation, nerve-stimulated relaxations were weaker in diabetic as compared to control animals. Weaker nerve-stimulated relaxations of diabetic fundus may reflect lower number of nNOS-ir neurons in myenteric ganglion (Table 3) and may impair the ability of the stomach to relax after a swallow.

Bethanechol

Bethanechol, a muscarinic receptor agonist resistant to enzymatic breakdown by endogenous AChE, can contract gastric smooth muscle by activating smooth muscle muscarinic receptors. Inherent differences in smooth muscle muscarinic receptor density may account for regional variability in strength of contraction to bethanechol in control animals. Stronger bethanechol-induced contractions of pylorus as compared to fundus and antrum may reflect higher smooth muscle muscarinic receptor density in this region but to our knowledge, the density of muscarinic receptors in pylorus has not been reported. Since fundus and antrum from control animals contracted similarly to bethanechol, even though the density of muscarinic receptors in guinea pig antrum may be greater than fundus as reported for rats (Morisset et al., 1981), receptor density may not be the sole factor contributing to strength of muscle contraction. Regional differences in efficiency of signal transduction after muscarinic receptor activation may contribute as well (Braverman et al., 2009). The decreased effectiveness of 4-DAMP to inhibit bethanechol-induced contraction of fundus but not antrum or pylorus provides some evidence for a varied efficacy of muscarinic signal transduction in this region as compared to antrum or pylorus.

Bethanechol-induced contractions of gastric regions were altered in diabetic mice. Diabetes did not alter the regional pattern of stronger bethanechol-induced contractions of pylorus than fundus or antrum (Fig. 2), yet there was a strong trend for contractions to be weaker in

diabetic regions. Similarly, the regional pattern of stronger bethanechol-induced contractions of fundus as compared to antrum or pylorus observed in control mice was preserved in diabetic db/db mice, yet bethanechol-induced contractions in diabetic mice were decreased in fundus, unchanged in the antrum, and increased in pylorus (James et al., 2004). Together data suggest that, even within the same diabetic animal, cholinergic contractions of distinct gastric regions were not uniformly affected by high plasma glucose and insufficient effective plasma insulin that characterize diabetes.

Neostigmine-induced contractions

In the presence of neostigmine, sufficient endogenous Ach accumulated at synaptic junctions to activate cholinergic receptors resulting in contraction of gastric smooth muscle. When preparations from fundus or antrum were cut on either the longitudinal or circular muscle axis, maximal neostigmine-induced contractions were similar within control or diabetic groups (Fig. 3) as reported previously (Milenov et al., 1983). Hence, data from fundic and antral preparations cut on the longitudinal or circular muscle axis were combined in subsequent analyses.

The strength of neostigmine-induced contractions varied with gastric region in control animals (Fig. 5A). Likewise, the enzymatic activity of AchE in rat longitudinal muscle with myenteric plexus (LMMP) varied in normal animals being higher in fundus than pylorus (Heitkemper et al., 1983). In addition, at least four isoforms of AchE with varying sensitivities to inhibitor were detected in guinea pig ileum LMMP preparations (Appleyard et al., 1989). Therefore, one could speculate that distinct gastric regions preferentially express different proportions of AchE isoforms with varying affinities for neostigmine. Indeed, the EC₅₀ for neostigmine was higher in fundus than pylorus. In fundus, a 10-fold higher concentration of AchE inhibitor was required to produce notable smooth muscle cholinergic contractions as compared to pylorus (Fig. 5A), suggesting that in control tissues, AchE activity may be higher in fundus than in pylorus. In addition, within control animals, only in fundus did a single dose of neostigmine produce weaker contractions than repetitive, increasing doses of neostigmine. Hence, the higher AchE activity in control fundus to rapidly break down Ach might be important in restraining cholinergic contractions given the high choline content in this region (Table 3).

The amount of Ach substrate available for enzymatic breakdown, as inferred from tissue choline (O'Regan et al., 1981), also varied in gastric regions from control animals. Density of autonomic innervation contributed to the amount of Ach substrate present in the region. Extrinsic vagal efferent innervation of the gastrointestinal tract demonstrated a proximal to distal gradient (Berthoud et al., 1990; Berthoud et al., 1991; Holst et al., 1997; Kirchgessner et al., 1989) and intrinsic ChAT-ir enteric neurons varied with gastric regions [corpus: 64% (Schemann et al., 1995) and antrum: 32%, (Vanden Berghe et al., 1999)]. Since there were significantly fewer HuC/D-ir myenteric neurons in fundus than antrum (Table 3), despite 6-fold higher choline content in fundus than antrum, data suggest that choline content more accurately reflected vagal efferent nerve fibers in fundus.

The regional variation in neostigmine-induced maximal contractions observed in control animals was altered in diabetic animals. Neostigmine-induced contractions of fundus and antrum were similar in control animals but in diabetic animals, neostigmine-induced contractions were stronger in antrum than fundus (Fig. 5). Neostigmine-induced contractions of diabetic tissues were weaker in fundus and pylorus, but similar in antrum, as compared to control tissues (Fig. 4).

Impaired muscarinic receptor signal transduction may contribute to diabetes-induced alterations in strength of muscarinic contractions. In diabetic gastric tissues, persistently

elevated intracellular calcium and altered PKC activity and translocation have been reported (Maruyama et al., 1999; Sakai et al., 1994; Takahashi et al., 1996) suggesting that impaired signal transduction may contribute to altered cholinergic contractions in animal models of diabetes. In these studies, greater accumulation of Ach above a critical concentration resulted in a decrease in force production in control fundus and control pylorus (Fig. 4). Since contractions to a single application of neostigmine were stronger than contractions elicited during dose-response experiments for control antrum and pylorus (Table 1), receptor desensitization may already be occurring at 10 μ M neostigmine in these tissues. In diabetic fundus, maximal neostigmine-induced contractions were likely not achieved since contractions elicited by bethanechol (Fig. 2) were approximately double those elicited by the highest dose of neostigmine (Fig. 4A) suggesting altered muscarinic receptor signal transduction at neostigmine doses even lower than 30 μ M. Therefore, muscarinic receptor desensitization may contribute to altered force production, especially at high neostigmine doses.

Diabetes-induced alteration in AchE enzymatic activity may also contribute to altered regional responses to neostigmine in diabetic animals. Carbachol activates both muscarinic and nicotinic receptors but is resistant to breakdown by AchE. In diabetic animals, maximal contractions to carbachol were decreased in fundus (Korolkiewicz et al., 1998) and corpus (Takahashi et al., 1996), but increased in antrum (Maruyama et al., 1999). Hence, in fundus, both neostigmine-induced contractions (Fig. 4) and carbachol-induced contractions (Korolkiewicz et al., 1998) were decreased in diabetic animals. Since carbachol and neostigmine had similar effects, data suggest that in fundus, diabetes did not alter the rate of endogenous Ach breakdown by AchE.

The amount of Ach precursor, choline, was altered in diabetic animals. Interestingly, the regional difference in choline between fundus and antrum was absent in diabetic animals (Table 3). Since autonomic vagal neuropathy has been reported in diabetic animal models (Kniel et al., 1986; Regalia et al., 2002; Robertson et al., 1980), decreased choline content may reflect reduced vagal cholinergic innervation of enteric neurons. Three weeks after total vagotomy (Gorny et al., 1983), despite increased ChAT activity, Ach content was increased in the pyloric region but not fundus suggesting that the enteric neurons of the pylorus responded to reduced vagal input by increasing enteric Ach content. However, six weeks after total vagotomy, after ChAT activity had returned to normal rates, Ach content was decreased in both fundus and pylorus (Gorny et al., 1983) reflecting the absence of vagal cholinergic nerve terminals. Since dense vagal innervation of all gastric regions (Berthoud et al., 1990; Berthoud et al., 1991; Holst et al., 1997) was provided by collaterals from vagal neurons (Hayakawa et al., 2003), and since tissue Ach content and ChAT activity can be altered by vagotomy (Gorny et al., 1983), the observed changes in tissue choline in diabetic fundus and antrum may result from decreased vagal efferent innervation or vagal neuropathy. The decrease in choline content of diabetic fundus may reflect reduced vagal efferent input (Kniel et al., 1986; Regalia et al., 2002; Robertson et al., 1980) whereas the increase in choline content of diabetic antrum may reflect an increased density of ChAT-ir enteric motor neurons (LePard, 2005) and/or an increased capacity to synthesize Ach in enteric neurons (Lincoln et al., 1984).

Taking data from bethanechol and neostigmine experiments together, the mechanisms contributing to altered cholinergic contraction of tissues from diabetic as compared to control animals varied with gastric region. In fundus, diabetes did not alter the rate of endogenous Ach breakdown but rather, rapid muscarinic receptor desensitization and reduced Ach content accounted for weaker neostigmine-induced contractions. In diabetic antrum, since there was a trend for bethanechol contractions to be weaker, yet tissue choline content was greater, similar neostigmine-induced contractions as compared to control most

likely reflected denser enteric cholinergic innervation of smooth muscle. In diabetic pylorus, since there was a trend for bethanechol contractions to be weaker, yet tissue choline content was similar, weaker neostigmine-induced contractions primarily reflected weaker muscarinic receptor activation by endogenous Ach.

Neostigmine: Role of muscarinic receptors

In the presence of 4-DAMP, only noncholinergic neurotransmitters, not Ach, released by enteric excitatory motor neurons could contribute to smooth muscle contractions. In addition, Ach released by vagal efferent nerves or enteric interneurons could activate enteric excitatory and inhibitory pathways through nicotinic cholinergic receptors to promote an increase or decrease, respectively, in smooth muscle force production. In all gastric regions from both control and diabetic animals, activation of smooth muscle muscarinic receptors by Ach accumulating at the neuroeffector junction was required for neostigmine-induced contractions.

Force production in the presence of 4-DAMP and neostigmine was stronger in diabetic as compared to control antrum (Fig. 6). Since 4-DAMP alone did not alter basal antral motility, data suggest that accumulated Ach was less effective in activating nicotinic cholinergic receptors on inhibitory motor neurons to promote smooth muscle relaxation in diabetic as compared to control antrum. Hence, despite similar strength of nerve-stimulated relaxations (Fig. 1) and number of nNOS-ir myenteric neurons (Table 3) in control and diabetic antrum, data suggest the vagal inhibitory pathway was functionally impaired in diabetic antrum.

Force production in the presence of 4-DAMP and neostigmine was weaker in diabetic as compared to control pylorus (Fig. 6). Since 4-DAMP alone did not alter basal pyloric motility, one explanation could be that accumulated Ach was less effective in activating nicotinic receptors on excitatory noncholinergic motor neurons to promote smooth muscle contraction in diabetic as compared to control pylorus. Since noncholinergic neurotransmitters have been reported to contract pyloric smooth muscle (Hasler, 2010; Mandrek et al., 1992; Milenov et al., 1983) and neuropeptides were altered in animal models of diabetes (Chandrasekharan et al., 2007), noncholinergic, excitatory neuropeptides may be reduced in diabetic pylorus.

4-DAMP and neostigmine: Role of enteric neural pathways (Fig. 6)

By comparing force production in the absence of any drug with force production by neostigmine after pretreatment with either 4-DAMP or hexamethonium, the role of enteric neural pathways were assessed in fundus and antrum from control and diabetic animals. Force production after 4D Neo was weaker than No Drug for control and diabetic fundus. Since 4-DAMP pretreatment alone did not alter basal gastric motility, data suggest that neostigmine-induced accumulated Ach activated nicotinic receptors on enteric inhibitory neurons to promote relaxation. Using fundus from STZ rats, pretreatment with the nitric oxide synthase (NOS) antagonist L-NAME normalized carbachol-induced contractions (Korolkiewicz et al., 1998) suggesting that prolonged activation of cholinergic receptors by carbachol enhanced nitric oxide production in fundus from diabetic animals by activating nicotinic receptors on enteric inhibitory motor neurons. Despite reduced nNOS-ir myenteric neurons and weaker nerve-stimulated relaxations of diabetic fundus suggesting autonomic neuropathy, this nitrergic pathway could be activated after sufficient accumulation of endogenous Ach by neostigmine in the neuro-neuronal synapse.

Neostigmine: Role of nicotinic receptors

In the presence of hexamethonium, neostigmine-induced accumulation of Ach allowed for activation of muscarinic receptors located postjunctionally on smooth muscle and

presynaptically on nitrergic and cholinergic enteric motor neurons. In gastric tissues, neostigmine-induced contractions in the presence of hexamethonium were weak and accumulation of Ach in the neuronal synapse was essential for strong neostigmine-induced contractions.

Despite weaker contractions to bethanechol and low basal cholinergic tone, muscarinic smooth muscle contractions after C6 Neo were stronger in diabetic pylorus as compared to control pylorus. Previous studies have demonstrated that the ability of presynaptic muscarinic autoreceptors to suppress Ach release from enteric motor nerve terminals was altered in diabetic ileum (Coulson et al., 2002). Therefore, data suggest that presynaptic inhibitory muscarinic autoreceptors were less effective at suppressing accumulation of Ach at the neuroeffector junction in diabetic pylorus, thereby prolonging the activation of smooth muscle muscarinic receptors and increasing force production. In the absence of neostigmine, muscarinic autoreceptor dysfunction was not apparent because of nominal basal release of Ach at the neuroeffector junction.

Hexamethonium and neostigmine: role of enteric neural pathways

Neostigmine-induced force production in the presence of hexamethonium was the same as that observed in the absence of drug for diabetic fundus and control pylorus. Since all experiments were performed in the absence of nerve stimulation, in some tissues, neuronal release of Ach at the neuroeffector junction was negligible. Indeed, in the absence of vagal stimulation, gastric motility was weak or even absent in guinea pigs (Berthoud et al., 2002). Without concurrent activation of nicotinic cholinergic receptors located on enteric neurons, accumulation of Ach at the neuroeffector junction was insufficient to activate a minimum number of muscarinic receptors located postsynaptically on smooth muscle or presynaptically on nitrergic neurons to generate measurable gastric contractions or relaxations, respectively.

Notably, neostigmine-induced contractions in the presence of hexamethonium were similar to no drug in diabetic fundus, but weaker than no drug in control fundus suggesting that in control, but not diabetic fundus, accumulated Ach activated presynaptic muscarinic receptors on enteric inhibitory motor neurons to decrease force production. Indeed, in the presence of hexamethonium, selective activation of presynaptic muscarinic receptors promoted nitrergic relaxation of gut smooth muscle in the absence (Olgart et al., 1999) and presence (Kortezova et al., 2004) of nerve stimulation. In addition, muscarinic receptor activation increased nitric oxide production in guinea pig fundus (Kortezova et al., 2004). Since the population of nNOS-ir neurons was reduced in diabetic fundus as compared to control (Table 3), in the presence of hexamethonium, accumulated Ach was less effective in generating nitric oxide by muscarinic receptor activation in diabetic fundus resulting in no measurable change in force production.

nNOS-ir myenteric neurons

Immunohistochemistry using HuC/D antibody, which stains neuronal cytoplasm, not processes, of all myenteric neurons resulted in similar myenteric cell counts as compared to previous studies. The numbers of HuC/D-ir myenteric neurons per ganglion in fundus (29 ± 1) and corpus (32 ± 2) were similar to the numbers of neuron-specific enolase-ir neurons per ganglion in fundus (25.2 ± 11.5) (Pfannkuche et al., 1998) and corpus (32.7 ± 18.4) (Schemann et al., 1995). In the antrum, when the total number of NSE-ir neurons was divided by the total number of ganglia evaluated, there were 21 ± 1 neurons per ganglion (Vanden Berghe et al., 1999), about half the number counted in these studies (50 ± 2). As acknowledged by Vanden Berghe *et al.* (Vanden Berghe et al., 1999), antral ganglia were difficult to define given their stellate shape and thick internodal strands which sometimes

contained cell bodies. Discrepancies in antral cell counts can be attributed to the different methods used to determine number of neurons per ganglion and the difficulty in defining the boundaries of individual ganglion.

Other studies have reported an overall reduction in number of enteric neurons in intestine from diabetic animal models (Guo et al., 2004; LePard, 2005) and antral biopsies from patients with diabetic gastroparesis (Harberson et al., 2010). In these studies, a decrease in the number of neurons per ganglion in diabetic as compared to control animals was observed in fundus and antrum (Table 3) providing evidence for myenteric neuronal apoptosis in diabetic animals (Guo et al., 2004; Li et al., 2010). Neuronal apoptosis may include nNOS-ir myenteric neurons in diabetic fundus, but probably also includes neurons not expressing nNOS-ir in diabetic antrum.

In control animals, the percentage of nNOS-ir myenteric neurons per ganglion was highest in fundus and corpus and lowest in antrum. In normal guinea pig, the percent of total myenteric neurons positive for nNOS was 29% in corpus (Schemann et al., 1995), similar to $34 \pm 2\%$ in this study, and 40.7% in antrum (Vanden Berghe et al., 1999), higher than $26 \pm 1\%$ in this study. In our study, each LMMP preparation was double-stained for HuC/D-ir and nNOS-ir and the nNOS percentage calculated for each ganglion. In other studies, each preparation was single-stained for either a pan-neuronal marker or nNOS, then the nNOS percentage calculated based on total numbers of cells (Schemann et al., 1995) or average numbers of cells per ganglion (Vanden Berghe et al., 1999) for all preparations. Combined with the difficulty of defining ganglionic barriers in antrum, these different methods in calculating percentage of nNOS-ir cells may account for the lower percentage of nNOS-ir neurons in the antrum observed in our study.

In fundus, the number of nNOS-ir neurons per ganglion was decreased in diabetic as compared to control animals (Table 3) and nerve-stimulated relaxations of preparations from diabetic animals were weaker than control animals (Fig. 1). As was observed in our study, a reduction in the number of nNOS-ir neurons in the gastric body (Fregonesi et al., 2001; Takahashi et al., 1997) was correlated with impaired nerve-stimulated relaxations (Takahashi et al., 1997) in diabetic animals. In vagotomized rats, the absence of vagal cholinergic input to gastric myenteric neurons was correlated with reduced nNOS mRNA expression, nNOS production, and NO synthesis (Nakamura et al., 1998). Therefore, diminished extrinsic cholinergic input to the myenteric plexus in diabetic guinea pigs, autonomic vagal neuropathy, supported by the decrease in tissue choline, may contribute to decreased nNOS-ir in fundus. In contrast, the number of nNOS-ir myenteric neurons was not altered in diabetic antrum. However, in STZ rat antrum, the percent of total myenteric neurons positive for nNOS was reduced (Wrzos et al., 1997), yet the amount of total nNOS protein in antral tissue was increased (Adeghate et al., 2003), resulting in normal nerve-stimulated relaxations (Gangula et al., 2007) suggesting an adaptation in the inhibitory pathway to maintain relaxation in this region. Since nitric oxide is a neurotransmitter not only of enteric inhibitory motor neurons innervating smooth muscle, but also of myenteric interneurons (Reiche et al., 2000), selective targeting to suppress enzymatic nitric oxide production only in inhibitory motor neurons but not in interneurons was not feasible.

In conclusion, in gastric tissues, the cholinergic neuro-neuronal and the neuromuscular pathways were mutually dependent in generating contractions in response to neostigmine. If either the nicotinic or muscarinic receptors were blocked, neostigmine-induced contractions were weak. These results would suggest that any prokinetic drug treatment aimed at enhancing endogenous Ach levels to promote stronger gastric contractions cannot also suppress cholinergic neurotransmission at neuronal synapses or at the neuroeffector junction. In addition, since diabetes-induced alterations in cholinergic contractions were

observed more frequently in fundus and pylorus as compared to antrum, prokinetic drugs which uniformly alter Ach release in all gastric regions may have confounding effects to either not improve or even exacerbate abnormal gastric emptying. Data also suggest that selective activation of presynaptic muscarinic receptors on enteric neurons may facilitate fundic storage to normalize gastric emptying as long as pyloric relaxation can be achieved. The demonstration of motor abnormalities in this animal model of diabetes suggests a strong role for insulin deficiency in contributing to autonomic neuropathy and gastric motor dysfunction.

Acknowledgments

Supported by R15 NS047106 to KJL and by ORSP at Midwestern University. JH was supported by the Kenneth A. Suarez Summer Research Fellowship provided through the Chicago College of Osteopathic Medicine.

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Figure 1.

Nerve-stimulated contractions and relaxations of gastric tissues from control and diabetic animals. A. Prevalence of nerve-stimulated contractions and relaxations. In control animals, nerve-stimulated contractions and relaxations were observed more frequently in fundus than antrum ($\dagger p < 0.05$). In diabetic animals, fewer contractions but more relaxations were evoked in fundus than antrum ($\dagger p < 0.05$). Nerve-stimulated contractions were less prevalent in diabetic fundus, but more prevalent in diabetic antrum, as compared to control animals ($* p < 0.05$). Nerve-stimulated relaxations were equally prevalent in fundus and antrum from control and diabetic animals. B. Strength of nerve-stimulated contractions and relaxations. Nerve-stimulated contractions of antrum were weaker than fundus within control animals,

but stronger than fundus within diabetic animals ($\dagger p < 0.05$). Nerve-stimulated contractions of fundus, but not antrum, were weaker in diabetic as compared to control animals ($*p < 0.05$). Regardless of region, relaxations of preparations cut on the longitudinal muscle axis, but not circular muscle axis, were weaker in gastric tissues from diabetic as compared to control animals ($*p < 0.05$). Legend: Black columns: control, White columns: diabetic. Positive area denotes contraction while negative area denotes relaxation. $*p < 0.05$, control versus diabetic; $\dagger p < 0.05$, within control or diabetic as compared to antrum or longitudinal muscle.

Figure 2.

Bethanechol-induced contractions of gastric regions from control and diabetic animals.

Overall, there was a strong trend for bethanechol-induced contractions of all gastric regions to be weaker in diabetic as compared to control animals ($p=0.053$). Overall, bethanechol-induced contractions of pylorus were stronger than antrum or fundus ($p<0.05$). Legend: Black columns: control, White columns: diabetic.

Figure 3.

Effect of longitudinal versus circular muscle axis on neostigmine-induced contractions of fundus and antrum from control and diabetic animals. A. Fundus. Within control or diabetic animals, preparations cut on the circular muscle (CM) or longitudinal muscle (LM) axis contracted similarly to neostigmine. Neostigmine-induced contractions of both fundic CM and LM were weaker in diabetic as compared to control animals (* $p < 0.05$). B. Antrum. Preparations from control or diabetic animals cut on either the longitudinal or circular muscle axis contracted similarly to neostigmine. Within diabetic animals, there was a trend for preparations cut on the circular axis to be weaker than those cut on the longitudinal axis ($p = 0.067$). Control: fundus CM $n = 3-12$ and LM $n = 2-15$, antrum CM $n = 3-4$ and LM $n = 2-5$;

Diabetic: fundus CM n=7–11 and LM n=7–14, antrum CM n=6–13 and LM n=5–9. Legend: Circles: longitudinal muscle, Triangles: circular muscle, Closed symbols: control, Open symbols: diabetic, Solid lines: curve fit for control, Dashed lines: curve fit for diabetic.

Figure 4.

Neostigmine-induced contractions of fundus, antrum, and pylorus from control and diabetic animals. A. Fundus. Contractions of diabetic fundus were weaker than control fundus at 10 and 30 μ M neostigmine (* p <0.05) with a trend observed at 1 μ M neostigmine (p =0.054). Within control fundus, weaker contractions were observed at 50 μ M as compared to 10 or 30 μ M neostigmine ($\dagger p$ <0.05). Control n =6–27, Diabetic n =14–25. B. Antrum. Neostigmine-induced contractions of control and diabetic antrum were similar at all doses. Control n =4–8, Diabetic n =11–22. C. Pylorus. Contractions of diabetic pylorus were weaker than control pylorus at 0.3, 0.5, and 1 μ M neostigmine (* p <0.05) with a trend observed at 10 μ M neostigmine (p =0.088). Within control but not diabetic pylorus, weaker contractions were observed at 50 μ M as compared to 10 μ M neostigmine ($\dagger p$ <0.05). Control n =3–7, Diabetic n =4–8. Legend: Closed circles with solid lines: control, Open circles with dashed lines: diabetic. * p <0.05, control vs. diabetic; $\dagger p$ <0.05, within control or diabetic as compared to 50 μ M neostigmine.

Figure 5.

Gastric regional differences in neostigmine-induced contractions within control or diabetic animals. Data from Fig. 4 was graphed to illustrate regional differences within control and diabetic animals. A. Control. Contraction of fundus and antrum were similar at all neostigmine doses. At 1 and 10 μM neostigmine, contractions of fundus and antrum were weaker than pylorus ($\dagger p < 0.05$). B. Diabetic. Contractions of fundus were significantly weaker than pylorus at 10, 30, and 50 μM neostigmine ($\dagger p < 0.05$) and antrum at 10 and 30 μM neostigmine ($\ddagger p < 0.05$). Legend: Closed circles: fundus, Open circles: antrum, Closed triangles: pylorus. $\dagger p < 0.05$, within control or diabetic as compared to pylorus; $\ddagger p < 0.05$, within control or diabetic as compared to antrum.

Figure 6.

Area of contractions in the absence of drug and after 4-DAMP neostigmine or hexamethonium neostigmine. The areas of contraction elicited by neostigmine (Neo, 10 μ M) after pretreatment with either hexamethonium (C6, 300 μ M) or 4-DAMP (4-D, 1 μ M) were compared among gastric regions from control and diabetic animals. Fundus: Within both control and diabetic animals, 4D Neo contractions were weaker than No Drug contractions ($\dagger p < 0.05$). For control but not diabetic animals, C6 Neo contractions were weaker than No Drug contractions ($\dagger p < 0.05$). Antrum: The area of 4D Neo contraction was stronger in diabetic as compared to control ($* p < 0.05$). Pylorus: Contractions in the presence of 4D Neo were weaker, but those in the presence of C6 Neo were stronger, in diabetic as compared to control animals ($* p < 0.05$). For diabetic but not control animals, C6 Neo contractions were stronger than No Drug contractions ($\dagger p < 0.05$). In the presence of 4D Neo, contractions of diabetic pylorus were significantly weaker than diabetic fundus or diabetic antrum ($\ddagger p < 0.05$). Control fundus $n = 18-27$, antrum $n = 8-11$, pylorus $n = 6$; Diabetic fundus $n = 23-25$, antrum $n = 18-22$, pylorus $n = 8-9$. Legend: Black columns: control, White columns: diabetic. $* p < 0.05$, control versus diabetic; $\dagger p < 0.05$, within control or diabetic as compared to No Drug; $\ddagger p < 0.05$, within control or diabetic as compared to fundus and antrum.

Figure 7.

Cartoon depicting the regional differences in excitatory and inhibitory pathways innervating gastric smooth muscle in control and diabetic animals. Control. Cholinergic contractions were stronger in pylorus and weaker in fundus and antrum. AChE was most efficient in breaking down endogenous ACh in fundus. Noncholinergic ENT contributed to smooth muscle contraction in pylorus. Activation of presynaptic M receptors on inhibitory motor neurons by endogenous ACh increased release of INT to relax fundus and antrum. Activation of muscarinic autoreceptors on cholinergic motor neurons suppressed release of ACh in pylorus to blunt contractions. The highest number of total myenteric neurons per ganglion and the lowest percentage of nNOS myenteric neurons per ganglion were in antrum.

Diabetic. In fundus, postjunctional smooth muscle M receptors desensitized rapidly to endogenous Ach. Cholinergic agonists were less effective in fundus, antrum and pylorus in activating postjunctional M receptors resulting in weaker contractions. Activation of presynaptic M receptors was less effective in increasing release of INT to relax fundus and antrum, reflecting the reduced number of nNOS neurons in fundus but not antrum. In pylorus, accumulated Ach was less effective in activating muscarinic autoreceptors to suppress additional Ach release from cholinergic enteric motor neurons resulting in stronger contractions. Choline tissue content was reduced in fundus, but increased in antrum, reflecting alterations in autonomic innervation by cholinergic extrinsic nerves and intrinsic neurons. Legend: Circle (cell body), grey: vagal preganglionic neuron; black: myenteric motor neuron; oval: gastric region with highest number of myenteric neurons; circle (upward diagonal): gastric region with lowest percentage of nNOS-ir neurons in ganglion; white: diabetes-induced reduced number of neurons. Line (axon): solid (thick, medium, thin), density or strength of neural innervation; dashed: neuropathy in diabetic regions. M square (muscarinic receptor), black: control regions; size (large, medium): relative strength of regional contraction after receptor activation; light grey: diabetes-induced decreased activation. White square: noncholinergic excitatory receptor. Grey rectangle: neuronal nicotinic receptor. AchE rectangle (acetylcholinesterase enzyme), size (large, small): relative regional activity of enzyme. Ach: acetylcholine, ENT: excitatory neurotransmitter, INT: inhibitory neurotransmitter, ↑: increase, ↓: decrease

Table 1

Comparison of area of neostigmine-induced contractions from multiple dose-response and single dose experiments.

	Control		Diabetic	
	MDR ^a	SD ^b	MDR	SD
Fundus	209±28 (n=27)	113±15 [†] (n=18)	81±10* (n=24)	156±21 (n=22)
Antrum	168±44 ⁺ (n=8)	324±46 ^{†‡+} (n=11)	227±35 [‡] (n=22)	262±34 ^{‡+} (n=18)
Pylorus	365±77 [‡] (n=7)	725±65 ^{‡‡} (n=6)	285±82 [‡] (n=8)	830±74 ^{‡‡} (n=8)

Area of contractions of control tissues and diabetic pylorus exposed to a single dose of neostigmine (^bSD: single dose) were different than those evoked by tissues previously exposed to lower doses of neostigmine (^aMDR: multiple dose-response, data from Fig. 4). Neostigmine dose was 10 μ M. Data are expressed as mean±SEM.

* p<0.05, control versus diabetic;

[†] p<0.05, within control or diabetic region SD as compared to MDR;

[‡] p<0.05, within each column as compared to fundus;

⁺ p<0.05, within each column as compared to pylorus.

Table 2

Effect of diabetes and gastric region on tissue choline content.

	Control	Diabetic
Fundus	22.2±4.6 (n=23)	13.0±1.7 ^{*‡} (n=26)
Antrum	3.7±0.6 [‡] (n=24)	13.4±2.7 ^{*‡} (n=19)
Pylorus	3.1±0.5 [‡] (n=13)	4.1 ±0.6 (n=19)

Tissue content of choline in gastric regions from control and diabetic animals was measured using an enzymatic assay. Data is represented as nM choline/100 µL/mg tissue wet weight. Data are expressed as mean±SEM.

* p<0.05 control versus diabetic,

[‡] p<0.05 within control or diabetic as compared to fundus,

[‡] p<0.05 within control or diabetic as compared to pylorus

Table 3
HuC/D-ir and nNOS-ir neurons in gastric myenteric plexus of control and diabetic animals.

	Fundus		Corpus		Antrum	
	Control	Diabetic	Control	Diabetic	Control	Diabetic
animals	6	5	6	6	5	5
total ganglia	129	124	103	139	100	109
HuC/D-ir	29±1	24±1 *	32±2	29±1 †	50±2 †‡	40±2 *†‡
nNOS-ir	10.4±0.4	8.7±0.4 *	9.6±0.5	10.1±0.5	11.9±0.6 ‡	10.4±0.6
nNOS-ir/HuC/D-ir	38±1 %	38±2 %	34±2 %	36±1 %	26±1 % †‡	26±1 % †‡

Longitudinal muscle with attached myenteric plexus was immunostained for HuC/D, a panneuronal marker, and nNOS, a marker for the enzyme neuronal nitric oxide synthase. Data are expressed as mean ±SEM of HuC/D-ir or nNOS-ir neurons per each ganglion or as percent nNOS-ir neurons per total HuC/D-ir neurons in each ganglion.

* p<0.05, control versus diabetic;

† p<0.05, within control or diabetic as compared to fundus,

‡ p<0.05 within control or diabetic as compared to corpus.