

Stomach-brain communication by vagal afferents in response to luminal acid backdiffusion, gastrin, and gastric acid secretion

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Danzer, Marion, Milana Jovic, Claudia Samberger, Evelin Painsipp, Elisabeth Bock, Maria-Anna Pabst, Karl Crailsheim, Rudolf Schicho, Irmgard T. Lippe, and Peter Holzer. Stomach-brain communication by vagal afferents in response to luminal acid backdiffusion, gastrin, and gastric acid secretion. *Am J Physiol Gastrointest Liver Physiol* 286: G403–G411, 2004. First published October 30, 2003; 10.1152/ajpgi.00308.2003.—Vagal afferents play a role in gut-brain signaling of physiological and pathological stimuli. Here, we investigated how backdiffusion of luminal HCl or NH₄OH and pentagastrin-stimulated acid secretion interact in the communication between rat stomach and brain stem. Rats were pretreated intraperitoneally with vehicle or appropriate doses of cimetidine, omeprazole, pentagastrin, dexloxiglumide (CCK₁ receptor antagonist), and itriglumide (CCK₂ receptor antagonist) before intragastric administration of saline or backdiffusing concentrations of HCl or NH₄OH. Two hours later, neuronal activation in the nucleus of the solitary tract (NTS) and area postrema was visualized by c-Fos immunohistochemistry. Exposure of the rat gastric mucosa to HCl (0.15–0.5 M) or NH₄OH (0.1–0.3 M) led to a concentration-dependent expression of c-Fos in the NTS, which was not related to gender, gastric mucosal injury, or gastropyloric motor alterations. The c-Fos response to HCl was diminished by cimetidine and omeprazole, enhanced by pentagastrin, and left unchanged by dexloxiglumide and itriglumide. Pentagastrin alone caused an omeprazole-resistant expression of c-fos, which in the NTS was attenuated by itriglumide and prevented by dexloxiglumide but in the area postrema was reduced by dexloxiglumide and abolished by itriglumide. We conclude that vagal afferents transmit physiological stimuli (gastrin) and pathological events (backdiffusion of luminal HCl or NH₄OH) from the stomach to the brain stem. These communication modalities interact because, firstly, acid secretion enhances afferent signaling of gastric acid backdiffusion and, secondly, gastrin activates NTS neurons through stimulation of CCK₁ receptors on vagal afferents and of CCK₂ receptors on area postrema neurons projecting to the NTS.

ammonium hydroxide; gastropyloric motility; expression of c-Fos in the nucleus of the solitary tract

VAGAL AFFERENT NEURONS PLAY an important role in gut-brain communication. They not only respond to a variety of physiological stimuli relevant to food intake and regulation of digestive activity, but are also activated by immunological and noxious stimuli (3, 15). For instance, painful distension of the rat stomach (38, 42) or exposure of the rat gastric mucosa to an injurious concentration of HCl (34) causes excitation of neurons in the nucleus of the solitary tract (NTS) as visualized by expression of c-Fos. A major participation of vagal afferents in the response to intragastric HCl exposure is deduced from the

finding that the c-Fos induction in the NTS is largely prevented by vagotomy (28, 34). The vagal afferent input from the acid-threatened stomach is further relayed to hypothalamic and limbic areas of the rat brain (28), which is consistent with the concept that vagal afferents contribute to the emotional-affective and autonomic aspects of abdominal nociception (3, 15, 38).

The first aim of this study was to characterize the range of intragastric HCl concentrations that are signaled to the NTS. To this end, the relationship among increasing concentrations of intraluminal HCl, gastric mucosal damage, and c-fos expression in the NTS at the mRNA and protein level was measured. Particular attention was paid to the question of whether physiological or supraphysiological concentrations of intragastric HCl are required to activate neurons in the NTS. The responses to intragastric HCl were compared with those of another noxious chemical, ammonia, administered in the form of NH₄OH. This product of *Helicobacter pylori* urease has been found to cause gastric inflammation and injury (39, 41) and to modify the pH-dependent regulation of gastrin and gastric acid secretion (17, 30).

Because intragastric HCl challenge alters gastric and pyloric motor activity (20) and entry of HCl into the duodenum inhibits gastric emptying and attenuates gastric motility (14, 25) the second aim was to record intragastric pressure and volume recovery following intragastric challenge with HCl or NH₄OH to find out whether different gastropyloric motor alterations could modify the afferent input to the NTS. Thirdly, possible gender differences in the HCl- and NH₄OH-evoked expression of c-Fos in the NTS were investigated, given that there are gender-related differences in the prevalence of functional bowel disorders and in the perceptual responses to gastrointestinal stimuli (9).

The fourth aim addressed the question of whether endogenous gastric acid secretion itself elicits vagal afferent input to the NTS and modulates the c-Fos expression evoked by intraluminal HCl or NH₄OH challenge. This possibility was thought of because stimulation of gastric acid secretion by pentagastrin (2) or administration of exogenous acid (35) can give rise to pain in humans. In our study, it was tested whether pharmacological inhibition or stimulation of gastric acid secretion would influence the expression of c-Fos in the NTS evoked by intragastric HCl or NH₄OH challenge. Furthermore, we analyzed whether the effect of systemic pentagastrin administration to evoke per se c-Fos expression in the NTS and

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area postrema is due to secreted acid as an afferent stimulus and/or to direct activation of CCK₁ (CCK_A) and CCK₂ (CCK_B) receptors on vagal afferents (6, 36) or in the brain stem (7, 37).

MATERIALS AND METHODS

Experiments with nonanesthetized animals. This study was approved by an ethics committee at the Federal Ministry of Education, Science, and Culture of the Republic of Austria. Unless otherwise stated, female age-matched Sprague-Dawley rats (Institut für Labor-tierkunde und -genetik, Himberg, Austria), weighing 150–180 g, were used. One set of experiments was performed with male Sprague-Dawley rats of 220–250 g body wt. All experimental treatments were carried out between 9 and 10 AM after the rats had been fasted for 20 h with free access to water. HCl, NH₄OH, or saline (0.15 M NaCl) was administered intragastrically at a volume of 10 ml/kg through a soft infant feeding tube (outer diameter 2.2 mm; Portex, Hythe, UK). After this intragastric treatment or the intraperitoneal administration of pentagastrin, the animals were no longer allowed to drink until tissue collection 45 min (for *in situ* hybridization) or 2 h (for immunohistochemistry) later. To this end, the rats were euthanized with an overdose of pentobarbital sodium (200 mg/kg ip). For immunohistochemistry (IHC), they were transcardially perfused with 0.1 M PBS (pH 7.4; 150 ml) followed by 4% buffered paraformaldehyde (250 ml). The brain and spinal cord were removed and postfixed overnight in 4% buffered paraformaldehyde at 4°C. Then the tissues were cryoprotected for 48 h in 20% sucrose at 4°C, frozen on dry ice, and stored at –70°C until use.

Six studies were carried out. In *study 1*, the relationship between the intragastric concentration of HCl or NH₄OH and the expression of c-Fos mRNA/c-Fos protein in the brain stem was examined. Animals were given intragastric saline, HCl (0.15, 0.25, 0.35, 0.5 M), or NH₄OH (0.1, 0.2, 0.3 M) 2 h before tissue collection for IHC or were treated intragastrically with saline, HCl (0.35, 0.5 M), or NH₄OH (0.2, 0.3 M) 45 min before tissue collection for *in situ* hybridization. In all further experiments, the expression of c-Fos was visualized by IHC 2 h after intragastric administration of saline, HCl (0.35 M), or NH₄OH (0.3 M) and after intraperitoneal injection of pentagastrin. *Study 2* examined whether pharmacological blockade of gastric acid secretion modifies the c-Fos response of the NTS to intragastric HCl or NH₄OH stimulation. Cimetidine (40 μmol/kg) or its vehicle was injected intraperitoneally 5 min before intragastric treatment with HCl or NH₄OH, whereas omeprazole (20 μmol/kg) or its vehicle was injected intraperitoneally 60 min before intragastric challenge with HCl.

In *study 3*, the influence of pentagastrin-stimulated acid secretion on the ability of an exogenous HCl stimulus to cause c-Fos expression in the brain stem was addressed. The animals received either an intraperitoneal injection of pentagastrin (195 μmol/kg) or of its vehicle 7 min before they were treated intragastrically with HCl. The possible implication of endogenous gastrin and CCK in the gastric acid-evoked c-Fos expression in the brain stem was assessed in *study 4*. For this purpose, rats were pretreated with the CCK₁ receptor antagonist dexloxiglumide (45 μmol/kg ip) (33) or the CCK₂ receptor antagonist itriglumide (10 μmol/kg ip) (27) 30 min before the intragastric administration of saline or HCl. *Study 5* tested the ability of pentagastrin to induce per se c-Fos in the brain stem. To this end, pentagastrin or its vehicle was given intraperitoneally 7 min before saline was administered intragastrically. In addition, the effect of pentagastrin was tested in rats that had been pretreated intraperitoneally with omeprazole (20 μmol/kg) or its vehicle 60 min before the intragastric treatment. In *study 6*, we examined whether CCK₁ and CCK₂ receptors mediate the omeprazole-resistant central c-Fos response to peripheral pentagastrin. Rats were first treated with intraperitoneal omeprazole (20 μmol/kg 53 min before pentagastrin) and then given an intraperitoneal injection of dexloxiglumide (45 μmol/

kg), itriglumide (10 μmol/kg), or dexloxiglumide plus itriglumide 23 min before the intraperitoneal administration of pentagastrin.

Evaluation of gross and histological injury of the gastric mucosa. The extent of injury was evaluated by an observer who was unaware of the experimental treatment (34). Gross gastric injury was assessed by computerized planimetry, and the mucosal area covered by visible hemorrhagic damage was expressed as a percentage of the total area of the glandular mucosa (34). For histological examination, 4-μm sections were cut from fixed and embedded stomachs and stained with a mixture of methylene blue-azure II and basic fuchsin (34). The sections were taken randomly from the gastric corpus and included areas of hemorrhagic damage, if present. The histological injury in each 10-μm segment of the sections was graded as follows: 0, no damage; 1, damage up to 10% of the mucosal depth; 2, damage involving 11–25% of the mucosal depth; and 3, damage involving >25% of the mucosal depth. The section length occupied by the respective injury grades was expressed as a percentage of the total section length (34).

***In situ* hybridization autoradiography.** The brain stems were quickly frozen on dry ice. Coronal sections (10 μm) were cut serially from the brain stem over the whole length of the area postrema and from the caudal thoracic (T8–T12) segments of the spinal cord with a cryostat (31, 34). Every sixth section was processed for *in situ* hybridization (ISH) with an oligodeoxyribonucleotide probe labeled at the 3'-end with [³⁵S]deoxyadenosine 5'-(α-thio)triphosphate as described previously (28). The sections were dipped in Ilford K5 photographic emulsion, and after 18–25 days of exposure in sealed boxes at 4°C, the autoradiograms were developed and the sections were counterstained with hematoxylin and placed on coverslips (28).

IHC. Serial sections of 35-μm thickness were cut from the brain stem over the whole length of the area postrema and from the caudal thoracic (T8–T12) segments of the spinal cord with a cryostat. Every second section was taken and processed as free float. Sections were washed three times in PBS and then incubated in 0.3% H₂O₂ for 30 min. After three washes (each for 10 min), tissues were first incubated for 1.5 h with a blocking serum (0.3% Triton X-100, 1% bovine serum albumin, 5% goat serum in PBS) at room temperature and then with the primary antibody (rabbit polyclonal anti-c-Fos; 1: 20,000) for 48 h at 4°C. Sections were washed again three times in PBS and then incubated for 1.5 h in a solution containing the biotinylated secondary antibody (goat anti-rabbit IgG; Vectastain Elite kit, Vector Laboratories, Burlingame, CA). After three washes in PBS, they were incubated for 1 h in avidin-biotin complex (Vectastain Elite kit). Tissues were rinsed afterward and developed with 3,3'-diaminobenzidine (DAB) substrate (Vector Elite kit) intensified with nickel sulfate for ~2 min. Tissues were subsequently mounted on gelatin-covered slides, air-dried overnight, and dehydrated through an alcohol series. Slides were placed on coverslips with Entellan. Preabsorption controls were performed by using a c-Fos-blocking peptide.

Measurement of intragastric pressure and volume recovery in anesthetized rats. Rats were anesthetized with phenobarbital sodium (230 mg/kg ip). After a midline laparotomy, an intragastric catheter (outer diameter, 2.2 mm) was inserted in the stomach via the esophagus, and the stomach was flushed (20). With its tip being positioned in the corpus region, the catheter was used to record the intragastric pressure (IGP) via a pressure transducer as well as to inject fluid into and drain it from the stomach (20). After an equilibration period of 30 min, a 2-ml fluid bolus was slowly injected into the stomach over a period of 5 s and left in the stomach for a period of 30 min, after which the stomach was drained, and the weight of the recovered fluid was determined. The recovery of fluid from the stomach (an indirect measure of gastric emptying) was expressed as a percentage of the weight of the fluid administered into the stomach (20). Each rat was subjected to four injections/recovery trials at intervals of 15 min during which the stomach was left empty. Two priming trials with saline were followed by a test trial with saline and a test trial with HCl (0.35 M) or NH₄OH (0.2 M). IGP was averaged for the periods of 2–3

min and 29–30 min postinjection. As described before (20), the IGP averaged during the period of 2- to 3-min postinjection was taken as 100% and the IGP taken during the 29- to 30-min postinjection period was expressed as a percentage of that reference value.

Substances and solutions. Cimetidine (Sigma, Vienna, Austria) was dissolved in 0.1 M HCl, then neutralized with 0.1 M NaOH to pH 7.4 and adjusted with sterile saline (Mayrhofer Pharmazeutika, Linz, Austria) to a final concentration of 40 mM cimetidine (21). Pentagastrin (Novabiochem, Läufelfingen, Switzerland) was dissolved in sterile saline to a final concentration of 150 $\mu\text{g/ml}$ (195 μM). Omeprazole was supplied by AstraZeneca (Möln dal, Sweden), dissolved in 30% polyethyleneglycol 400 (Merck-Schuchardt, Hohenbrunn, Germany), and diluted in a weak NaHCO_3 buffer (0.56 mg/ml) to a concentration of 20 mM. The rabbit polyclonal anti-c-Fos antibody and its blocking peptide were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Dexloxiglumide (CR 2017) and itriglumide (CR 2945; gifts of Rotta Research Laboratories, Monza, Italy) were suspended in distilled water and subsequently dissolved by addition of 0.1 M NaOH. The pH of the solutions (45 mM dexloxiglumide and 10 mM itriglumide) was adjusted to 8 by the use of 0.1 M HCl.

Data analysis and statistics. Immunohistochemically processed brain stem and spinal cord sections stained with DAB were examined in a coded manner with a light microscope (Axiophot, Zeiss, Oberkochen, Germany). Eight sections (35 μm) per region and animal were analyzed, and all c-Fos protein-positive cells (nuclei) on one side of the brain stem and dorsal horn of the spinal cord were counted. In situ autoradiograms were also examined in a coded manner with the microscope coupled to a computerized image analysis system (Imaging, St. Catharines, ON, Canada). Cells were considered c-Fos mRNA positive when their grain density was at least 10 times higher than the background (28, 34). Five sections of the brain stem and spinal cord were examined in each animal, and the unilateral counts of c-Fos mRNA-positive cells were determined. To avoid the same cells being counted twice, only every second (IHC) or sixth section (ISH) was analyzed. All counts for a given area in each animal were averaged to give the number of c-Fos mRNA-positive or c-Fos-positive cells in that particular area. These average values from each animal were then used to calculate the mean number of positive cells in the respective areas of each experimental group. All data are presented as means \pm SE, n referring to the number of rats in the respective group. Statistical evaluation of the results was performed with Student's t -test, one-way ANOVA, or ANOVA for repeated measures followed by Dunnett's test, as appropriate. Probability values of $P < 0.05$ were regarded as significant.

RESULTS

Relationship among intragastric HCl concentration, gastric damage, and c-Fos mRNA/c-Fos protein expression in the brain stem and spinal cord. The gastric mucosa of rats was exposed to different concentrations of HCl (0.15, 0.25, 0.35, 0.5 M), and the nuclear c-Fos protein in the NTS was measured immunohistochemically 2 h later. Relative to saline, intragastric HCl increased the total number of c-Fos protein-expressing cells (nuclei) in a concentration-dependent manner (Fig. 1A). The lowest concentration of HCl tested (0.15 M) augmented the number of c-Fos-positive cells by a factor of 1.85 ($P < 0.05$), whereas the highest concentration of HCl (0.5 M) enhanced it by a factor of 12.2 ($P < 0.01$). The number of c-Fos mRNA-positive cells visualized 45 min after intragastric treatment by ISH was roughly equivalent to that of c-Fos protein-positive cells, although after intragastric challenge with 0.35 M HCl, IHC seemed to reveal more activated cells than ISH (Fig. 1A). Gastric exposure to 0.35 and 0.5 M HCl also caused some neurons in the area postrema to express c-Fos

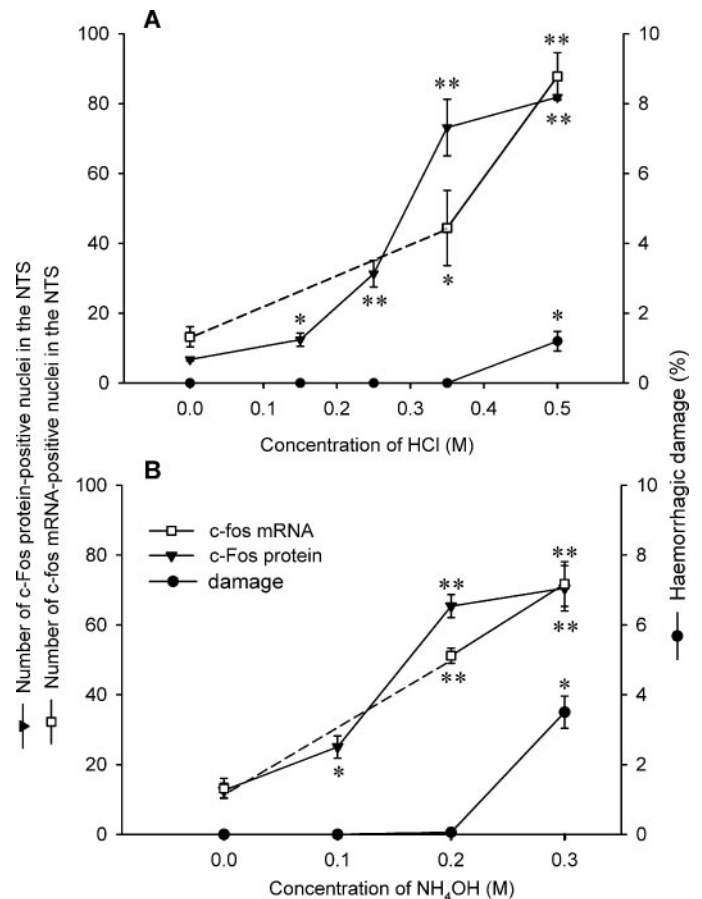


Fig. 1. Relationship between the intragastric concentration of HCl (A) and NH_4OH (B), the number of c-Fos protein-positive cells (nuclei) and the number of c-Fos mRNA-positive cells in the nucleus of the solitary tract (NTS), and the extent of hemorrhagic damage in the stomach (expressed as a percentage of the total area of the glandular mucosa). The measurements were taken 45 min after intragastric administration of HCl, NH_4OH , or saline (0.0) for in situ hybridization (ISH) autoradiography or 2 h posttreatment for immunohistochemistry (IHC) and gastric damage assessment. The data obtained with ISH were calculated relative to a section thickness of 35 μm so as to be comparable with the data obtained with IHC. Values are means \pm SE; $n = 5$ –11. * $P < 0.05$; ** $P < 0.01$ vs. saline.

protein (data not shown) but failed to induce any c-Fos protein in the dorsal horn of the posterior thoracic spinal cord. Thus the number of c-Fos protein-positive cells in saline-treated rats was 3.5 ± 0.2 ($n = 4$) compared with 4.6 ± 0.6 ($n = 4$) cells in animals treated with 0.35 M HCl.

As noted previously (34), only 0.5 M HCl led to formation of hemorrhagic lesions, which covered $1.2 \pm 0.28\%$ ($n = 6$) of the glandular area of the stomach, whereas 0.15–0.35 M HCl failed to induce any macroscopically visible damage (Fig. 1A). Histology revealed that, after exposure to 0.5 M HCl, ~25% of the section length was afflicted with microscopic damage that involved only the superficial 10% of the mucosal depth (Table 1). The characteristics of the acid-induced injury included damaged parietal cells, vacuoles in the superficial epithelial cells, and dilated blood vessels underneath the surface epithelium (34).

Relationship among intragastric NH_4OH concentration, gastric damage, and c-Fos mRNA/c-Fos protein expression in the brain stem and spinal cord. Animals were treated intragastrically with NH_4OH (0.1, 0.2, 0.3 M) for 45 min and 2 h

Table 1. Gross and histological injury in the gastric mucosa after intragastric treatment with NH_4OH and HCl

Type of Injury	Intragastric NH_4OH , 0.3 M	Intragastric HCl, 0.5 M
Gross injury	$3.2 \pm 0.4\%$	$1.2 \pm 0.3\%$
Histological injury grade 0	$59.0 \pm 6.7\%$	$75.0 \pm 4.8\%$
Histological injury grade 1	$32.2 \pm 4.2\%$	$22.8 \pm 4.6\%$
Histological injury grade 2	$3.5 \pm 1.8\%$	$1.8 \pm 0.2\%$
Histological injury grade 3	$5.3 \pm 2.5\%$	$0.3 \pm 0.2\%$

Values are means \pm SE. Stomachs were examined 2 h after the intragastric administration of NH_4OH ($n=9$) and HCl ($n=6$). The mucosal area covered by gross injury was expressed as a percentage of the total area of the glandular mucosa. Histological injury was divided into 4 categories as defined in MATERIALS AND METHODS. Each histological injury grade was expressed as a percentage of the section length covered by the respective grade.

before tissue removal for ISH and IHC, respectively. Relative to saline, NH_4OH led to a concentration-dependent expression of c-Fos mRNA and c-Fos protein in the NTS (Fig. 1B), whereas the spinal cord failed to respond to intragastric NH_4OH with synthesis of c-Fos mRNA and c-Fos protein. Relative to a section thickness of 35 μm , 4.6 ± 0.9 ($n = 4$) and 6.1 ± 0.3 ($n = 4$) cells in the dorsal horn of the posterior thoracic spinal cord were positive for c-Fos mRNA and c-Fos protein after intragastric treatment with saline compared with 4.7 ± 0.6 ($n = 4$) and 5.1 ± 0.3 ($n = 4$) cells after intragastric treatment with 0.3 M NH_4OH , respectively. Appreciable hemorrhagic damage in the stomach was seen only in response to the highest concentration of NH_4OH (0.3 M) tested (Fig. 1B). Histological examination of the injury revealed that $\sim 40\%$ of the section length was covered by lesions, which mostly involved the superficial 10% of the mucosal depth but occasionally also affected deeper levels of the mucosa (Table 1). Compared with the HCl-injured mucosa, the NH_4OH -injured mucosa was characterized by a larger number of widely dilated blood vessels underneath the surface epithelium and fewer vacuoles in the superficial epithelial cells. Unlike the HCl-exposed mucosa, the NH_4OH -injured mucosa did not exhibit damaged parietal cells.

Lack of gender differences in the effects of intragastric HCl and NH_4OH to induce c-Fos in the NTS. Male and female rats did not significantly differ in the number of cells that in the NTS expressed c-Fos after intragastric administration of saline, 0.35 M HCl, or 0.3 M NH_4OH (Table 2).

Effects of intragastric HCl and NH_4OH on intragastric pressure and fluid recovery. The time course of IGP, which increased after intragastric injection of a 2-ml fluid bolus and then began to decline, depended on the nature of the intragastric medium. After administration of HCl (0.35 M), IGP decreased at a significantly slower rate than after injection of saline as determined 29–30 min postinjection, whereas NH_4OH (0.2 M) failed to change the time course of IGP relative to saline (Fig. 2A). Another effect of HCl was to enhance gastric fluid retention as deduced from a 100% recovery of the injected fluid volume from the stomach 30 min postinjection (Fig. 2B). In contrast, 30 min after administration of saline or NH_4OH , only $\sim 50\%$ of the injected fluid volume could be regained (Fig. 2B).

Effects of cimetidine and omeprazole on intragastric NH_4OH - and/or HCl-induced expression of c-Fos in the NTS.

Cimetidine (40 $\mu\text{mol/kg}$ ip) failed to alter the effect of NH_4OH (0.3 M) to cause expression of c-Fos in the NTS (Fig. 3A). The baseline expression of c-Fos in the NTS of rats treated intragastrically with saline remained either unchanged (Fig. 3A) or was slightly enhanced (Fig. 3B) by cimetidine. In contrast, the effect of intragastric HCl (0.35 M) to induce c-Fos in the NTS was significantly reduced by cimetidine (Fig. 3B). Omeprazole (20 $\mu\text{mol/kg}$ ip) likewise decreased the HCl-evoked c-Fos induction in the NTS to a significant extent but had no effect on c-Fos expression in animals treated intragastrically with saline (Fig. 4).

Effect of pentagastrin on intragastric HCl-induced expression of c-Fos in the NTS. Whereas inhibition of gastric acid secretion attenuated the medullary c-Fos response to exogenous HCl challenge, stimulation of gastric acid secretion with pentagastrin (195 $\mu\text{mol/kg}$ ip) enhanced the induction of c-Fos in the NTS by intragastric HCl (0.35 M) to a significant extent (Fig. 4). In addition, the number of c-Fos protein-positive cells in the NTS of rats treated intragastrically with saline was also augmented by pentagastrin (Fig. 4). The action of pentagastrin to enhance the HCl-induced expression of c-Fos in the NTS was significantly reduced in rats pretreated with omeprazole (20 $\mu\text{mol/kg}$), although in these rats, pentagastrin was still able to enhance the medullary c-Fos response to HCl when compared with omeprazole-pretreated rats exposed to intragastric HCl (Fig. 4).

Effects of dexloxiglumide and itriglumide on intragastric HCl-induced expression of c-Fos in the NTS. Intraperitoneal pretreatment of rats with the CCK_1 receptor antagonist dexloxiglumide (45 $\mu\text{mol/kg}$ ip) and the CCK_2 receptor antagonist itriglumide (10 $\mu\text{mol/kg}$ ip) 30 min before intragastric challenge with saline or 0.35 M HCl did not significantly change the expression of c-Fos in the NTS relative to the c-Fos response seen in vehicle-pretreated rats (Table 3).

Analysis of the effect of pentagastrin to induce c-Fos in the brain stem. Omeprazole (20 $\mu\text{mol/kg}$ ip) failed to significantly reduce the ability of pentagastrin (195 $\mu\text{mol/kg}$ ip) to induce c-Fos in the NTS (Fig. 4). The omeprazole-resistant c-Fos response of the NTS to pentagastrin was suppressed by dexloxiglumide (45 $\mu\text{mol/kg}$ ip) to a level that was no longer different from the c-Fos expression seen after intraperitoneal injection of saline (Fig. 5A). Itriglumide (10 $\mu\text{mol/kg}$ ip) reduced the effect of pentagastrin in the NTS to a smaller extent than dexloxiglumide, because the pentagastrin-evoked c-Fos expression in the NTS of itriglumide-pretreated rats was still significantly higher than the saline-evoked c-Fos induction (Fig. 5A). The combination of dexloxiglumide plus itriglumide was as effective as dexloxiglumide alone in reducing the c-Fos response of the NTS to pentagastrin (Fig. 5A).

Table 2. Number of c-Fos protein-positive cells (nuclei) in the NTS after intragastric administration of saline, HCl, and NH_4OH to male and female rats

Intragastric Treatment	Male Rats	Female Rats	P Value
Saline (0.15 M NaCl)	6.5 ± 0.83	7.4 ± 1.0	0.490
HCl (0.35 M)	77.0 ± 3.5	70.0 ± 2.6	0.126
NH_4OH (0.3 M)	69.1 ± 5.1	75.1 ± 5.3	0.427

Values are means \pm SE; $n = 11$. The expression of c-Fos in the nucleus of the solitary tract (NTS) was measured 2 h after the intragastric treatment. There were no significant differences between male and female rats.

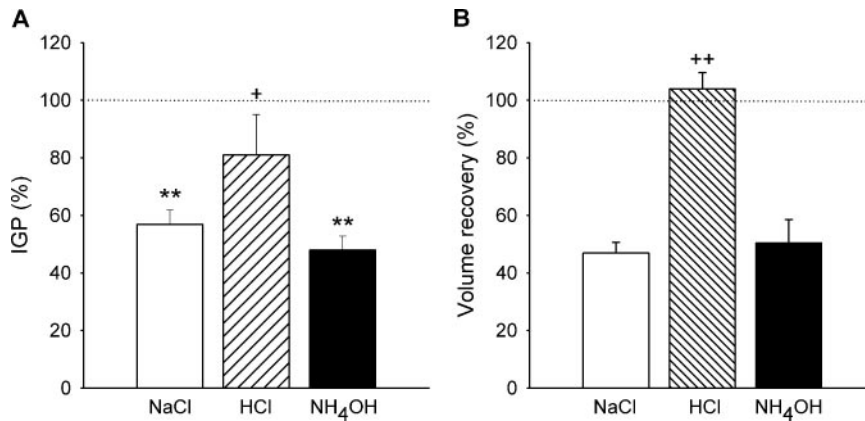


Fig. 2. Effects of intragastric injection of saline (NaCl, 0.15 M), HCl (0.35 M), and NH₄OH (0.2 M) on intragastric pressure (IGP; A) and fluid recovery (B) from the stomach. Saline, HCl, and NH₄OH were injected as a 2-ml bolus. The IGP recorded 29–30 min postinjection is expressed as a percentage of the initial IGP rise recorded 2–3 min postinjection. The volume recovery measured 30 min after bolus injection is expressed as a percentage of the injection volume (2 ml). Values are means + SE; *n* = 6. ***P* < 0.01 vs. respective values recorded 2–3 min postbolus; +*P* < 0.05, ++*P* < 0.01 vs. respective values under NaCl treatment.

Pentagastrin increased the expression of c-Fos not only in the NTS but also in the area postrema, in which it was studied only in omeprazole-pretreated rats (Fig. 5B). The effect of dexloxiglumide and itriglumide on the c-Fos response to pentagastrin in the area postrema of omeprazole-pretreated rats was distinct from that in the NTS, because in the area postrema, itriglumide was more effective than dexloxiglumide in blocking the c-Fos induction due to pentagastrin (Fig. 5B). Whereas itriglumide and the combination of itriglumide plus dexloxiglumide suppressed the pentagastrin-evoked c-Fos re-

sponse in the area postrema to a level that was indifferent from the c-Fos response evoked by saline, dexloxiglumide alone caused only a partial inhibition of the pentagastrin-evoked c-Fos expression in this brain stem region (Fig. 5B).

DISCUSSION

The current study shows that exposure of the rat stomach to HCl or NH₄OH and intraperitoneal administration of pentagastrin stimulate neurons in the brain stem as visualized by expression of the inducible gene c-Fos at the mRNA and protein level, a method that has been established as a standard tool in functional neuroanatomy to delineate the stimulus-evoked activation of neurons (23). Because c-Fos transcription begins within a few minutes after neuronal excitation (23) and in the rat NTS and area postrema seems to be maximal 45 min after gastric acid challenge (34), the induction of c-Fos mRNA was determined by ISH 45 min poststimulus. The appearance of c-Fos protein was measured by IHC 2 h posttreatment, given

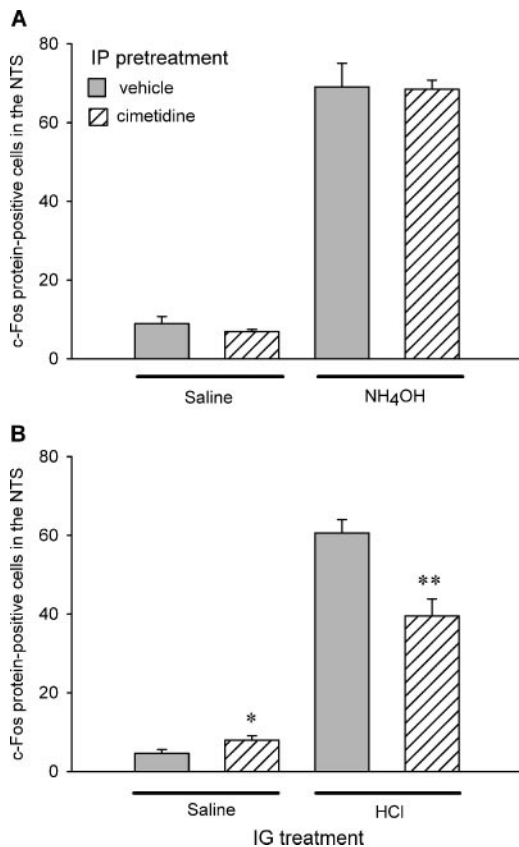


Fig. 3. Effect of cimetidine on the number of c-Fos protein-positive cells (nuclei) counted in the NTS after intragastric treatment with saline, 0.3 M NH₄OH (A), or 0.35 M HCl (B). Cimetidine (40 μmol/kg) or its vehicle was given intraperitoneally 5 min before the intragastric treatment. The expression of c-Fos in the NTS was examined 2 h after the intragastric treatment. Values are means + SE; *n* = 5–7. **P* < 0.05; ***P* < 0.01 vs. vehicle.

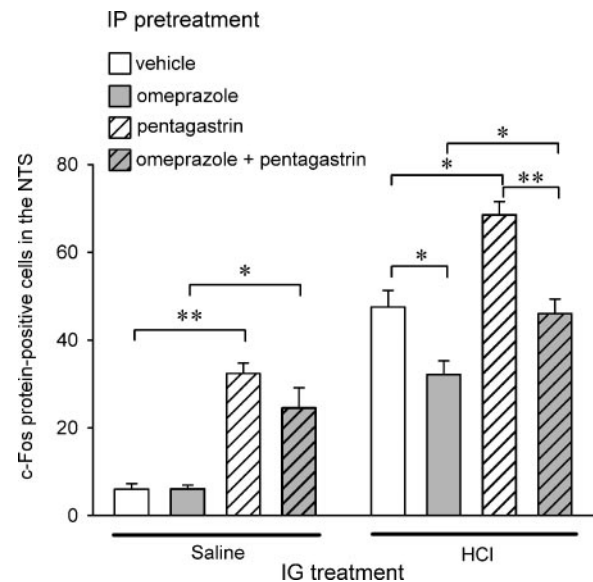


Fig. 4. Effects of omeprazole, pentagastrin, and their combination on the number of c-Fos protein-positive cells (nuclei) counted in the NTS after intragastric treatment with saline or 0.35 M HCl. Vehicle, omeprazole (20 μmol/kg), and pentagastrin (195 μmol/kg) were given intraperitoneally 60 and 7 min, respectively, before the intragastric treatment. The expression of c-Fos in the NTS was examined 2 h after the intragastric treatment. Values are means + SE; *n* = 6–11. **P* < 0.05; ***P* < 0.01 as indicated.

Table 3. Effect of dexloxiglumide and itriglumide, relative to vehicle, on the expression of c-Fos in the NTS following intragastric administration of saline and HCl

Intraperitoneal Pretreatment	Intragastric Pretreatment	Number of c-Fos-Positive Nuclei in the NTS	n
Vehicle	saline	9.4±1.9	6
Dexloxiglumide	saline	13.9±2.7	6
Itriglumide	saline	7.9±1.0	5
Vehicle	HCl (0.35 M)	47.4±7.5	6
Dexloxiglumide	HCl (0.35 M)	40.0±4.8	6
Itriglumide	HCl (0.35 M)	42.1±7.0	6

Values are means ± SE. Vehicle, dexloxiglumide (45 μmol/kg ip), and itriglumide (10 μmol/kg ip) were injected 30 min before intragastric challenge with saline or 0.35 M HCl. The expression of c-Fos in the NTS was measured 2 h postintra-gastric challenge. There were no significant differences.

that the translation of c-Fos mRNA into c-Fos protein reaches its maximum between 1 and 3 h poststimulus (23, 38, 42). We limited our analysis to the brain stem because exposure of the rat stomach to HCl or NH₄OH failed to induce any c-Fos mRNA and c-Fos protein in the dorsal horn of the posterior thoracic spinal cord, which receives gastric input via spinal afferent neurons (16, 29). These data reinforce the concept that intragastric challenge with HCl or NH₄OH is signaled to the brain stem via vagal afferents. In contrast, distension of the rat stomach induces c-Fos both in the brain stem and spinal cord (38, 42), the expression of c-Fos in the NTS being considerably more abundant than in the spinal cord (38).

The induction of c-Fos mRNA and c-Fos protein in the NTS, the central projection area of vagal afferents (1), was related to the intragastrically administered HCl concentration. In addition, it was found that the expression of the c-Fos protein increased at lower concentrations of intragastric HCl than the induction of c-Fos mRNA, which demonstrates that the sensitivity of c-Fos IHC is superior to that of c-Fos mRNA ISH. A comparative analysis of the medullary c-Fos induction and gastric damage indicates that the afferent signaling of gastric acid challenge is not directly related to the formation of overt mucosal injury, because c-Fos expression in the NTS can be evoked by HCl concentrations (0.15–0.35 M) that do not induce any macroscopic lesions and that cause little histological damage (34). Because supraphysiological concentrations of intragastric HCl (0.15 M or higher) are required to induce c-Fos in the NTS, we infer that only a massive increase of the proton gradient across the acid-tight gastric mucosal barrier is able to drive sufficient protons into the lamina propria where they can excite vagal afferent nerve fibers either directly (10, 19) or indirectly via neuroactive factors released in the tissue. Our experimental setup is thus thought to model pathophysiological circumstances where backdiffusion of luminal acid stimulates vagal afferents.

Similar to HCl, intragastrically administered NH₄OH (0.1–0.3 M) led to a concentration-related rise of the c-Fos mRNA and c-Fos protein expression in the NTS but not in the spinal cord. The hemorrhagic lesions seen after exposure to the highest concentration of NH₄OH under study (0.3 M) were histologically different from those induced by HCl, inasmuch as they were characterized by a large number of widely dilated blood vessels underneath the surface epithelium, whereas vacuoles in the superficial epithelial cells were rare and any

appreciable damage to the parietal cells was absent. Because the medullary c-Fos response to lower NH₄OH concentrations (0.1–0.2 M) was not associated with gross mucosal damage, ammonia-induced injury does not seem to be a prerequisite for afferent signaling from the NH₄OH-threatened gastric mucosa. This inference is consistent with the ability of ammonia to stimulate vagal afferents as shown in studies of the rat respiratory tract (22, 40). We hypothesize, therefore, that excess luminal NH₄OH drives NH₄⁺ and OH⁻ across the gastric epithelium into the lamina propria where they can lead to excitation of vagal afferents at concentrations that are too low to damage the mucosa. Whether our findings with acute intragastric NH₄OH challenge have any bearing on the gastric pathology and symptoms induced by chronic infection with *Helicobacter pylori*, which produces ammonia from urea to survive in the acidic environment of the stomach (39, 41) remains to be explored.

The afferent signaling of an intragastric challenge with HCl or NH₄OH is likely to be determined not only by the

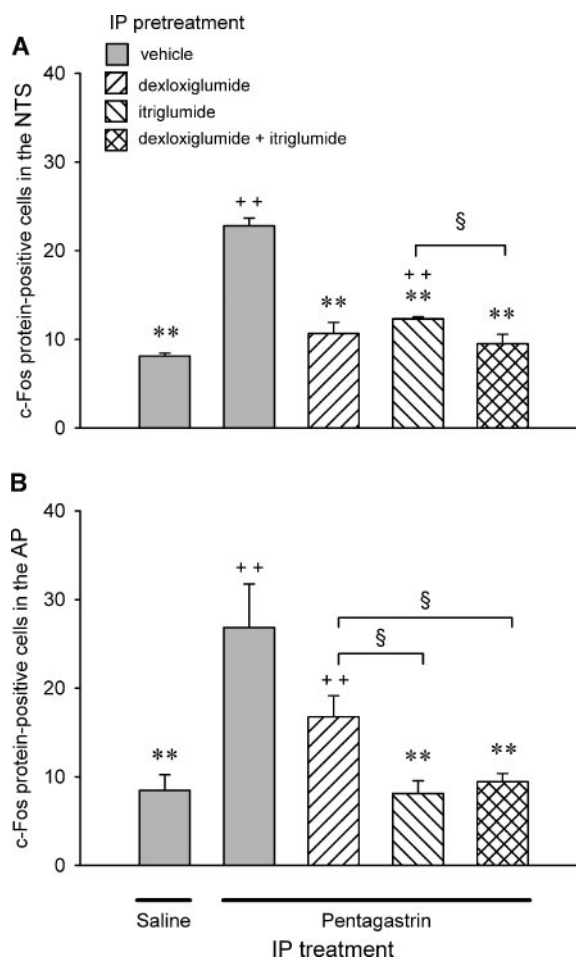


Fig. 5. Effects of vehicle, dexloxiglumide, and/or itriglumide on the expression of c-Fos protein evoked by intraperitoneal pentagastrin in the NTS (A) and the area postrema (AP; B) of omeprazole-pretreated rats. All rats were pretreated intraperitoneally with omeprazole (20 μmol/kg) 180 min before examination. Dexloxiglumide (45 μmol/kg) and/or itriglumide (10 μmol/kg) were given intraperitoneally 150 min and pentagastrin (195 μmol/kg) 127 min before the expression of c-Fos in the brain stem was examined. Values are means ± SE; n = 6. **P < 0.01 vs. vehicle + pentagastrin; ++P < 0.01 vs. saline; §P < 0.05 as indicated.

concentration of the noxious chemical but also by the duration of its presence in the gastric lumen and by alterations in gastropyloric motor activity. For this reason, IGP and gastric fluid recovery after intragastric exposure to HCl or NH₄OH were recorded. As has been shown before (20), intragastric administration of an HCl (0.35 M) bolus delayed the adaptation of IGP and enhanced gastric fluid recovery when compared with the responses to a saline bolus. The HCl-induced fluid retention results from inhibition of gastric emptying and enhanced gastric fluid secretion (20), and the underlying gastropyloric motor effects of HCl are mediated by neural reflexes that are initiated both in the stomach and duodenum (14, 20, 25). Unlike HCl, NH₄OH (0.2 M) failed to change the time course of IGP adaptation and to alter gastric fluid recovery. Because the maximal c-Fos response to intragastric HCl and NH₄OH was very similar, it would appear that the afferent input to the NTS is little influenced by the differential gastropyloric motor alterations elicited by intragastric HCl and NH₄OH. It can also be ruled out that the hyperosmolarity of the intragastric HCl and NH₄OH stimuli contributed to the c-Fos response in the NTS, because intragastric administration of 0.5 M NaCl fails to induce c-Fos mRNA in the NTS (28).

Because most experiments in this study were conducted with female rats, it was proven in a separate experiment that male and female rats did not differ in the medullary expression of c-Fos after intragastric exposure to HCl or NH₄OH. Such a gender difference appeared conceivable because somatic pain processing and antinociceptive mechanisms can differ between male and female rats (8, 13) and irritable bowel syndrome is more common in women than in men (9). Whereas it is not known at which level in the gut-brain axis these gender differences occur, the present study shows that the vagal afferent signaling from the HCl- or NH₄OH-threatened stomach to the rat brain stem is not subject to a gender difference.

In addressing the question of whether gastric acid secretion modifies chemosensory afferent inputs from the gastric lumen, we used cimetidine and omeprazole at doses known to effectively suppress basal and stimulated gastric acid secretion in the rat: cimetidine at the dose of 40 μmol/kg (5), omeprazole at the dose of 20 μmol/kg (24), and pentagastrin at a dose (195 μmol/kg) that maximally increases gastric acid secretion in the rat (26). Because cimetidine failed to alter the NTS response to intragastric NH₄OH, it can be ruled out that NH₄OH led to stimulation of vagal afferents because it facilitated the backdiffusion of luminal acid through the NH₄OH-compromised gastric mucosa or because it increased gastrin and gastric acid secretion (30). These arguments reinforce our contention that ammonia per se is able to excite vagal afferents as suggested by other studies (22, 40).

In contrast, cimetidine significantly diminished the NTS response to intragastric HCl. It is very improbable that cimetidine acted directly on vagal afferents or NTS neurons, because the c-Fos response to intragastric NH₄OH was not attenuated by cimetidine, and the inhibitory effect of cimetidine on the HCl-evoked expression of c-Fos was shared by omeprazole. We conclude, therefore, that endogenous acid secretion plays a role in the central c-Fos response to backdiffusing HCl and propose that endogenously secreted acid sensitizes vagal afferents to luminal HCl backdiffusion, much as luminal acid sensitizes mucosal mechanoreceptors

(11). It remains to be explored whether gastric acid secretion is at all maintained when supraphysiological HCl concentrations are present in the gastric lumen, how endogenously secreted acid reaches vagal afferent nerve endings in the lamina propria, and whether the parietal cell damage caused by exogenous HCl but not NH₄OH has a bearing on the afferent signaling of gastric HCl challenge. Although a CCK₁ receptor antagonist can reduce vagal afferent excitation by exogenous acid (32), our data negate an involvement of gastrin or CCK, because the intragastric HCl-evoked expression of c-Fos in the NTS remained unaltered by dexloxiglumide and itriglumide.

Our conjecture that endogenous acid boosts the medullary input from the acid-threatened stomach was further corroborated by the observation that stimulation of gastric acid secretion with pentagastrin enhanced the c-Fos response to excess HCl in an omeprazole-sensitive manner. In addition, intraperitoneal administration of pentagastrin was found to induce c-Fos in the NTS in the absence of luminal HCl exposure. Because the latter effect was not significantly diminished by omeprazole, two mechanisms of pentagastrin action can be differentiated. On the one hand, pentagastrin augments the gastric afferent input caused by luminal HCl backdiffusion through stimulation of endogenous acid secretion, and on the other hand, it excites NTS neurons through an action that is largely independent of gastric acid secretion and mediated by CCK₁ and/or CCK₂ receptors on vagal afferents (6, 36) or neurons in the brain stem (7, 37).

The omeprazole-resistant activation of NTS neurons by pentagastrin was further analyzed with effective and receptor-selective doses of dexloxiglumide (45 μmol/kg) (33) and itriglumide (10 μmol/kg) (27). The finding that the acid-independent NTS response to pentagastrin was suppressed by dexloxiglumide and partially inhibited by itriglumide is in keeping with the presence of many CCK₁ and some CCK₂ receptors on nodose ganglion neurons (6, 36). It follows that pentagastrin at the dose used here activates both CCK₁ and CCK₂ receptors and that the acid-independent NTS reaction to peripheral pentagastrin is primarily brought about by stimulation of CCK₁ receptors on vagal afferents. However, the ability of the CCK₂ receptor antagonist itriglumide to markedly attenuate the pentagastrin-evoked stimulation of the NTS is difficult to reconcile with reports that stimulation of vagal afferents by CCK-like peptides is exclusively mediated by CCK₁ receptors (12, 32). Because the pentagastrin-evoked c-Fos expression in the area postrema, unlike that in the NTS, was more sensitive to blockade by itriglumide than by dexloxiglumide, we postulate a second pathway of pentagastrin-induced stimulation of the NTS. This pathway involves the area postrema, which, similar to other circumventricular organs, is exempt from the blood-brain barrier and thus directly accessible to circulating peptides. Because the area postrema contains many CCK₂ and some CCK₁ receptors (7) and the CCK-induced activation of area postrema neurons is mediated by CCK₂ receptors (37), it is inferred that intraperitoneally administered pentagastrin can enter this brain stem region and induce c-Fos expression primarily via activation of CCK₂ receptors. Importantly, neurons in the area postrema issue output to the NTS and have been shown to facilitate the processing of vagal afferent input to the NTS (4, 18).

In conclusion, vagal afferents transmit both physiological stimuli (gastrin) and pathological events (backdiffusion of luminal HCl or NH₄OH) from the stomach to the brain stem. These communication modalities interact with each other, given that the gastric acid secretory system modulates vagal afferent nerve traffic through multiple mechanisms. Firstly, vagal afferents are stimulated by gastrin/CCK acting on CCK₁ receptors. Secondly, endogenously secreted acid augments the gastric afferent input evoked by luminal backdiffusion. Thirdly, circulating gastrin can excite area postrema neurons that bear CCK₂ receptors and project to the NTS. The implication of endogenous acid and circulating gastrin in gut-brain communication may have an important bearing on the understanding of acid-related disorders and their symptoms.

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GRANTS

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