

Gastric Acid-Evoked c-fos Messenger RNA Expression in Rat Brainstem Is Signaled by Capsaicin-Resistant Vagal Afferents

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Background & Aims: Gastric acid is known to contribute to ulcer pain, but the mechanisms of gastric chemoreception are poorly understood. This study set out to investigate the pathways and mechanisms by which gastric acid challenge is signaled to the brain. **Methods:** Neuronal excitation in the rat brainstem and spinal cord after intragastric administration of HCl (0.35–0.7 mol/L) was examined by in situ hybridization autoradiography for the immediate early gene c-fos. **Results:** Gastric acid challenge did not induce c-fos transcription in the spinal cord but caused many neurons in the nucleus tractus solitarius and area postrema to express c-fos messenger RNA (mRNA). The HCl concentration-dependent excitation of medullary neurons was in part associated with behavioral manifestations of pain but not directly related to the acid-induced injury and contraction of the stomach. Subdiaphragmatic vagotomy suppressed the c-fos mRNA response to intragastric acid, and morphine inhibited it in a naloxone-reversible manner, whereas pretreatment of rats with capsaicin was without effect. **Conclusions:** Gastric acid challenge is signaled to the brainstem, but not the spinal cord, through vagal afferents that are sensitive to acid but resistant to capsaicin. It is hypothesized that the gastric acid-induced c-fos transcription in the brainstem is related to gastric chemoreception.

Nonulcer dyspepsia and peptic ulcer are frequently associated with pain, yet the mechanisms of gastric nociception are poorly understood.^{1,2} Although pain caused by excessive distention of the stomach has been studied to some extent,^{3,4} little is known as to how a chemical insult of the gastric mucosa is signaled to the brain. We therefore set out to monitor the afferent input, which the spinal cord and brainstem receives after acute acid challenge of the rat stomach, via expression of messenger RNA (mRNA) for the immediate early gene c-fos. The induction of this gene reflects neuronal excitation and can hence be used to map the central somata that are activated by primary afferent input from the periphery.⁴⁻⁶

Although intragastric (IG) administration of noxious chemicals has been reported to induce c-fos expression in certain brain areas, but not the spinal cord,⁷⁻¹⁰ the primary afferent pathways that signal a chemical insult of the gastric mucosa to the central nervous system remain to be identified. The overall aim of the current study was therefore to investigate whether gastric acid challenge activates the c-fos gene in the dorsal horn of the caudal thoracic spinal cord and in the nucleus tractus solitarius (NTS) and area postrema (AP) of the brainstem. Hydrochloric acid (HCl) was chosen as the test stimulus because, first, this chemical is the primary aggressive factor in the stomach. Second, the noxious action of acid is largely confined to the stomach and duodenum, because any absorbed acid will be neutralized, whereas injurious factors such as ethanol or acetylsalicylic acid may have direct effects on the sensory processing in the central nervous system. Third, acid has been shown to induce ulcer pain by a mechanism other than causing muscle spasm.^{11,12}

Five specific aims were pursued in the present study. First, we set out to investigate which concentrations of IG acid give rise to c-fos mRNA expression in the spinal cord and brainstem and to establish the time course of the c-fos mRNA response. The second aim was to examine whether central activation of the c-fos gene relates to acid-induced damage of the gastric mucosa or gastric motor activity. Third, we went on to probe the implication of some mediators, such as prostanoids and bradykinin, which may be released from the gastric mucosa in response to acid challenge and in turn could contribute to the activation of nociceptive afferents. The fourth aim of the study was to characterize the afferent neurons that convey the message of gastric acid challenge to the brainstem with regard to their sensitivity to capsaicin¹³ and their course within the vagus nerve. Fifth and last, we sought to address the question of whether the central

Abbreviations used in this paper: AP, area postrema; NTS, nucleus tractus solitarius.

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c-fos transcription in response to gastric acid challenge is related to nociception. This problem was approached by examining whether gastric acid-evoked c-fos mRNA expression takes place in parallel with behavioral manifestations of pain such as writhing^{14,15} or somatic hyperalgesia¹⁶ and whether morphine is able to inhibit gastric acid-evoked c-fos mRNA expression in the brainstem in a naloxone-reversible manner.

Materials and Methods

Animals and Drug Treatment

The study, which was approved by an ethical committee at the Austrian Ministry of Science, was carried out on female Sprague-Dawley rats weighing 180–220 g. The rats were fasted for 16 hours but had free access to water before their stomachs were challenged by IG administration of saline or HCl (0.35, 0.5, or 0.7 mol/L) at a volume of 10 mL · kg⁻¹ through a soft infant feeding tube (outer diameter, 2.2 mm; Portex, Hythe, England) between 9 and 10 AM. At various time intervals (45 minutes, 2 hours, or 24 hours) thereafter, the rats were killed by intraperitoneal (IP) injection of an overdose of pentobarbital (250 mg · kg⁻¹) to collect the tissues under study. When the survival time was 24 hours, the rats were offered food until 5 PM on the day of IG treatment, followed by another period of 16 hours of fasting. In some experiments, rats were anesthetized with pentobarbital (50 mg · kg⁻¹ IP) 45 minutes after the gastric acid challenge for blood (0.1 mL) to be collected from a carotid artery for the determination of blood pH and bicarbonate content with a blood gas analyzer (AVL, Graz, Austria).

All drugs to be tested were injected subcutaneously at volumes of 1–2 mL · kg⁻¹. To defunctionalize capsaicin-sensitive afferent neurons, rats anesthetized with ether were pretreated with a neurotoxic dose of capsaicin (125 mg · kg⁻¹ subcutaneously) or its vehicle 2 weeks before the experiments.¹⁷ The effectiveness of capsaicin pretreatment was assessed by measuring the calcitonin gene-related peptide (CGRP) content in specimens of the gastric corpus full-thickness wall (100–200 mg wet wt) with a radioimmunoassay whose sensitivity was 1–2 fmol per tube.¹⁷

Subdiaphragmatic Vagotomy

After induction of anesthesia with pentobarbital (50 mg · kg⁻¹ IP), the abdomen was opened by a midline incision. The dorsal and ventral trunks of the vagus nerve attached to the subdiaphragmatic portion of the esophagus were transected, and the cut nerve stumps were retracted^{4,16}; control rats were sham-operated. After closure of the incision, the rats were given 4 days to recover from the surgery. The effectiveness of vagotomy was assessed by radioimmunologic determination¹⁷ of the CGRP content in specimens (100–200 mg wet wt) of the distal esophagus.

Gross and Histological Injury of the Gastric Mucosa

The extent of injury was evaluated by an observer who was unaware of the experimental treatment. The mucosal area covered by gross hemorrhagic damage was assessed by computerized planimetry and expressed as a percentage of the total area of the glandular mucosa of the rat stomach.¹⁸ For histological examination, tissue blocks of the fixed (2.5% glutaraldehyde and 2% paraformaldehyde in 0.1 mol/L cacodylate buffer of pH 7.2) stomachs were embedded in Historesin (LKB, Bromma, Sweden) and cut to obtain 4- μ m sections, which were stained with a mixture of methylene blue-azure II and basic fuchsin.¹⁹ The sections were taken randomly from the gastric corpus and included areas of hemorrhagic damage, if present. Histological injury was quantified by dividing the sections lengthwise into 10- μ m segments and determining the grade of injury for each segment as follows: I, damage involving up to 25% of the mucosal depth; II, damage involving 26%–50% of the mucosal depth; III, damage involving 51%–75% of the mucosal depth; and IV, damage involving 76%–100% of the mucosal depth. The 10- μ m segments that were completely deprived of the surface epithelium (surface ablation) and those showing vascular dilation were also counted, and the section length occupied by the respective injury grades was expressed as a percentage of the total section length.¹⁹

Measurement of IG Pressure

Rats anesthetized with pentobarbital (50 mg · kg⁻¹ IP) were placed on a heating pad to maintain their rectal temperature at 37°C. After laparotomy was performed, a catheter (inner diameter, 3 mm) was inserted in the gastric lumen via an incision in the forestomach and held in place by a ligature. The catheter was connected, via a T piece, with inflow and outflow cannulas each equipped with a valve whereby the gastric lumen could repeatedly be filled with fluid and then emptied. IG pressure was recorded with a pressure transducer (HSE, March-Hugstetten, Germany) in the inflow cannula. The pressure signal was digitized and fed into a personal computer for quantitative evaluation.

Plantar Test and Writhing Response

The thermosensitivity of the hindpaw plantar skin was determined with a Plantar Test Apparatus (Ugo Basile, Comerio-Varese, Italy).²⁰ The intensity of radiant heat emitted by the apparatus was adjusted to a level at which the paw-withdrawal latency was, on average, 7–9 seconds. Three measurements of each hindpaw were taken at 10-minute intervals to determine the average paw-withdrawal latency 15 minutes before (i.e., the average of the recordings made 25, 15, and 5 minutes before) and 45 minutes, 2 hours, and 24 hours after IG administration of saline or HCl (0.5 mol/L). In other experiments, rats treated with IG saline or HCl (0.35 and 0.5 mol/L) were placed in cages with a plastic cover and observed for 12 consecutive periods of 10 minutes (total observation time, 2 hours) to monitor the incidence and number of writhings (abdominal contractions).^{14,15}

In Situ Hybridization Autoradiography

The brainstem and spinal cord were quickly removed and frozen on powdered dry ice. After transfer of the tissues to a cryostat (Microm, Walldorf, Germany), coronal sections (10 μm) were cut from the brainstem over the whole length of the AP and from the caudal thoracic (T8–T12) segments of the spinal cord, which were identified according to the atlas of Paxinos and Watson.²¹ Expression of *c-fos* mRNA was visualized by in situ hybridization with an oligodeoxyribonucleotide probe (48 bases, British Biotechnology, Oxford, England) labeled at the 3' end with [³⁵S]deoxyadenosine 5'-(α -thio)triphosphate.^{10,22} The autoradiograms were examined with a computerized image-analysis system (Imaging, St. Catharines, Ontario, Canada) by an individual who was blinded to the experimental conditions. Cells were considered *c-fos* mRNA positive when their grain density was at least 10 times higher than the background and counted on one side of the NTS, AP, and spinal dorsal horn. The area of AP and NTS was also determined to standardize the sections in the rostrocaudal axis such that only those sections were counted in which the AP covered an area of $\geq 100,000 \mu\text{m}^2$, unless stated otherwise. Particular care was taken to evaluate equivalent sections in the rostrocaudal axis when different treatment groups were compared with each other. To enhance the reliability of the quantitative results, 6 sections of the brainstem and 10 sections of the spinal cord from each animal were counted. The brainstem sections that were evaluated were 50 μm apart and the spinal cord sections 30 μm apart from each other to avoid the same cells being counted twice. The number of positive cells on one side of the AP was expressed relative to an area of 50,000 μm^2 , whereas that counted in one NTS was expressed relative to an area of 250,000 μm^2 . All section counts for each animal were averaged to give the number of *c-fos* mRNA-positive cells in the unilateral NTS, AP, and dorsal spinal cord of the respective rat. The average figures were then used to calculate the mean number of *c-fos* mRNA-positive cells characteristic of each experimental group.

Drugs, Solutions, and Statistics

HOE-140 (icatibant; 100 $\mu\text{mol/L}$; Hoechst, Frankfurt, Germany), morphine hydrochloride (10 $\text{mg} \cdot \text{mL}^{-1}$; Diosynth, Apeldoorn, The Netherlands), and naloxone hydrochloride (2 $\text{mg} \cdot \text{mL}^{-1}$; Du Pont, Geneva, Switzerland) were dissolved in saline (0.15 mol/L NaCl). Indomethacin (Sigma, Vienna, Austria) was dissolved in 2% (wt/wt) Na_2CO_3 at a concentration of 30 $\text{mg} \cdot \text{mL}^{-1}$ and diluted with phosphate-buffered saline at pH 7.4 to give a 10 $\text{mg} \cdot \text{mL}^{-1}$ solution for injection. Capsaicin (Serva, Heidelberg, Germany) was first dissolved in two equal parts of ethanol and Tween 80 and then diluted with saline to obtain a solution of 12.5 $\text{mg} \cdot \text{mL}^{-1}$ capsaicin in a medium containing 10% ethanol, 10% Tween 80, and 80% saline (by volume).¹⁷

All data are presented as means \pm SEM, *n* referring to the number of rats in the respective group. Statistical evaluation of the results was performed with the Mann–Whitney *U* test or, if multiple comparisons were made, with the Kruskal–Wallis *H*

test followed by the Mann–Whitney *U* test. Probability values of <0.05 were regarded as significant.

Results

Gastric Acid–Induced Transcription of *c-fos* in the Brainstem and Spinal Cord

The initial experiments involved IG administration of 0.7 mol/L HCl and unilateral examination of the brainstem and spinal cord 45 minutes after treatment.¹⁰ Compared with saline, 0.7 mol/L HCl caused a more than 30-fold increase in the number of *c-fos* mRNA-expressing cells in the NTS and a more than 200-fold increase in the AP but did not induce any *c-fos* mRNA in the dorsal horn of the caudal thoracic spinal cord (Figure 1). Gastric acid (0.7 mol/L HCl)-evoked *c-fos* mRNA expression in the NTS extended over a long distance in the rostrocaudal axis, because the number of *c-fos* mRNA-positive NTS cells did not differ between sections taken immediately rostral to the AP (35.7 ± 4.1 cells per 250,000 μm^2 ; *n* = 5), immediately caudal to the AP (36.4 ± 3.7 cells per 250,000 μm^2 ; *n* = 6) or in the middle of the AP (42.5 ± 3.0 cells per 250,000 μm^2 ; *n* = 6). The time course with which IG HCl (0.7 mol/L) induced *c-fos* mRNA in the NTS and AP was rapid and transient. Among the time points examined, the expression of *c-fos* mRNA was highest 45 minutes after gastric acid challenge, had markedly declined by 2 hours after challenge, and was completely gone 24 hours later (Figure 2A and C). The average number of cells that 45 minutes after IG administration of 0.7 mol/L HCl expressed *c-fos* mRNA in the AP and bilateral NTS was 42.3 ± 7.1 and 112.7 ± 10.2 per section, respectively (*n* = 6).

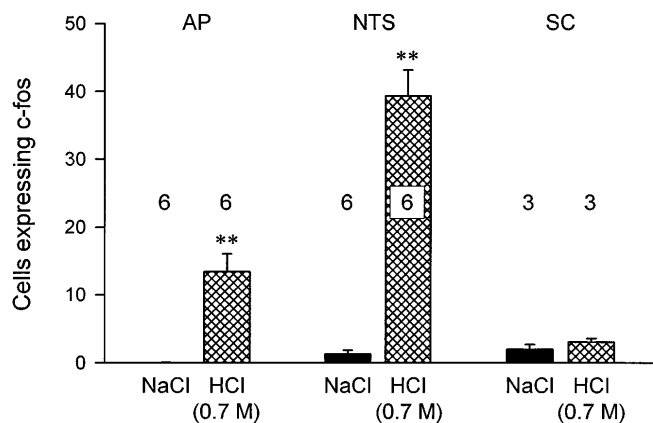


Figure 1. Unilateral expression of *c-fos* mRNA in the AP (no. of cells/50,000 μm^2) and NTS (no. of cells/250,000 μm^2) of the brainstem and in the dorsal horn of the caudal thoracic spinal cord (SC) as examined 45 minutes after IG administration of saline (NaCl) or HCl. Data given as means \pm SEM; number of rats is given within or above the columns. ***P* < 0.01 vs. NaCl.

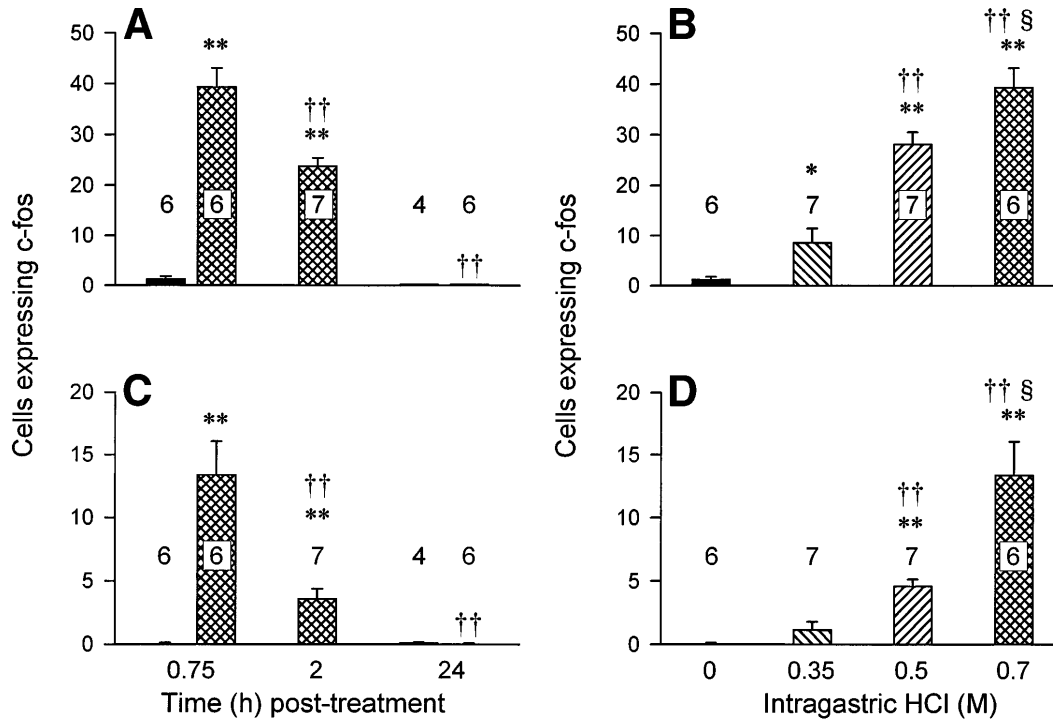


Figure 2. Unilateral expression of c-fos mRNA in the NTS (no. of cells/250,000 μm^2) and AP (no. of cells/50,000 μm^2) of the brainstem after IG administration of saline or acid. (A and C) Time course of c-fos mRNA induction in the NTS and AP, respectively, after IG treatment with saline (NaCl; ■) or HCl (0.7 mol/L; ▨). (B and D) Relationship between IG HCl concentration and c-fos mRNA expression in the NTS and AP, respectively, as examined 45 minutes after administration of NaCl (0) or HCl. Data given as means \pm SEM; number of rats is given within or above the columns. * $P < 0.05$, ** $P < 0.01$ vs. NaCl; †† $P < 0.01$ vs. HCl at (A and C) 0.75 hours or (B and D) 0.35 mol/L HCl; § $P < 0.05$ vs. 0.5 mol/L HCl (B and D).

Relationship Between IG HCl Concentration, c-fos Transcription in the Brainstem, and Gastric Injury

A comparison of the responses to saline and 0.35, 0.5, and 0.7 mol/L HCl revealed that activation of the c-fos gene in the brainstem depended on the concentration of acid administered into the stomach 45 minutes before examination (Figure 2B and D). The mucosal injury seen 45 minutes after treatment was likewise related to the IG HCl concentration, although macroscopically visible damage became apparent only with 0.5 mol/L or higher concentrations of HCl (Table 1). Quantitative histological assessment revealed, however, that even 0.35 mol/L HCl induced mucosal damage (Table 1), whereas saline did not induce any appreciable injury (data not shown).¹⁹

Table 1 shows that with increasing concentrations of acid, damage progressed deeper into the mucosa. Remarkably, the acid-evoked formation of injury in the mucosa did not go in parallel with ablation of the surface epithelium. Whereas 0.7 mol/L HCl led to denudation of more than 90% of the mucosal surface, 0.35 and 0.5 mol/L HCl caused only 1.5% and 11% of the mucosa, respectively, to lose its surface epithelium (Table 1 and Figure 3A). Characteristic of the acid-injured mucosa was the presence of damaged parietal cells, which were

swollen and contained vacuoles (Figure 3B), and of extremely dilated vessels underneath a partly continuous surface epithelium (Figure 3A). The incidence of this vascular abnormality correlated with the extent of histological tissue damage (Table 1). Despite these pathological alterations, IG administration of 0.35 and 0.5 mol/L HCl did not cause substantial destruction of the mucosal

Table 1. Concentration-Related Effect of IG HCl to Induce Gross and Histological Injury in the Gastric Mucosa

Type of injury	0.35 mol/L HCl	0.5 mol/L HCl	0.7 mol/L HCl
Gross injury	0.01 \pm 0.01	1.4 \pm 0.31 ^a	10.3 \pm 2.6 ^{a,b}
Histological injury			
Grade I	4.8 \pm 2.3	0.87 \pm 0.79	0 ^c
Grade II	43.5 \pm 6.2	23.5 \pm 6.0 ^c	2.5 \pm 2.1 ^{a,d}
Grade III	30.4 \pm 4.3	35.2 \pm 4.5	6.8 \pm 2.1 ^{a,b}
Grade IV	21.3 \pm 7.3	40.4 \pm 9.3	90.7 \pm 4.1 ^{a,b}
Surface ablation	1.5 \pm 0.30	10.9 \pm 1.9 ^a	92.4 \pm 6.6 ^{a,b}
Vascular dilation	23.1 \pm 4.6	44.7 \pm 11.3	98.9 \pm 1.0 ^{a,b}

NOTE. The gastric mucosa was examined 45 minutes after IG administration of HCl (0.35, 0.5, or 0.7 mol/L). The area occupied by gross injury was expressed as a percentage of the total area of the glandular mucosa. Six categories of histological injury (for definition see Materials and Methods) were recorded, and the section length occupied by the respective injury grades was expressed as a percentage of the total section length. Data given as means \pm SEM (n = 6).

^a $P < 0.01$ vs. 0.35 mol/L HCl.

^b $P < 0.01$ vs. 0.5 mol/L HCl.

^c $P < 0.05$ vs. 0.35 mol/L HCl.

^d $P < 0.05$ vs. 0.5 mol/L HCl.

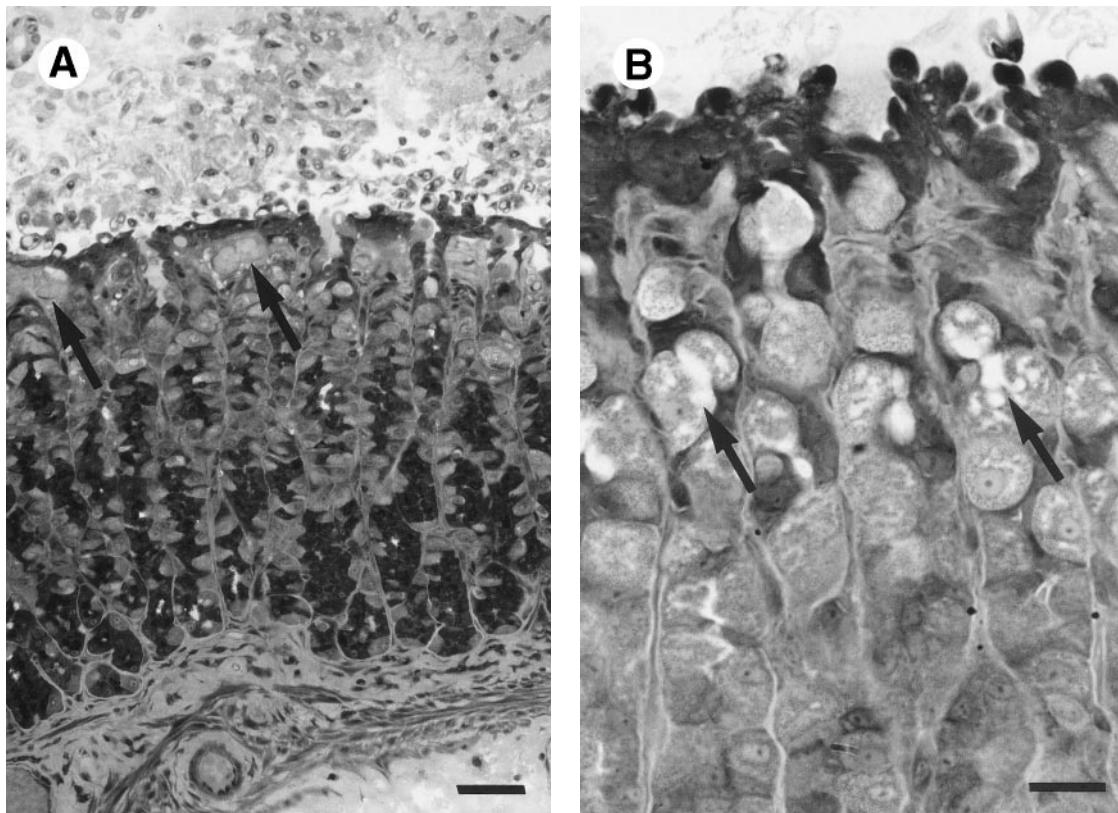


Figure 3. Light-microscopic appearance of the gastric corpus mucosa as examined 45 minutes after exposure to 0.5 mol/L HCl. Morphological details characteristic of acid injury can be seen, such as excessive vascular dilation underneath a partly continuous surface epithelium (arrows in A) and damage to parietal cells (arrows in B) (bars = 50 μ m in A, 20 μ m in B).

architecture, which was typical of the damage induced by 0.7 mol/L HCl (not shown).

Effect of Gastric Acid Challenge on Blood pH, Blood Bicarbonate, and IG Pressure

Blood pH was not altered 45 minutes after IG administration of 0.5 mol/L HCl, whereas blood bicarbonate was significantly ($P < 0.01$) reduced. The respective figures in saline- and acid-treated rats were as follows: blood pH, 7.33 ± 0.01 and 7.27 ± 0.03 ; blood bicarbonate, 25.8 ± 0.3 mmol/L and 20.2 ± 0.9 mmol/L ($n = 4$).

Slow injection of fluid ($10 \text{ mL} \cdot \text{kg}^{-1}$) into the stomach led to an initial increase in IG pressure by >200 Pa, a response that did not differ between stomachs exposed to saline or acid. After the initial peak, the intraluminal pressure in the saline-treated stomachs declined so that 9–10 minutes after treatment IG pressure was only $\sim 50\%$ of that recorded during the period of 2–3 minutes (Figure 4A and C). The decrease in IG pressure reflected emptying of the stomach, because hardly any fluid could be recovered from the gastric lumen after an observation period of 10 minutes. In contrast, stomachs filled with acid did not empty readily, because a considerable amount of fluid could be recovered 10 minutes after

treatment. Accordingly, IG pressure decreased only during the first few minutes after application of HCl (0.15–0.7 mol/L) and then increased in an acid concentration-related manner. This secondary increase in IG pressure peaked ~ 10 minutes after treatment (Figure 4B) and did not significantly differ if 0.35 mol/L, 0.5 mol/L or 0.7 mol/L HCl was administered to the stomach (Figure 4C). Thereafter, the IG pressure became variable but in essence remained high during the observation period of 45 minutes (Figure 4B).

Gastric Acid Challenge and Pain Behavior

The latency with which rats withdrew their hindpaws from exposure to radiant heat was 8.2 ± 0.37 seconds ($n = 12$) before they received any treatment. This latency did not significantly change after IG administration of saline ($n = 6$) or 0.5 mol/L HCl ($n = 6$) as measured 45 minutes, 2 hours, and 24 hours after treatment ($n = 6$ for each treatment; data not shown). None of the rats treated with saline ($n = 11$) or 0.35 mol/L HCl ($n = 10$) showed any writhing (abdominal contractions) during an observation period of 2 hours, whereas 0.5 mol/L HCl caused 15 of 36 rats (41.7%) to respond with this behavior. Analysis of the time course revealed that the writhing response could start as early as

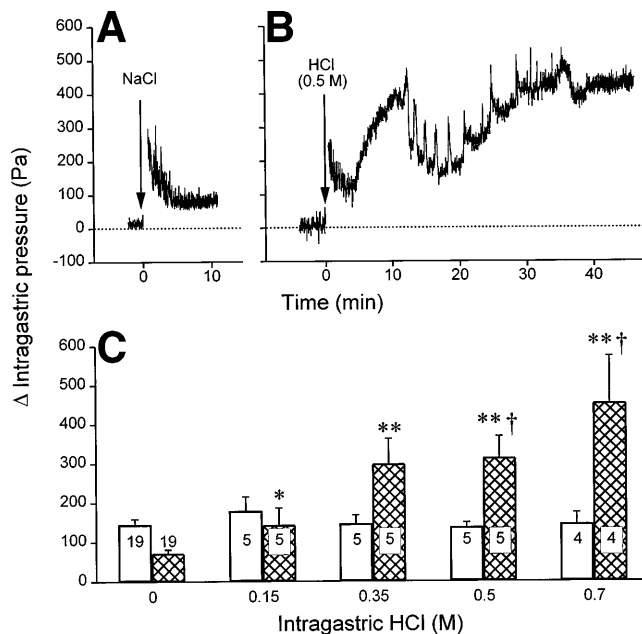


Figure 4. IG pressure changes in response to intraluminal administration of HCl. (A and B) Recording of the effect of IG application of saline (NaCl, 10 mL · kg⁻¹) and acid (HCl), respectively. (C) Relationship between intraluminal HCl concentration and the increase in IG pressure as recorded during 2–3 minutes (□) and 9–10 minutes (▨) after treatment. The abscissa (Δ Intra-gastric pressure) refers to the change in IG pressure relative to the baseline pressure recorded before administration of HCl or saline (0). Data given as means ± SEM; number of rats is given within or above the columns. **P* < 0.05, ***P* < 0.01 vs. respective value recorded after administration of saline (0); †*P* < 0.05 vs. respective value recorded after administration of 0.15 mol/L HCl.

5 minutes after treatment, reached a peak ~45 minutes after treatment, and then gradually dwindled in incidence and intensity (Table 2).

Effects of Capsaicin and Vagotomy on Gastric Acid-Induced Transcription of c-fos in the Brainstem

Pretreatment of rats with 125 mg · kg⁻¹ capsaicin 2 weeks before the experiments did not significantly alter the effect of gastric acid challenge on c-fos transcription in the brainstem. When examined 45 minutes after IG administration of 0.5 mol/L HCl, the number of c-fos mRNA-positive cells was 26.8 ± 2.1 per 250,000 μm² in the NTS of vehicle-pretreated rats (n = 5), 29.5 ± 2.4 per 250,000 μm² in the NTS of capsaicin-pretreated rats (n = 6), 3.5 ± 0.29 per 50,000 μm² in the AP of vehicle-pretreated rats (n = 5), and 3.1 ± 0.34 per 50,000 μm² in the AP of capsaicin-pretreated rats (n = 6). In contrast, the area of acid-induced hemorrhagic lesions in the gastric mucosa of capsaicin-pretreated rats (4.5% ± 1.4% of the total area of the glandular mucosa; n = 6) was significantly (*P* < 0.01) higher than that in vehicle-pretreated rats (0.47% ± 0.10%; n = 6). In

addition, capsaicin pretreatment reduced the concentration of immunoreactive CGRP in the gastric corpus wall (5.1 ± 0.32 fmol · g⁻¹, n = 6) in vehicle-pretreated rats to a level below the detection limit of the radioimmunoassay (n = 6), which reflects a >87% depletion of the peptide.¹⁷

Bilateral subdiaphragmatic vagotomy 5 days before the experiments reduced the ability of IG HCl (0.5 mol/L) to activate the c-fos gene in the NTS and AP by 75%–80% (Figures 5 and 6A and B). In contrast, the failure of IG HCl to induce c-fos mRNA in the caudal thoracic spinal cord (Figure 1) was not changed by vagotomy, and the number of dorsal horn cells expressing c-fos mRNA in vagotomized rats (0.58 ± 0.10; n = 6) did not differ from that in sham-operated animals (1.1 ± 0.37; n = 6). The baseline expression of c-fos mRNA in the NTS and AP after IG saline treatment was likewise indistinguishable between sham-operated and vagotomized rats (Figure 6A and B). Vagotomy, however, reduced the area of acid-induced hemorrhagic damage in the gastric mucosa (Figure 6C) and the concentration of immunoreactive CGRP in the wall of the lower esophagus (Figure 6D).

Effects of HOE-140, Indomethacin, Morphine, and Naloxone on Gastric Acid-Induced Transcription of c-fos in the Brainstem

As shown in Table 3, the ability of IG HCl (0.5 mol/L) to activate the c-fos gene in the brainstem remained unchanged by HOE-140 (100 nmol · kg⁻¹) and indomethacin (10 mg · kg⁻¹). Whereas HOE-140 did not change the acid-induced hemorrhagic damage in the

Table 2. Abdominal Contractions (Writhings) in Response to IG Administration of 0.5 mol/L HCl

Time posttreatment (min)	No. of responding rats	No. of contractions
0–10	2	0.47 ± 0.40
10–20	4	1.3 ± 0.93
20–30	7	1.8 ± 0.69
30–40	11	3.4 ± 0.97
40–50	14	4.7 ± 0.98
50–60	13	3.5 ± 0.67
60–70	10	2.5 ± 0.62
70–80	11	2.9 ± 1.1
80–90	11	2.3 ± 0.83
90–100	7	1.6 ± 0.66
100–110	7	1.3 ± 0.50
110–120	4	1.3 ± 0.61

NOTE. The middle column gives the number of rats (out of a total of 36) that responded with writhing to IG administration of 0.5 mol/L HCl at the specified time periods after treatment. The right column gives the number of writhings per 10 minutes that were counted in the responders (means ± SEM of 15 rats).

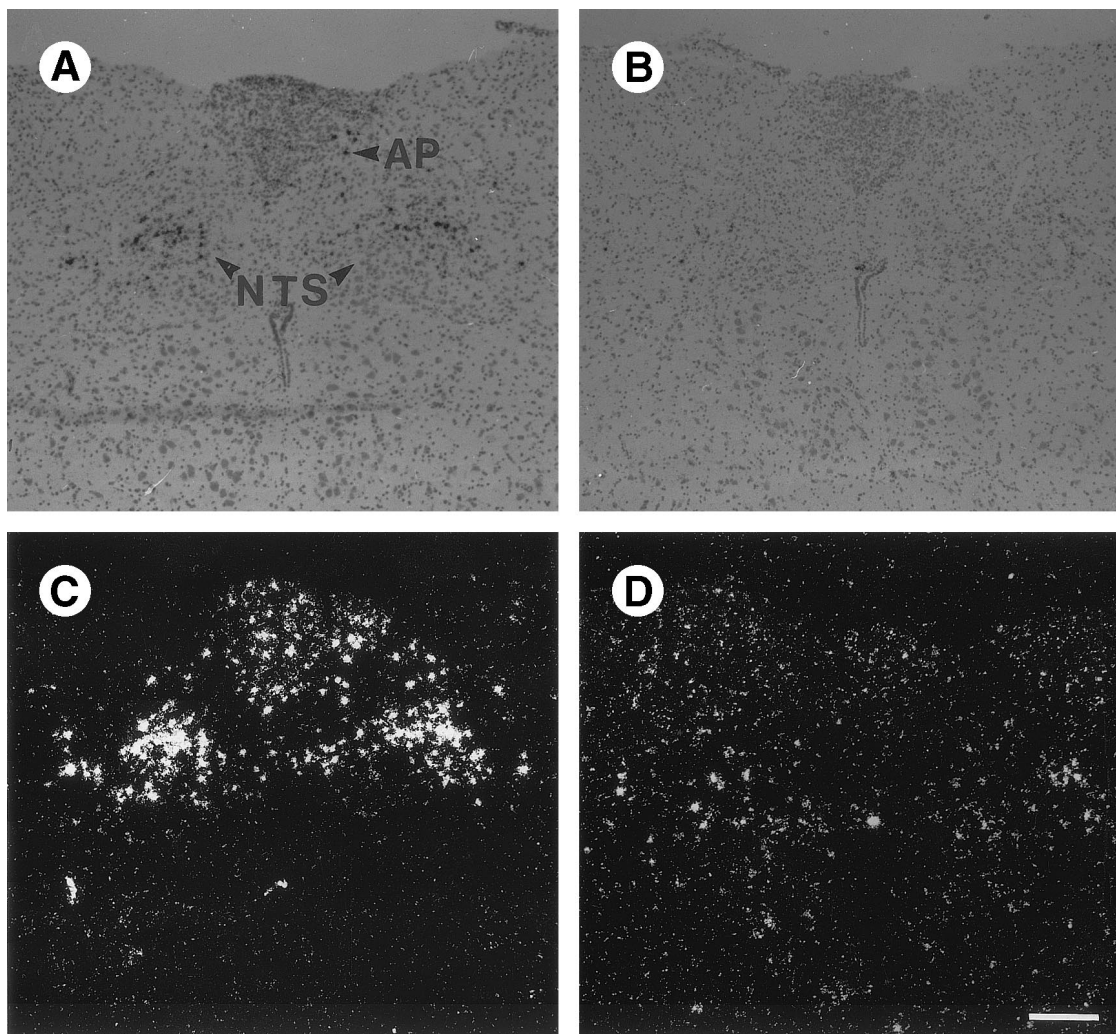


Figure 5. Bright-field and dark-field photographs of brainstem sections taken 45 minutes after exposure of the gastric mucosa to 0.5 mol/L HCl. (A and C) Brainstem of a sham-operated rat; (B and D) brainstem of a rat subjected to bilateral subdiaphragmatic vagotomy 5 days before the experiment. The sham-operated but not the vagotomized rat shows many c-fos mRNA-positive cells in the NTS and AP (bar = 200 μ m).

stomach either, indomethacin enhanced gross injury by a factor of 15 (Table 3). Further analysis showed that in rats receiving IG saline, indomethacin led to 5- and 15-fold increases in the number of cells expressing c-fos mRNA in the NTS and AP, respectively, although the drug did not cause gross mucosal injury under these conditions (Table 3).

Morphine (10 mg \cdot kg⁻¹) attenuated the stimulant action of IG HCl (0.5 mol/L) on c-fos mRNA expression in the NTS and AP by 53% and 73%, respectively, whereas naloxone (4 mg \cdot kg⁻¹) was without significant effect (Figure 7A and B). Naloxone, however, prevented the inhibitory action of morphine on gastric acid-evoked c-fos mRNA expression in the brainstem (Figure 7A and B). The acid-evoked formation of hemorrhagic injury in the gastric mucosa remained unchanged by morphine but was significantly attenuated by naloxone given either alone or in combination with morphine (Figure 7C).

Discussion

The current study shows that acid challenge of the rat gastric mucosa is signaled to the NTS and AP as deduced from a significant activation of the c-fos gene in these nuclei. Calculated relative to a section thickness of 10 μ m, the average number of c-fos mRNA-positive cells in the AP (n = 42) and bilateral NTS (n = 113) of rats treated IG with 0.7 mol/L HCl was as large as, or even larger than, that found in rats treated IP with 300 mg \cdot kg⁻¹ aspirin (AP, 3; NTS, 87)²³ or 100 μ g \cdot kg⁻¹ cholecystinin octapeptide (AP, 43; NTS, 88).²⁴ Being an immediate early gene, c-fos is transcribed within minutes after neuronal excitation.⁴⁻⁶ A similar time course applies to gastric acid-evoked c-fos transcription in the NTS and AP because, among the time points examined here, c-fos mRNA induction was highest 45 minutes after gastric acid challenge and had substantially

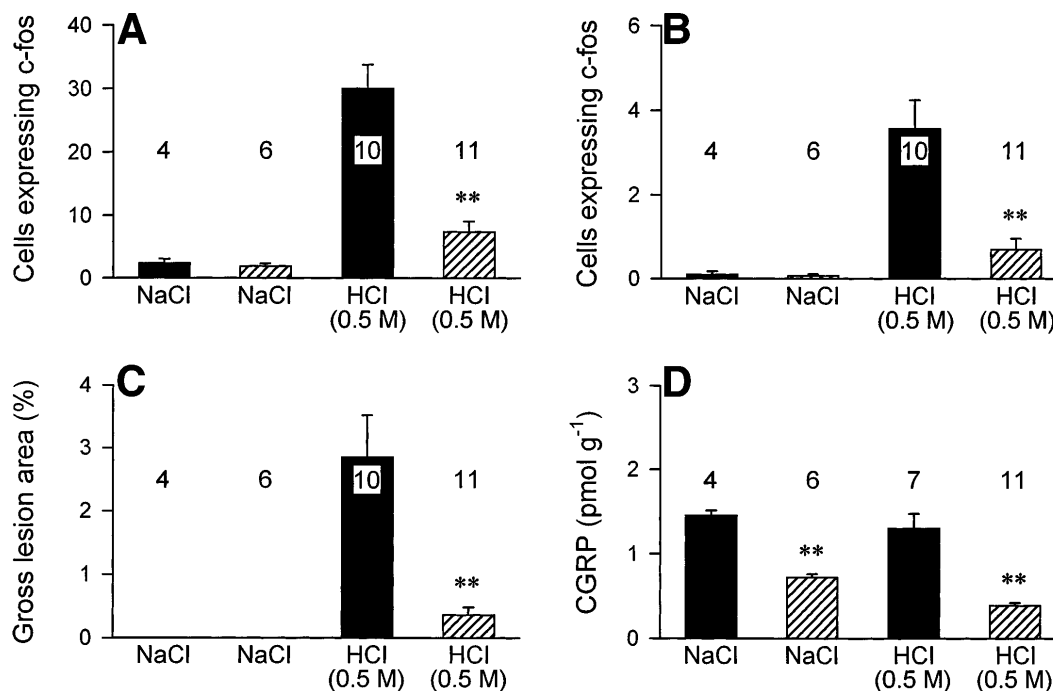


Figure 6. Effect of IG saline (NaCl) and HCl on the unilateral expression of c-fos mRNA in the (A) NTS (no. of cells/250,000 μm^2) and (B) AP (no. of cells/50,000 μm^2) of the brainstem, on (C) gross hemorrhagic damage in the gastric mucosa, and on (D) the CGRP concentration in the esophageal wall of rats subjected to bilateral subdiaphragmatic vagotomy or sham operation 5 days before the experiments. ■, Pretreatment; ▨, vagotomy. Parameters were measured 45 minutes after IG treatment with saline or acid. Gross injury was expressed as a percentage of the total area of the glandular mucosa and the CGRP content related to the wet weight of the tissue. Data given as means \pm SEM; number of rats is given within or above the columns. ** $P < 0.01$ vs. sham operation.

declined 2 hours after treatment. Although transcription of c-fos can start within 5 minutes of cellular activation,⁶ formalin-evoked expression of c-fos mRNA in the spinal cord takes 30–120 minutes to reach a maximum,^{5,10} which was the reason why we chose 45 minutes as the shortest after-treatment interval in our experiments.

As reported previously,¹⁰ gastric acid challenge did not induce any c-fos transcription in the caudal thoracic spinal cord, which receives the densest afferent input from the stomach.^{25,26} It would hence appear that splanchnic afferents do not signal acid challenge in the gastric lumen to the spinal cord. In contrast, the brainstem responded to gastric acid challenge with

abundant expression of c-fos mRNA in the NTS and AP, two nuclei that receive a prominent input from vagal afferent neurons.²⁷ This relationship was affirmed by the ability of bilateral subdiaphragmatic vagotomy to largely prevent gastric acid-evoked transcription of c-fos in the NTS and AP. The effectiveness of vagotomy was proved by a substantial depletion of CGRP from the esophagus, given that part of the peptide present in this tissue is derived from vagal afferents.²⁸ It is inferred, therefore, that primarily vagal afferents signal gastric acid challenge to the brainstem. We do not think that the suppression of afferent input to the brainstem of vagotomized rats is related to the attenuation of gastric mucosal injury found

Table 3. Effect of Indomethacin and HOE-140 on c-fos mRNA Expression in the Brainstem and on Gross Lesion Formation in the Gastric Mucosa Exposed to Saline or HCl

SC treatment	IG treatment	Cells expressing c-fos in NTS	Cells expressing c-fos in AP	Gross lesion area (%)
Vehicle	Saline	1.3 \pm 0.58 (6)	0.06 \pm 0.06 (6)	0.03 \pm 0.03 (6)
Indomethacin	Saline	6.1 \pm 1.1 (6) ^a	0.90 \pm 0.19 (6) ^a	0.03 \pm 0.03 (6)
Vehicle	HCl	29 \pm 2.4 (6)	4.3 \pm 0.51 (6)	1.0 \pm 0.44 (6)
Indomethacin	HCl	28 \pm 1.7 (6)	5.2 \pm 0.55 (6)	15 \pm 1.9 (6) ^a
Vehicle	HCl	30 \pm 4.1 (4)	4.0 \pm 0.89 (4)	1.5 \pm 0.56 (4)
HOE-140	HCl	28 \pm 1.7 (6)	3.0 \pm 0.12 (6)	2.0 \pm 1.1 (6)

NOTE. Indomethacin (10 mg \cdot kg⁻¹) or its vehicle was injected subcutaneously 60 minutes before or HOE-140 (100 nmol \cdot kg⁻¹) or its vehicle 15 minutes before IG administration of HCl (0.5 mol/L) or saline. The unilateral expression of c-fos mRNA in the NTS (no. of cells/250,000 μm^2) and AP (no. of cells/50,000 μm^2) and the area of hemorrhagic damage in the gastric mucosa were determined 45 minutes later. Data given as means \pm SEM with the number of rats given in parentheses.

^a $P < 0.01$ vs. vehicle.

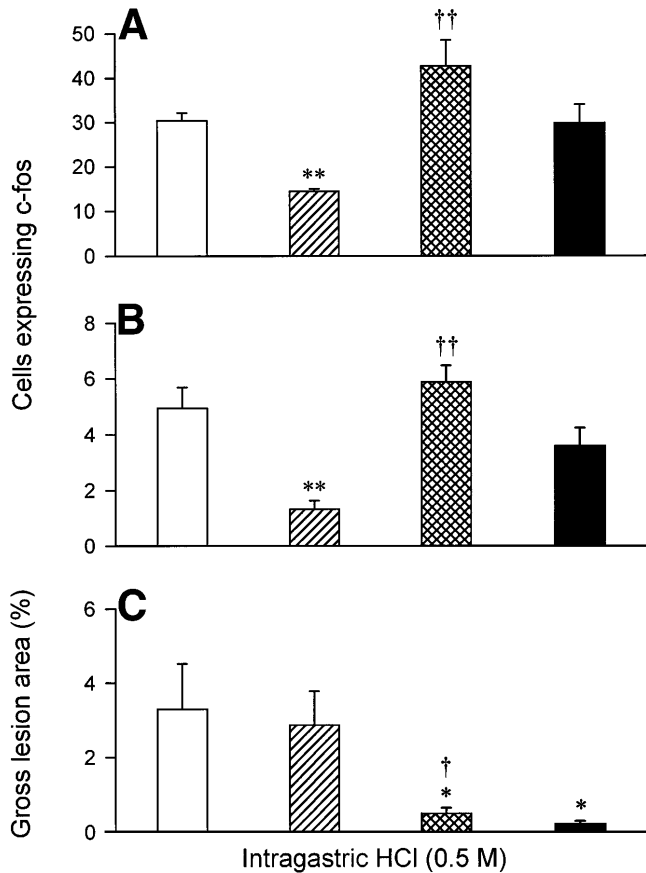


Figure 7. Effect of IG HCl on the unilateral expression of c-fos mRNA in the (A) NTS (no. of cells/250,000 μm^2) and (B) AP (no. of cells/50,000 μm^2) of the brainstem and on (C) gross hemorrhagic damage in the gastric mucosa of rats pretreated with vehicle (\square), morphine (\square), naloxone (\blacksquare), or a combination of the two drugs (\boxtimes). Morphine (10 mg \cdot kg $^{-1}$) was injected 30 minutes and naloxone (2 mg \cdot kg $^{-1}$) 35 and 5 minutes before the IG acid challenge, and the rats were examined 45 minutes later. Gross injury was expressed as a percentage of the total area of the glandular mucosa. Data given as means \pm SEM (n = 6). * P < 0.05, ** P < 0.01 vs. vehicle; † P < 0.05, †† P < 0.01 vs. morphine.

in these animals, because naloxone, which had a similar gastroprotective action as vagotomy, did not alter the acid-evoked transcription of c-fos in the NTS and AP. Vagotomy is known to enhance the size of the stomach, to reduce gastric acid secretion, motility, and emptying,^{29,30} and, depending on the experimental conditions, to attenuate³¹ or aggravate³² experimental gastric injury. In view of this dual role of the vagus nerve in gastric mucosal homeostasis,³³ it appears improbable that vagotomy inhibits gastric afferent input to the brainstem by blunting the noxious activity of acid in the stomach.

In explaining the small gastric acid-induced expression of c-fos mRNA that persisted in the brainstem of vagotomized rats, it needs to be considered that the AP, like other circumventricular organs, lies outside the blood-brain barrier and hence may directly respond to blood-borne substances^{34,35} and in turn stimulate the

NTS. Because gastric acid challenge caused metabolic acidosis as reflected by a lowered plasma level of bicarbonate, it cannot be ruled out that alteration of the acid-base balance or generation of circulating factors in the acid-threatened gastric mucosa acted directly on the AP to cause some transcription of c-fos.

The neurons that signal gastric acid challenge to the brainstem were characterized as being resistant to capsaicin, an excitotoxin that acts selectively on a group of C- and A δ -fiber afferents with pronounced chemosensitivity.¹³ The effectiveness of capsaicin pretreatment was confirmed by gastric depletion of CGRP, which in the rat stomach is almost totally contained in capsaicin-sensitive spinal afferents,^{16,26} and by aggravation of gastric damage, as observed in other experimental ulcer studies.^{18,36} Although the vagus nerve also comprises capsaicin-sensitive afferents that respond to nutrients and cholecystokinin,^{24,37,38} the present study corroborates the existence of an important population of capsaicin-resistant vagal afferents that signal gastric acid challenge (present study) and painful gastric distention³⁸ to the brainstem. Insensitivity to capsaicin discriminates the vagal afferents that mediate gastric acid-evoked c-fos mRNA expression in the brainstem from the capsaicin-sensitive splanchnic afferents that trigger local homeostatic reactions in the acid-threatened stomach but do not signal to the spinal cord.^{10,18,36} The failure of IG HCl to induce c-fos expression in the spinal cord cannot be explained by the reported ability of vagal afferents to activate descending pathways and thereby inhibit spinal nociception,³⁹ because the spinal transcription of c-fos was in no way boosted by vagotomy. Further consistent with this argument is the observation that the vagal afferents that lead to depression of spinal nociception are sensitive to capsaicin,⁴⁰ whereas the vagal afferents that mediate gastric acid-evoked c-fos mRNA expression in the brainstem are resistant to capsaicin (present study).

The expression of c-fos mRNA in the NTS and AP was related to the IG concentration of HCl (0.35–0.7 mol/L) and to the extent of mucosal damage that comprised vascular dilation and hemorrhagic erosions. However, there is good reason to assume that acid itself is the relevant stimulus that excites vagal afferent neurons, given that pH-sensitive vagal afferents are present in the gastric mucosa^{41–43} and the formation of hemorrhagic injury and afferent input to the brainstem dissociated in a number of experimental situations. Thus, the transcription of c-fos in the NTS and AP did not change when gastric injury was attenuated by naloxone or aggravated by capsaicin or indomethacin, whereas morphine reduced c-fos mRNA expression but did not alter gastric damage. These divergent findings negate the notion that gastric

injury is the primary stimulus for vagal afferent neurons that signal gastric acid challenge to the NTS and AP and advocate the hypothesis that c-fos mRNA induction in the brainstem is a response to acid or acid-generated factors. This concept receives additional support if the gastric motor responses to IG HCl are taken into account. Excess acid entering the duodenum closes the pylorus and prevents further emptying of the stomach.⁴⁴ The current measurements of IG pressure indicate that acid-treated stomachs not only failed to empty to a substantial degree but in fact also contracted in response to intraluminal acid administration, as deduced from the swift secondary increase in IG pressure. Although the vagus nerve contains mechanoreceptors that can respond to both contraction and distention,⁴³ it is unlikely that mechanoreceptor signaling contributed to gastric acid-evoked transcription of c-fos in the brainstem, because the HCl concentrations necessary to induce medullary c-fos expression were higher than those required to cause gastric contraction.

Taken together, all observations signify that the primary acid insult and not the subsequent lesion formation and gastric contraction determines the vagal afferent input to the NTS and AP. There is little reason to conjure that acid-generated factors such as bradykinin, which is formed in the acid-threatened stomach,⁴⁵ and prostanooids, which are ubiquitously generated in response to tissue irritation, contribute to the acid-induced stimulation of vagal afferents, because the gastric acid-evoked transcription of c-fos in the brainstem remained unaltered by an effective dose of the bradykinin B₂-receptor antagonist HOE-140⁴⁶ and the cyclooxygenase inhibitor indomethacin.⁴⁷ The ability of indomethacin to induce per se some medullary expression of c-fos mRNA may be related to the drug's adverse influence on the gastric mucosa, an action that was not further analyzed in the current study.

In examining the question of whether gastric acid challenge gives rise to pain, we found that morphine depressed the gastric acid-evoked transcription of c-fos in the NTS and AP in a naloxone-reversible manner. This result shows that μ -opioid receptors control the gastric acid-evoked input to the brainstem, which is in keeping with the presence of μ -opioid receptors on vagal afferents in the NTS.⁴⁸ In analogy with other studies,^{15,49} we hypothesize that the opiate-sensitive expression of c-fos mRNA in the NTS and AP is a molecular correlate of gastric chemonociception, although it needs to be considered that not all neural pathways can be mapped via c-fos transcription,⁶ and certain neurons in the central nervous system may be inhibited, rather than excited, by afferent neuron stimulation.^{39,40,43,44} Further circumstantial evi-

dence that the medullary transcription of c-fos relates to gastric pain comes from its association with writhing, a behavior that is widely used to study visceral pain in response to IP injection of noxious chemicals.^{14,15} Writhing was observed only with a concentration of acid (0.5 mol/L) that was high enough to cause substantial expression of c-fos mRNA in the brainstem. Remarkably, the 42% incidence of writhing triggered by 0.5 mol/L HCl in the rat stomach matches the 37%–42% incidence of pain that is produced if ulcer craters of patients with symptomatic peptic ulcers are superfused with 0.1 mol/L HCl.^{11,12} It remains to be determined why writhing is evoked by HCl in only 42% of the rats, although the afferent input to the brainstem, as measured by c-fos transcription, is reproducible in every rat. Heat-evoked nociception in the skin was not altered by exposure of the stomach to 0.5 mol/L HCl, a possibility that was envisaged because IP injection of bacterial lipopolysaccharide causes cutaneous hypersensitivity to noxious heat through an action involving the vagus nerve.¹⁶

The exclusive signaling of a chemical insult in the gastric mucosa to the NTS and AP but not the spinal cord attributes vagal afferents and their projection nuclei in the brainstem a potential role in visceral nociception. Our findings are in accord with those of an increasing number of studies that underline the importance of the vagus nerve in the central transmission of noxious conditions in the stomach and intestine, including painful gastric distention^{4,38} and exposure to bacterial lipopolysaccharide.^{16,30} In an attempt to reconcile this role of vagal afferents with the traditional view that gastrointestinal nociception is a domain of spinal, but not vagal, afferents,^{1,2,43} it has been proposed that nociceptive input through the vagus nerve is particularly important for the emotional-affective component of pain.⁴

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