Pyloric Sphincter Dysfunction in nNOS$^{-/-}$ and W/W^{v} Mutant Mice: Animal Models of Gastroparesis and Duodenogastric Reflux

DIGAVALLI V. SIVARAO, HIROSHI MASHIMO, and RAJ K. GOYAL

Center for Swallowing and Motility Disorders, Department of Veterans Affairs Medical Center, West Roxbury, Massachusetts; and Harvard Medical School, Boston, Massachusetts

Background & Aims: **Nitrergic nerves and interstitial cells of Cajal (ICC) have been implicated in the regulation of pyloric motility. The purpose of these studies was to define their roles in pyloric function in vivo.** ***Methods:*** **Pyloric sphincter manometry was performed in wild-type controls, neuronal nitric oxide synthase–deficient (nNOS$^{-/-}$) mice, and ICC-deficient W/W^{v} mice, and the effect of deafferented cervical vagal stimulation was examined.** ***Results:*** **Mice showed a distinct ~0.6-mm-wide zone of high pressure at the antroduodenal junction, representing the pyloric sphincter. In wild-type controls, the pylorus exhibited tonic active pressure of 12.4 ± 1.6 mm Hg with superimposed phasic contractions. The motility indices, minute motility index, and total myogenic activity were reduced by vagal stimulation, and the reduction was antagonized by the nitric oxide synthase inhibitor N^{G}-nitro-L-arginine methyl ester (L-NAME). In nNOS$^{-/-}$ mice, pyloric basal tone, minute motility index, and total myogenic activity were not significantly different from those in controls, but vagal stimulation paradoxically increased pyloric motility. In contrast, the W/W^{v} mice had significantly reduced resting pyloric pressure that was suppressed by vagal stimulation in an L-NAME–sensitive manner. The stomachs of fasted nNOS$^{-/-}$ mice showed solid food residue and bezoar formation, while W/W^{v} mice showed bile reflux.** ***Conclusions:*** **In nNOS$^{-/-}$ mice, loss of nitrergic pyloric inhibition leads to gastric stasis and bezoars. In contrast, basal pyloric hypotension with normal nitrergic inhibition predisposes W/W^{v} mice to duodenogastric bile reflux.**

BASIC–ALIMENTARY TRACT

Disorders of gastric motility, including gastric stasis and reflux of duodenal contents into the stomach, are important clinical problems.[1] Although these disorders have complex pathophysiology, pyloric sphincter dysfunction is believed to play a key role in their pathogenesis.[2–5] Recently, the nitrergic inhibitory nerves and the interstitial cells of Cajal (ICC) along with the cholinergic nerves have been reported to be involved in the regulation of pyloric sphincter function.[5–10]

Nitrergic neurons of the myenteric plexus mediate inhibition in different parts of the stomach, including the pyloric sphincter.[5] Genetically engineered mice lacking neuronal nitric oxide synthase (nNOS$^{-/-}$)[11] have been shown to have dilated stomachs, delayed gastric emptying of liquids as well as solids, and gastric bezoars.[6] The gastric abnormality in nNOS$^{-/-}$ mice has been likened to hypertrophic pyloric stenosis[11] and diabetic gastroparesis.[6,7] These phenotypic abnormalities are caused by multiple factors, including impaired nitrergic inhibition of the pyloric sphincter.[12,13] Studies on muscle strips in vitro have shown that the normal relaxation of the pylorus to electrical field stimulation is lacking in nNOS$^{-/-}$ mice.[7] However, data on pyloric sphincter dysfunction in intact nNOS$^{-/-}$ mice are not currently available.

Recently, ICC have been reported to regulate motility of the stomach, including the pyloric sphincter.[8,9] In the gut, many types of ICC that differ in function and regional distribution have been recognized. The 2 main types in the stomach are the intramuscular ICC (ICC-IM) and the myenteric ICC (ICC-MY).[8,9] It has been proposed that ICC-IM, interposed between enteric nerves and the smooth muscle cells, transduce nitrergic and cholinergic signals to the smooth muscle cells of the gut, including the pyloric sphincter.[14,15] Moreover, the network of ICC-MY regulates frequency and propagation of the slow waves.[8,9] The W/W^{v} mutant mice have a partial deficiency of the c-kit receptor and show a loss of ICC-IM in the stomach, including the pylorus.[8,14] Thus, these mutants have been used to define the role of ICC-IM in the stomach. Because ICC-IM is believed to transduce neural signals to smooth muscle, loss of the ICC-IM may disrupt cholinergic as well as nitrergic neurotransmission. Disruption of cholinergic neurotransmission may lead to impaired gastric contractions and abnormal compliance.[15] However, the effect of presumed deficit of nitrergic neurotransmission on the pyloric function and its

Abbreviations used in this paper: ICC, interstitial cells of Cajal; ICC-IM, intramuscular interstitial cells of Cajal; ICC-MY, myenteric interstitial cells of Cajal; L-NAME, N^{G}-nitro-L-arginine methyl ester; MMI, minute motility index; nNOS, neuronal nitric oxide synthase; TMA, total myogenic activity; WT, wild-type.

0016-5085/08/$34.00
doi:10.1053/j.gastro.2008.06.039

contribution to gastric motor abnormality in W/W^{v} mutants in vivo remain unclear.

The gastric phenotype in W/W^{v} mice differs from that of nNOS$^{-/-}$ mutants. nNOS$^{-/-}$ mutants primarily have gastroparesis, whereas W/W^{v} mutants exhibit reflux of duodenal contents into the stomach, bile gastritis, and associated complications.[16,17] Enhanced duodenogastric reflux is also a feature in the W^{s}/W^{s} rat, which has a 12–base pair deletion in its c-kit gene and lacks a functional c-kit receptor.[18,19] These differences in the gastric phenotype of nNOS$^{-/-}$ and W/W^{v} mice may be related, at least in part, to the differences in pyloric function.

Therefore, the aim of the current study was to compare pyloric sphincter function in nNOS$^{-/-}$ and W/W^{v} mice in vivo using a miniaturized perfusion manometry system. We found that fasting anesthetized mice have a manometric high-pressure zone at the pylorus that exhibits both tonic and phasic contractions that are suppressed by vagal stimulation–mediated nitric oxide release. nNOS$^{-/-}$ mice have a normotensive pyloric sphincter but lack nitrergic inhibition. The residual gastric contents in these mice show food remnants along with bezoars but not bile. On the other hand, the pylorus in W/W^{v} mice is hypotensive but with preserved nitrergic inhibition. W/W^{v} mice show gastric bile but no bezoars. These results show that distinct patterns of pyloric sphincter motility in nNOS$^{-/-}$ and W/W^{v} mice contribute to the distinct gastric phenotypes of these strains.

Materials and Methods

General Methods

Site-bred C57BL/6J wild-type (WT) mice of either sex weighing between 25 and 31 g (mean ± SEM, 29 ± 1 g; n = 10) served as controls. Site-bred nNOS$^{-/-}$ mice, originally generated using targeted disruption of exon 2 by homologous recombination,[11] were of either sex and weighed between 15 and 27 g (22 ± 1 g; n = 10). W/W^{v} male mice weighing between 23 and 29 g (24 ± 1 g; n = 18) were purchased from Jackson Laboratories (Bar Harbor, ME). For gastric content analysis, age-matched male Kit^{+}/Kit^{+} mice (29 ± 1 g; n = 8) served as WT controls. All mice used in this study were in the age range of 3–6 months. They were housed 5 per cage at an ambient temperature of 25°C in a 12-hour light/dark cycle with unlimited access to food and water. They were denied food but not water overnight before surgery.

Surgical Methods

The Institutional Animal Care and Use Committee of the Brockton/West Roxbury VA Medical Center approved all experimental procedures. Mice were anesthetized with pentobarbital (50 mg/kg intraperitoneally) and core body temperature was maintained at 36 ± 1°C using a heating pad. A midline incision was made below the mandible, and the left jugular vein was cannulated with PE-10 tubing (Becton Dickinson, Parsippany, NJ) for intravenous infusions. The left vagus was isolated and tied loosely with a silk thread and transected rostral to the knot such that the peripheral stump could be manipulated using the thread. In some mice, the left carotid artery was cannulated using PE-10 tubing connected to a BP-100 pressure transducer (CB Science, Dover, NH) for continuous mean arterial pressure recording. The trachea was cannulated using a 0.5-cm-long PE-50 tubing and, in general, the mice breathed spontaneously.

Manometry

Intraluminal pyloric manometry was performed using a custom-designed catheter assembly (Dent Sleeve, Adelaide, Australia) of 3 silicon channels of ~0.3-mm internal and 0.6-mm external diameter each. The length of the manometry probe was 6.2 cm. The pressure-sensing side openings were located 2, 4, and 6 mm from the tip of the catheter assembly. The in-flow port of each channel was connected to a flow-through pressure transducer (CB Science) that was perfused with distilled water at the rate of 7 μL/min. The pressure rise rate for each channel exceeded 400 mm Hg/s. At this flow rate, the compliance for each channel was ~1.2 μL for the first 100–mm Hg increase in pressure. The pressure transducers were connected to a Maclab data acquisition system (MacLab/8e; AD Instruments, Castle Hill, Australia) through a preamplifier (ETH-400; CB Sciences).

A midline incision into the abdomen was made, and a short piece of PE-260 tubing was inserted through the abdominal wall to serve as a guide tube. The gastroduodenal junction was visually identified, and a small incision was made approximately 1 cm below the junction. A 10-cm-long PE-50 tubing moistened in saline and carrying a piece of silk thread at one end was introduced into the stomach through the duodenal incision. The other end of the silk thread was tied to the tip of the flexible manometry probe. The thread was then used to guide the flexible silicone assembly through the abdominal guide tube and through the duodenal incision into the stomach. A small hole made in the fundus of the stomach allowed the manometry probe to be pulled in if necessary. All ports were initially positioned inside the stomach, and a slow withdrawal of the probe (~1 mm at a time) enabled clear identification of the high-pressure zone recorded by all 3 ports consecutively. After identifying the high-pressure zone, the manometry probe was positioned to record the high pressure through the middle port and record the antral and duodenal pressures 2 mm proximal and distal to the middle port through the first and third ports, respectively. Once in position, the manometry probe was sutured to the gastroduodenal junction to minimize catheter movement relative to the lumen.

Intraluminal pressures were recorded continuously. The pressure profile showed tonic and phasic components. Tonic pressure was the pressure from baseline to

the bottom of the phasic contractions. The pressure due to passive factors such as a relatively large diameter of the catheter assembly vis-à-vis the pyloric lumen was determined by the residual pressure after maximal relaxation with sodium nitroprusside (1 mg/kg intravenously). Injection of sodium nitroprusside caused a sharp but transient decrease in the high-pressure zone, and the baseline pressure so obtained was regarded as passive pressure of the pylorus (Figure 1). Subtracting passive pressure from total pressure gave myogenic tone of the pylorus.[20]

Mean pylorus pressure for each mouse was calculated by registering pressure at the bottom of the phasic waves every 5 minutes for 1 hour. Phasic pyloric activity was calculated as minute motility index (MMI). Briefly, amplitudes of phasic waves of magnitude ≥10 mm Hg were measured and multiplied by the number of contractions to obtain MMI. Such observations were made every 5 minutes for 60 minutes, and mean MMI was calculated for each animal. Total myogenic activity (TMA) was computed by determining the area enclosed by a line that excluded the passive pressure (pressure persisting after administration of sodium nitroprusside; see Figure 1) and that included all contractions using MacLab/8e software. As with MMI, TMA was computed every 5 minutes for 60 minutes and a mean value was determined.

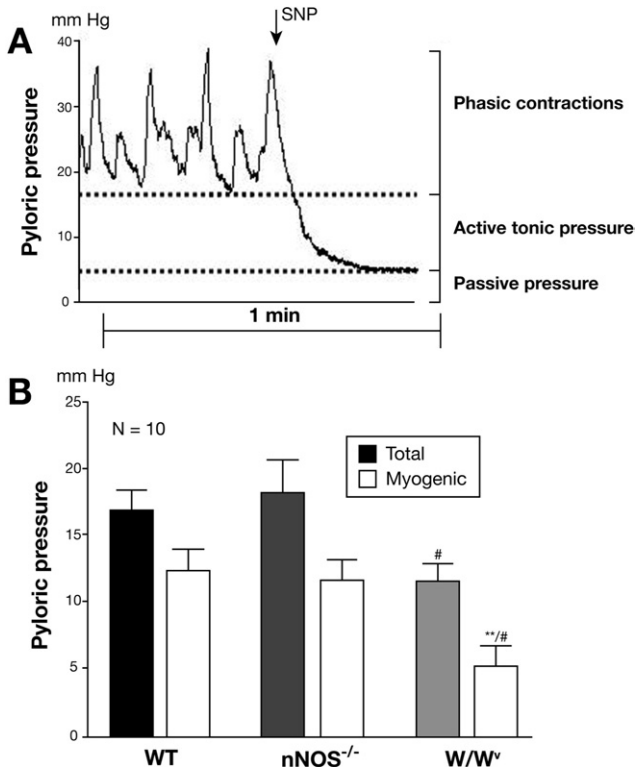

Figure 1. Myogenic and passive components of the pyloric sphincter. *A* is a representative pyloric pressure trace recorded using perfusion manometry. Note phasic contractions superimposed on tonic pressure. Intravenous sodium nitroprusside (1 mg/kg; *arrow*) injection abolished both pressure components. The residual passive pressure was due to luminal stretch by the manometric catheter assembly. The passive pressure was subtracted from the total pressure to obtain active myogenic pressure. *B* shows a summary of total and active myogenic tonic pressures in the 3 strains of mice. Note that the active pyloric sphincter pressure in nNOS$^{-/-}$ mice was similar to that in the controls. On the other hand, the active myogenic component of the pyloric sphincter pressure in W/W^{v} mice was significantly less than the WT (**$P < .01$) as well as the nNOS$^{-/-}$ mice ($^{\#}P < .05$).

Electrical Vagal Stimulation

The cut end of the left vagal trunk was placed on elevated bipolar platinum electrodes mounted on a micromanipulator (Leica Microsystems, Banockburn, IL) and connected to a Grass S11 stimulator (Grass Instruments, Quincy, MA). The nerve was kept insulated and hydrated by submerging in a viscous mixture of mineral oil and petrolatum jelly. The elevated position of the electrodes ensured that electrical contact was restricted to the nerve only. In preliminary studies, vagal stimulation produced a frequency, voltage, and stimulus train-dependent inhibition of the pylorus. In preliminary studies, a stimulus of square wave pulses at 8 V with a 0.5-millisecond pulse width and 10-Hz frequency applied for 1 minute were chosen as optimal stimulus. At least 3 minutes was allowed between successive electrical stimuli. To determine whether vagal-mediated inhibition was mediated by nitrergic nerves, the effect of the NOS inhibitor N^{G}-nitro-L-arginine methyl ester (L-NAME) was examined. The effect of vagal stimulation on pyloric activity was determined before and after L-NAME treatment. Skeletal muscle artifact could be easily identified and isolated from normal responses by observing the neck and thoracic muscles during the intrastimulus period. In some experiments, 2 platinum wire electrodes 0.5 cm long and 0.5 cm apart were inserted subserosally into the antrum, about 0.5 cm orad to the gastroduodenal junction.

Residual Gastric Contents

Residual contents were examined to determine gastric stasis, bezoars, and duodenal reflux. Duodenal reflux was inferred by bile staining of the gastric mucosa and bile acid content. The methods used for gastric pH and total bile acids estimation were modified from Azuma et al.[16] Briefly, overnight fasted male adult W/W^{v} mice and their age-matched WT cohorts were killed in pairs by cervical dislocation and the stomachs were rapidly excised and cut open along the greater curvature. The contents were flushed using 2 aliquots of 0.75 mL distilled water. The stomach was blotted off any excess fluids and weighed to record the wet weight. The pH of the gastric contents was recorded and adjusted to a neutral value using 0.1N NaOH. The total volume of the contents was then adjusted to 2.5 mL and filtered, and 50 μL of the filtrate was assayed for total bile acids. A commercially available kit for bile acids (#450-A; Sigma Chemical Co, St Louis, MO) was used to quantify the total bile acids.[21] Briefly, the bile acids are added to a

reaction mixture containing 3α-hydroxysteroid dehydrogenase and nicotinamide adenine dinucleotide. The ensuing enzymatic reaction oxidizes the bile acids to 3-oxo bile acids with a concomitant reduction of an equimolar quantity of nicotinamide adenine dinucleotide to reduced nicotinamide adenine dinucleotide. The reduced nicotinamide adenine dinucleotide is subsequently reoxidized to nicotinamide adenine dinucleotide with a simultaneous reduction of nitroblue tetrazolium salt to formazan by the enzyme diaphorase. The absorbance maxim of formazan is at 530 nm and is directly proportional to the total bile acid content of the sample.

Drugs

Drugs were injected via the jugular vein in a volume of 0.15 mL, followed by an equal volume of normal saline (0.9% wt/vol) flush over a 3- to 4-minute period. L-NAME (100 mg/kg) and sodium nitroprusside (1 mg/kg) (Sigma Chemical Co) were freshly prepared in normal saline before use. Saline was used as a vehicle control. The effect of L-NAME on vagal-stimulated pyloric activity was studied within 20 minutes after its injection.[22]

Data Analysis

The mean total tonic pyloric pressure for each mouse was averaged to obtain a group mean. The pyloric motility, which was calculated as MMI and TMA, and which included both phasic and tonic activity, was compared among the 3 strains of mice. MMI and TMA were determined before and during vagal stimulation and after dosing with L-NAME. Comparison across groups was made using one-way analysis of variance, with the difference assessed by Tukey's posttest or Student *t* test when applicable. $P < .05$ was deemed significant. Values are expressed as mean ± SEM, with n indicating the number of mice per group.

Results

Pyloric Sphincter Manometry: General Observations

In all 3 groups of mice tested, a short (~0.6-mm) pyloric high-pressure zone, flanked by a relatively low-pressured antrum proximally and the duodenum distally, was easily identified. The high-pressure zone was generally superimposed by phasic pressure waves (Figure 1*A*).

In fasted mice, phasic pyloric contractions were prominent and occurred for the most part continuously, whereas antral or duodenal contractions were of lower amplitude and occurred with periods of activity interposed by periods of inactivity. During periods of antral activity, frequency, regularity, and the relation of pyloric contractions with antral contractions could be estimated. In general, antral contractions occurred at a rate similar to the rate of pyloric contractions, whereas duodenal contractions occurred at a much faster rate. In our preparation, the effect of respiration on intraluminal pressures was small and respiratory artifact could be easily distinguished from the contractile activity.

Tonic Activity

In WT mice, the mean intraluminal pressure was 16.7 ± 1.5 mm Hg. After a bolus injection of sodium nitroprusside, the pyloric pressure decreased to 4.3 ± 0.4 mm Hg, suggesting that the mean myogenic pyloric tone was 12.4 ± 1.6 mm Hg (Figure 1*A* and *B*). In nNOS$^{-/-}$ mice, mean intraluminal pressure was 18.3 ± 2.3 mm Hg and decreased to 6.7 ± 1.5 mm Hg in response to administration of sodium nitroprusside, indicating that the myogenic component of the high-pressure zone was 11.6 ± 1.5 mm Hg. This value was not significantly different from that in the WT mice ($P > .05$). In W/W^{v} mice, the myogenic component was 5.3 ± 1.4 mm Hg, a value that was significantly less than the WT ($P < .01$) and the nNOS$^{-/-}$ mice ($P < .05$). On the other hand, the passive component of 6.2 ± 0.4 mm Hg in W/W^{v} mice was not different from the WT or the nNOS$^{-/-}$ mice ($P > .05$). These results are summarized in Figure 1*B*.

Phasic Activity

In the WT mice, the phasic activity was superimposed on the basal tone of the pylorus. The phasic activity had a rate of 5.6 ± 0.4 (range, 5–7) contractions per minute. At times, the pylorus showed a fast phasic activity, resembling the duodenal rhythm, superimposed on tonic contraction. This observation is similar to that previously noted in the canine pylorus.[10] In nNOS$^{-/-}$ mice, the high-pressure zone was superimposed with phasic contractions with a rate of 7.0 ± 1.3 (range, 4–14) cycles per minute. In W/W^{v} mice, the phasic contractions occurred at a mean rate of 6.1 ± 0.9 (range, 4–11) per minute. Amplitude of pyloric phasic contractions was 16.9 ± 2.8 mm Hg in WT, 17.4 ± 5.7 mm Hg in nNOS$^{-/-}$, and 11.6 ± 2.0 mm Hg in W/W^{v} mice; mean duration of pyloric contractions was 6.8 ± 0.8 seconds in WT, 7.6 ± 1.4 seconds in nNOS$^{-/-}$, and 9.2 ± 1.1 seconds in W/W^{v} mice. Neither phasic amplitude nor duration was significantly different across strains ($P > .05$).

Effect of Vagal Stimulation

In WT mice, vagal stimulation significantly inhibited ($P < .01$) both phasic and tonic contractions (Figure 2, *top panel*, and Figure 3). The inhibition sometimes persisted for 30–45 seconds after termination of the stimulus. The end of the stimulation was often marked by a prominent "off" contraction whose amplitude exceeded the average phasic activity before the stimulus (Figure 4, *top left panel*). In contrast to WT mice, efferent vagal stimulation caused no inhibition of the pyloric tonic or phasic contractions in the nNOS$^{-/-}$ mice; rather, a significant increase in motility was noted ($P <$

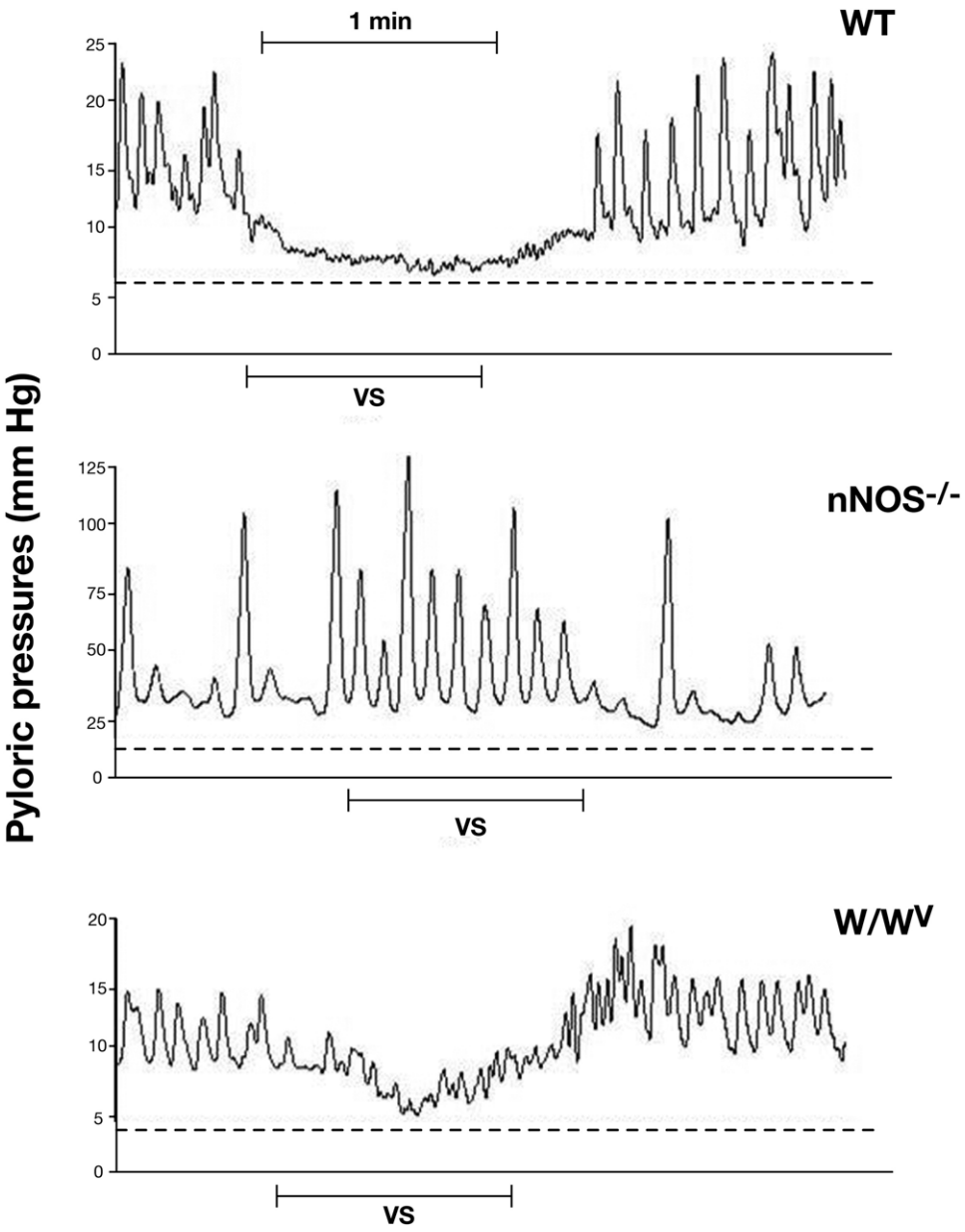

Figure 2. Examples of the effects of efferent vagal stimulation on pyloric pressures in the 3 strains of mice. The *top panel* shows the response in WT mice. Note that vagal stimulation produced inhibition of both tonic and phasic pyloric activity through the period of stimulation. The *middle panel* shows the effect of vagal stimulation on pyloric pressures in an nNOS$^{-/-}$ mouse. Note that vagal stimulation caused a paradoxical increase in the motility. The *bottom panel* shows the effect of vagal stimulation on pyloric activity in a W/W^{v} mouse. Note that vagal stimulation caused a normal inhibition in pyloric motility. The *dotted line* in each panel represents the sodium nitroprusside–resistant passive pressure in that experiment.

.05; Figure 3). In contrast to nNOS$^{-/-}$ mice, and similar to WT mice, vagal stimulation produced inhibition ($P <$ .01) of both phasic and tonic components of pyloric motility in the W/W^{v} mice (Figure 2, *bottom panel*, and and Figure 3).

Effect of L-NAME on Vagal Stimulation

In WT mice, administration of L-NAME had no consistent effect on basal pyloric motility. However, L-NAME abolished vagal inhibition of pyloric motility as illustrated in Figure 4 (*top right panel*) ($P > .05$ compared with prestimulation baseline). Pretreatment with L-NAME also abolished vagal stimulation–induced inhibition of pyloric motility in W/W^{v} mice (Figure 4, *bottom right panel*) ($P > .05$, compared with prestimulation baseline). Moreover, no consistent effect of L-NAME was noted on the basal pyloric motility. Vagal stimulation–induced change in motility indices with or without L-NAME are summarized in Figure 3.

Antral and Duodenal Contractions

In the control mice, the antral contractions ranged narrowly between 4 and 6 per minute, with a mean value of 5.0 ± 0.4 cycles per minute. When both pyloric and antral contractions were present at the same time, they appeared regular and phase locked in a nearly 1:1 ratio (0.96 ± 0.04). Compared with the antral contractions, duodenal contractions occurred at a higher frequency of 24.1 ± 2.8 contractions per minute (range, 14–29; n = 6). In nNOS$^{-/-}$ mice, the antrum showed a variable and irregular rate of contractions. The contractions occurred at a mean rate of 4.4 ± 0.8 per minute but

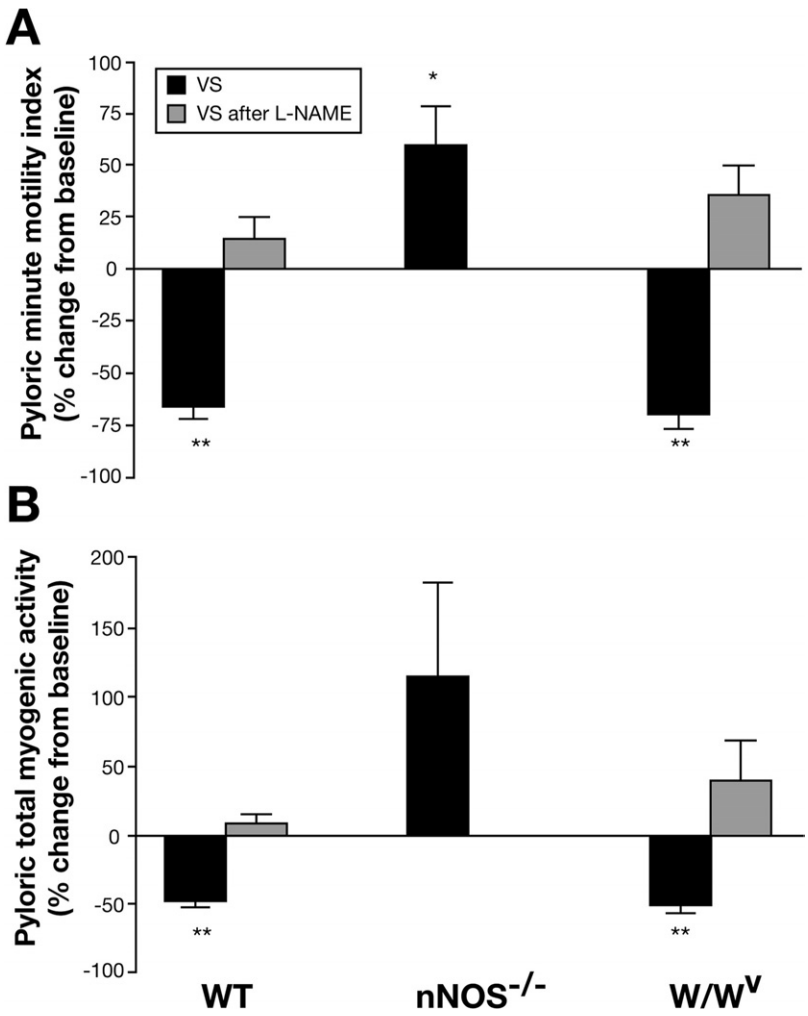

Figure 3. Quantitative data on vagal stimulation–induced changes in pyloric motility indices in 3 strains of mice. Data on the MMI are shown in the *top panel* (*A*) and that on the TMA/min are summarized in the *bottom panel* (*B*). Note that in WT mice, vagal stimulation significantly reduced both motility indices and the effect of vagal stimulation was neutralized by L-NAME pretreatment. In the W/W^{v} mice, MMI and TMA were significantly suppressed by vagal stimulation and the inhibitory effect of vagal stimulation was antagonized by L-NAME. In contrast, vagal stimulation evoked a significant increase in motility indices in the nNOS$^{-/-}$ mice. *$P < .05$; **$P < .01$.

with a wide range of 1 to 9 contractions per minute (n = 8) ($P > .05$, compared with WT). Moreover, when antral contractions occurred contemporaneous with pyloric contractions, they were poorly coordinated. The antral/pyloric contraction ratio was 0.71 ± 16 ($P > .05$, compared with WT). Duodenal contractions occurred at a rate of 38 ± 10 cycles per minute (range, 18–104; n = 8) ($P > .05$, compared with WT). In W/W^{v} mice, antral contractions occurred at a rate of 2.0 ± 0.7 per minute (range, 0–4; n = 8), with nearly half the mice showing no antral contractions. This reduction in antral motility was significant compared with WT ($P < .05$). The antral/pyloric contraction frequency ratio was 0.40 ± 19 ($P > .05$, compared with WT). The rate of duodenal contractions varied widely with an average rate of 15 ± 5 (range, 0–43; n = 8) ($P > .05$, compared with WT).

Residual Gastric Contents

Mice underwent postmortem examination, and gastric contents were carefully examined. In WT mice, gastric bezoars were not seen but bile staining was seen occasionally. Three out of 10 nNOS$^{-/-}$ mice showed one or more bezoars in the stomach; food residues were commonly seen in these mice. Bile staining of the gastric mucosa was not seen in any nNOS$^{-/-}$ animal. In contrast, none of the W/W^{v} mice showed bezoars but all had lemon yellow–colored gastric contents. To document the presence of bile in the stomach, we studied age-matched overnight-fasted W/W^{v} mice and their wild-type cohorts for total bile acid content. The total bile acid content in nanomoles was 7.08 ± 1.7 (n = 5) in the WT and 12.94 ± 0.98 (n = 5) in the W/W^{v} mutant mice. This difference was statistically significant ($P < .05$). Consistent with the increase in bile acids, the gastric pH was significantly higher in the W/W^{v} mice (3.96 ± 0.44) compared with their WT cohorts (2.94 ± 0.14) ($P < .05$). The mean stomach weights before or after normalizing to body weight did not differ between the 2 groups ($P > .05$).

Discussion

The current studies show the following. (1) In mice, the pyloric sphincter is identified as a zone of high pressure, interposed between gastric antrum and duodenum, and a nitric oxide–mediated mechanism is involved in inhibition of pyloric activity in response to vagal stim-

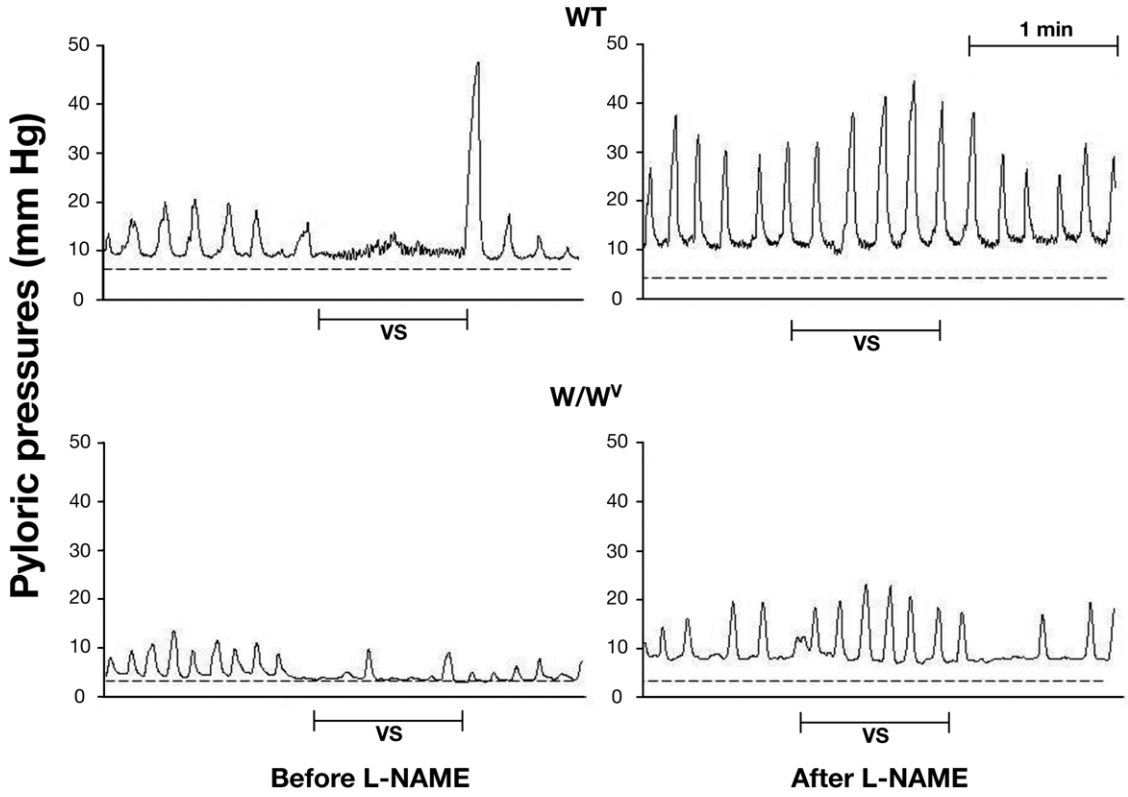

Figure 4. Examples of the effect of L-NAME pretreatment on vagal inhibition of pyloric activity. The *top panels* show WT pyloric response to vagal stimulation before (*left*) and after (*right*) L-NAME. The *bottom panels* show W/W^v pyloric response to vagal stimulation before (*left*) and after (*right*) L-NAME. Note the abolition of inhibition with L-NAME in both strains of mice. The effect of L-NAME (100 mg/kg intravenously) was examined 20 minutes after administration. Stimulus parameters for vagal stimulation were as described in Materials and Methods.

ulation. (2) In nNOS$^{-/-}$ mice, the pyloric sphincter is normotensive but without nitrergic inhibition. (3) In W/W^v mice, the pyloric sphincter is hypotensive but with preserved nitrergic inhibition. (4) nNOS$^{-/-}$ mice show gastric residual contents, while W/W^v mice show increased duodenal reflux. These studies show that nNOS$^{-/-}$ and W/W^v mice exhibit distinct gastric phenotypes associated with different patterns of pyloric sphincter abnormalities.

Although manometric studies of pylorus in the mice have not been reported in the past, studies in other animal species have yielded contradictory results regarding the presence of a tonic high-pressure zone in the pylorus.[5] Some early investigators, who used relatively thin manometric catheters, failed to demonstrate a zone of high pressure at the pylorus. However, other investigators, using balloon or perfusion manometry with larger catheters, were able to demonstrate a clear zone of high pressure at the pylorus[23] that was inhibited by efferent vagal as well as local antral stimulation.[5,10,20] In mice, we found a ~0.6-mm-wide zone of high pressure at the pylorus that showed both tonic and phasic activity. Efferent vagal stimulation caused inhibition of sphincter activity. Because vagal stimulation could excite both excitatory and inhibitory nerves,[10] this observation suggests that predominantly inhibitory innervation was invoked under the stimulus conditions used in this study. These findings in mice are similar to those in the cat, opossum, dog, and human.[5,10,20] Nitrergic inhibitory modulation of lumen and the force of closure of the pyloric sphincter are key to reducing pyloric resistance, facilitating transpyloric flow and gastric emptying of liquids and chyme with solid particles.[3,13]

nNOS$^{-/-}$ mice showed pyloric basal pressures similar to the WT mice. However, in contrast to the controls, the pyloric sphincter in nNOS$^{-/-}$ mice failed to be inhibited with vagal stimulation, but instead demonstrated a paradoxical excitation. The lack of nitrergic pyloric inhibition during transpyloric flow may explain residual contents and delayed emptying, particularly of solids.[3,4,6,13] Dilated stomach, delayed gastric emptying, gastric stasis, and gastric bezoars have been previously described in nNOS$^{-/-}$ mice.[6,7,11] Apart from impaired pyloric relaxation, loss of nitrergic innervation may have other adverse effects on gastric motility. Deficiency in nitrergic neurotransmission has been shown to impair gastroduodenal coordination.[12,24] In addition, inhibitors of NOS have been shown to disrupt migrating motor complexes.[25] These gastric motor abnormalities may also contribute to gastroparesis in nNOS$^{-/-}$ mice.

The gastric phenotype in nNOS$^{-/-}$ mice has been proposed to resemble human hypertrophic pyloric steno-

sis.[11] This view is supported by the fact that pyloric tissue samples from patients with hypertrophic pyloric stenosis show deficiency of nitrergic innervation.[26] However, it is unknown if nNOS$^{-/-}$ mice fully support phenotype of hypertrophic pyloric stenosis. The gastric phenotype in nNOS$^{-/-}$ mice has also been proposed to resemble diabetic gastroparesis,[6,7] and deficiency of nitrergic neurotransmission has been reported in experimental diabetic gastroparesis.[6,7,27] The clinical importance of pyloric resistance in causing gastroparesis is also demonstrated by the reported usefulness of intrapyloric injection of botulinum toxin in the treatment of diabetic gastroparesis.[12] Moreover, delayed gastric emptying, particularly of solids with gastric bezoars but no bile reflux, which characterizes diabetic gastroparesis,[12] is also observed in nNOS$^{-/-}$ mice.[6] Although pyloric function has not been studied in animal models of diabetes mellitus, morphological and functional studies of the stomach in nondiabetic obese (NOD) mice[28] have reported focal losses of ICC-IM and ICC-MY and impaired enteric neurotransmission. Further studies are needed to fully define pyloric sphincter dysfunction in diabetic gastroparesis.

Our study suggests that the difference in the pattern of pyloric sphincter motor activity in W/W^{v} and nNOS$^{-/-}$ mice may explain differences in gastric phenotypes in W/W^{v} and nNOS$^{-/-}$ mice. Hypotensive pyloric sphincter with preserved nitrergic inhibition may predispose to duodenogastric reflux and gastritis.[16,17] It has also been shown that W/W^{v} mice have markedly disrupted duodenal slow waves and contractions due to the loss of ICC-MY in the small bowel that are associated with duodenal stasis and duodenogastric reflux.[29] Moreover, weak contractions in these mutants[15] may lead to poor gastric clearing of refluxed bile, leading to the high incidence of antral erosions and ulceration.[16,17] Duodenogastric bile reflux is an important clinical problem with implications for gastritis, bile esophagitis, and cancer. W/W^{v} mice may represent a nonsurgical animal model of duodenogastric reflux.

One of the complicating factors in distinguishing specific roles of nitrergic nerves and ICC-IM in clinical disorders is the fact that the two are often present together. For instance, deficiency of nitrergic innervation, as in hypertrophic pyloric stenosis and diabetic gastroparesis, may also be associated with loss of ICC.[30] It has been recently shown that NO helps in maintenance of the ICC[31] and that loss of nitrergic innervation may lead to secondary loss of ICC.[12] However, contribution of this secondary loss of ICC to the pathogenesis of gastric motor abnormalities requires further investigation.

The present results have important implications in understanding the physiology and pathophysiology of gastric stasis and bile gastritis. The view that nitrergic neurotransmission to the gastric smooth muscles is transduced by ICC-IM and that loss of ICC-IM leading to a loss of nitrergic neurotransmission to the smooth muscles[9,14,15] has not been supported by other studies.[22] The present study also fails to support the view that ICC-IM is involved in nitrergic neurotransmission in vivo in the pyloric sphincter.

In summary, our studies show the feasibility of studying pyloric sphincter function in vivo in the mouse. This is important because genetic changes can be easily engineered in this species, allowing for the studies on specific genetic mutational effects on pyloric function. The current studies show that nNOS$^{-/-}$ pylorus lacks nitrergic inhibition, which contributes to gastric stasis and development of gastric bezoars. On the other hand, W/W^{v} mice that lack ICC-IM have hypotensive pylorus with preserved nitrergic neural inhibition that predisposes to duodenogastric bile reflux.

References

1. Fisher RS. Gastroduodenal motility disturbances in man. Scand J Gastroenterol Suppl 1985;109:59–68.
2. Mearin F, Camilleri M, Malagelada JR. Pyloric dysfunction in diabetics with recurrent nausea and vomiting. Gastroenterology 1986;90:1919–1925.
3. Indireshkumar K, Brasseur JG, Faas H, et al. Relative contributions of "pressure pump" and "peristaltic pump" to gastric emptying. Am J Physiol Gastrointest Liver Physiol 2000;278:G604–G616.
4. Anvari M, Yu P, Dent J, et al. Role of antral intramural neural pathways in control of gastric emptying in the pig. J Physiol 1995;488:203–209.
5. Ramkumar D, Schulze KS. The pylorus. Neurogastroenterol Motil 2005;17(Suppl 1):22–30.
6. Mashimo H, Kjellin A, Goyal RK. Gastric stasis in neuronal nitric oxide synthase-deficient knockout mice. Gastroenterology 2000; 119:766–773.
7. Watkins CC, Sawa A, Jaffrey S, et al. Insulin restores neuronal nitric oxide synthase expression and function that is lost in diabetic gastropathy. J Clin Invest 2000;106:803.
8. Huizinga JD. Physiology and pathophysiology of the interstitial cell of Cajal: from bench to bedside. II. Gastric motility: lessons from mutant mice on slow waves and innervation. Am J Physiol Gastrointest Liver Physiol 2001;281:G1129–G1134.
9. Sanders KM, Ordog T, Ward SM. Physiology and pathophysiology of the interstitial cells of Cajal: from bench to bedside IV. Genetic and animal models of GI motility disorders caused by loss of interstitial cells of Cajal. Am J Physiol Gastrointest Liver Physiol 2002;282:G747–G756.
10. Allescher HD, Daniel EE, Dent J, et al. Extrinsic and intrinsic neural control of pyloric sphincter pressure in the dog. J Physiol 1988;401:17–38.
11. Huang PL, Dawson TM, Bredt DS, et al. Targeted disruption of the neuronal nitric oxide synthase gene. Cell 1993;75:1273–1286.
12. Vittal H, Farrugia G, Gomez G, et al. Mechanisms of disease: the pathological basis of gastroparesis—a review of experimental and clinical studies. Nat Clin Pract Gastroenterol Hepatol 2007; 4:336–346.
13. Ueno T, Uemura K, Harris MB, et al. Role of vagus nerve in postprandial antropyloric coordination in conscious dogs. Am J Physiol Gastrointest Liver Physiol 2005;288:G487–G495.
14. Ward SM, Morris G, Reese L, et al. Interstitial cells of Cajal mediate enteric inhibitory neurotransmission in the lower esophageal and pyloric sphincters. Gastroenterology 1998;115:314–329.

15. Ward SM, Beckett EA, Wang X, et al. Interstitial cells of Cajal mediate cholinergic neurotransmission from enteric motor neurons. J Neurosci 2000;20:1393–1403.
16. Azuma T, Dojyo M, Ito S, et al. Bile reflux due to disturbed gastric movement is a cause of spontaneous gastric ulcer in W/Wv mice. Dig Dis Sci 1999;44:1177–1183.
17. Yokoyama M, Tatsuta M, Baba M, et al. Bile reflux: a possible cause of stomach ulcer in nontreated mutant mice of W/WV genotype. Gastroenterology 1982;82:857–863.
18. Isozaki K, Hirota S, Nakama A, et al. Disturbed intestinal movement, bile reflux to the stomach, and deficiency of c-kit-expressing cells in Ws/Ws mutant rats. Gastroenterology 1995;109: 456–464.
19. Nakama A, Hirota S, Okazaki T, et al. Disturbed pyloric motility in Ws/Ws mutant rats due to deficiency of c-kit-expressing interstitial cells of Cajal. Pathol Int 1998;48:843–849.
20. Behar J, Biancani P, Zabinski MP. Characterization of feline gastroduodenal junction by neural and hormonal stimulation. Am J Physiol 1979;236:E45–E51.
21. Mashige F, Tanaka N, Maki A, et al. Direct spectrophotometry of total bile acids in serum. Clin Chem 1981;27:1352–1356.
22. Sivarao DV, Mashimo HL, Thatte HS, et al. Lower esophageal sphincter is achalasic in nNOS(−/−) and hypotensive in W/W(v) mutant mice. Gastroenterology 2001;121:34–42.
23. Desipio J, Friedenberg FK, Korimilli A, et al. High-resolution solid-state manometry of the antropyloroduodenal region. Neurogastroenterol Motil 2007;19:188–195.
24. Orihata M, Sarna SK. Inhibition of nitric oxide synthase delays gastric emptying of solid meals. J Pharmacol Exp Ther 1994;271: 660–670.
25. Sarna SK, Otterson MF, Ryan RP, et al. Nitric oxide regulates migrating motor complex cycling and its postprandial disruption. Am J Physiol 1993;265:G749–G766.
26. Vanderwinden JM, Mailleux P, Schiffmann SN, et al. Nitric oxide synthase activity in infantile hypertrophic pyloric sphincter. N Engl J Med 1992;327:511–515.
27. Takahashi T, Nakamura K, Itoh H, et al. Impaired expression of nitric oxide synthase in the gastric myenteric plexus of spontaneously diabetic rats. Gastroenterology 1997;113:1535–1544.
28. Ordog T, Takayama I, Cheung WK, et al. Remodeling of networks of interstitial cells of Cajal in a murine model of diabetic gastroparesis. Diabetes 2000;49:1731–1739.
29. Der-Silaphet T, Malysz J, Hagel S, et al. Interstitial cells of Cajal direct normal propulsive contractile activity in the mouse small intestine. Gastroenterology 1998;114:724–736.
30. Vanderwinden JM, Liu H, De Laet MH, et al. Study of the interstitial cells of Cajal in infantile hypertrophic pyloric stenosis. Gastroenterology 1996;111:279–288.
31. Choi KM, Gibbons SJ, Roeder JL, et al. Regulation of interstitial cells of Cajal in the mouse gastric body by neuronal nitric oxide. Neurogastroenterol Motil 2007;19:585–595.

Received September 26, 2007. Accepted June 12, 2008.

Address requests for reprints to: Raj K. Goyal, MD, Research and Development Service (151), West Roxbury VA Medical Center, 1400 VFW Parkway, West Roxbury, Massachusetts 02132. e-mail: raj_goyal@hms.harvard.edu; fax: (857) 203-5596.

Supported in part by research grants DK 062867 (R.K.G.) and DK 002462 (H.M.) from the National Institute of Diabetes and Digestive and Kidney Diseases and a VA Merit Review Award from the Office of Research and Development, Medical Research Service, Department of Veterans Affairs (R.K.G.).

The authors have no conflict of interest to disclose.

The authors thank Drs John Dent and Arun Chaudhury for helpful suggestions.