Gastric distension-induced release of 5-HT stimulates c-fos expression in specific brain nuclei via 5-HT3 receptors in conscious rats

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Mazda, Takayuki, Hiroshi Yamamoto, Masaki Fujimura, and Mineko Fujimiya. Gastric distension-induced release of 5-HT stimulates c-fos expression in specific brain nuclei via 5-HT3 receptors in conscious rats. Am J Physiol Gastrointest Liver Physiol 287: G228–G235, 2004. First published December 18, 2003; 10.1152/ajpgi.00373.2003.—We examined c-fos expression in specific brain nuclei in response to gastric distension and investigated whether 5-HT released from enterochromaffin (EC) cells was involved in this response. The role of 5-HT3 receptors in this mechanism was also addressed. Release of 5-HT was examined in an ex vivo-perfused stomach model, whereas c-fos expression in brain nuclei induced by gastric distension was examined in a freely moving conscious rat model. Physiological levels of gastric distension stimulated the vascular release of 5-HT more than luminal release of 5-HT, and induced c-fos expression in the nucleus of the solitary tract (NTS), area postrema (AP), paraventricular nucleus (PVN), and supraoptic nucleus (SON). The c-fos expression in all these brain nuclei was blocked by truncal vagotomy as well as by perivagal capsaicin treatment, suggesting that vagal afferent pathways may mediate this response. Intravenous injection of 5-HT3 receptor antagonist granisetron blocked c-fos expression in all brain nuclei examined, although intracerebroventricular injection of granisetron had no effect, suggesting that 5-HT released from the stomach may activate 5-HT3 receptors located in the peripheral vagal afferent nerve terminals and then induce brain c-fos expression. c-fos Positive cells in the NTS were labeled with retrograde tracer fluorogold injected in the PVN, suggesting that neurons in the NTS activated by gastric distension project axons to the PVN. The present results suggest that gastric distension stimulates 5-HT release from the EC cells and the released 5-HT may activate 5-HT3 receptors located on the vagal afferent nerve terminals in the gastric wall leading to neuron activation in the NTS and AP and subsequent activation of neurons in the PVN and SON.

paraventricular nucleus

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Address for reprint requests and other correspondence: M. Fujimiya, Dept. of Anatomy, Shiga Univ. of Medical Science, Seta, Otsu, Shiga 520-2192 (E-mail: fujimiya@belle.shiga-med.ac.jp).
by a chronically implanted balloon in freely moving conscious rat models to avoid the suppressive effects of anesthesia on c-Fos protein synthesis (30). We examined c-fos expression in the nuclei of the medulla and hypothalamus because these brain regions are important in the autonomic regulation of food intake (2, 3, 41). We also used truncal vagotomy as well as perivagal capsaicin treatment to examine the pathways mediating brain response to peripheral stimulation. We further employed the retrograde tracer experiments to determine whether c-fos-expressing neurons in the brain stem nuclei directly project axons to the hypothalamic nuclei.

MATERIALS AND METHODS

Male Wistar rats (Clea Japan, Tokyo, Japan) weighing 250–300 g were housed under controlled temperature (21–24°C) and light (lights on 800–2000) conditions with free access to laboratory chow pellets (CE-2; Clea Japan) and water. Care of animals was conducted in accordance with the Guide for Use of Experimental Animals (Shiga University of Medical Science).

Ex vivo-perfused rat stomach. Animals were anesthetized with pentobarbital sodium (60 mg/kg) and the stomach was perfused vascularly and luminaly as described previously (37). In brief, arterial perfusion was achieved with a cannula inserted into the aorta with the tip lying adjacent to the celiac artery, and the vascular effluent was collected through a portal vein cannula. Luminal perfusion was performed through a cannula inserted into the cardia and effluent perfusate was collected through a cannula with a side hole placed at the pylorus. A catheter (3-Fr, 1-mm diameter; ATOM, Tokyo, Japan) with a balloon made from a latex condom (15–17 mm in diameter when inflated with 3.0 ml of air) was inserted through the side hole and a balloon was placed at the gastric corpus. The flow rate for vascular and luminal perfusion was 3 and 1 ml/min, respectively. Both vascular and luminal samples were collected at 3-min intervals for 30 min. After a 12-min perfusion period, gastric distension was given for 2 min by inflating the balloon with 3 ml of air. 5-HT determination was carried out with HPLC as described in the previous study (37). Statistical analysis of the data was performed by using one-way ANOVA.

Preparation of animals for in vivo experiment on gastric distension. Rats were anesthetized with pentobarbital sodium (50 mg/kg). The stomach was exposed via a midline abdominal incision and a small incision was made on the stomach fundus. A balloon made from a latex condom (15–17 mm in diameter when inflated with 3.0 ml of air) connected to a catheter (3-Fr, 1-mm diameter; ATOM) was inserted through the cervical neck incision. A strip of paraffin was placed under the vagal trunk, and gauze was used to cover the operative field to protect surrounding tissues. A small piece of cotton soaked in capsaicin (Sigma, St. Louis, MO) (10 mg/ml vehicle, 10% Tween 80 in olive oil) was placed on the vagal nerve for 30 min. One to two additional drops of capsaicin were added every 5 min; the maximum amount applied was 0.1 ml (1 mg) for each nerve. After treatment, the area was thoroughly rinsed with 0.9% NaCl and dried with cotton swabs. The procedure was then repeated for the opposite vagal trunk, and followed by closing the neck incision with sutures.

Immunohistochemistry of c-fos expression. Rats were anesthetized with an injection of pentobarbital sodium (50 mg/kg ip; Nembutal, Abbott Laboratories), perfused for 10 min via the left ventricle with 0.01 M PBS to wash out the blood and then perfused with a fixative containing 4% paraformaldehyde, 0.5% glutaraldehyde, and 0.2% picric acid in 0.1 M phosphate buffer at 4°C for 10 min at a speed of 30 ml/min. The brain was removed from the skull and immersed for 24 h in the postfixative containing 4% paraformaldehyde and 0.2% picric acid in 0.1 M phosphate buffer at 4°C and washed for 4 days with several changes of 0.1 M phosphate buffer containing 15% sucrose. The brain was cut into 20-μm coronal sections in a cryostat and collected in 0.1 M PBS containing 0.3% Triton X-100 (PBST). The sections were incubated with c-fos antibody (Ab5; Oncogene, San Diego, CA) diluted 1:5,000 in PBST for 2 days at 4°C. After being washed for 30 min with PBST, the sections were incubated at room temperature for 2 h in biotinylated anti-rabbit IgG (Vector Laboratories, Burlingame, CA) diluted 1:1,000 in PBST. Sections were then washed and placed in avidin-biotin peroxidase complex (Elite; Vector Laboratories) diluted 1:2,000 in PBST for 1 h at room temperature. Immunoreactivity was then visualized by incubation with 0.05 M Tris-HCl buffer (pH 7.6) containing 0.01% diaminobenzidine, 1%
ammonium nickel sulfate, and 0.0003% H$_2$O$_2$ for 30 min at room temperature. The stained sections were mounted on gelatin-coated glass slides, dehydrated with graded ethanol, and coveredslipped with Entellan (Merck, Darmstadt, Germany). Quantitative analysis for the number of c-fos positive cells was performed as follows. Under the light microscopy at ×200 magnification, the total number of c-fos positive cells was counted in each brain nucleus. A mean value of each brain nucleus was determined by sampling from three randomly selected sections cut through the specific brain nuclei, and results were expressed as means ± SD from three animals. Value was compared by Student’s t-test and P < 0.05 was considered statistically significant.

Retrograde tracing with fluorogold and double staining for fluorogold/c-fos. One microliter of fluorogold (5% solution in distilled water; Chemicon, Temecula, CA) was injected stereotaxically into the unilateral paraventricular nucleus (2.0 mm posterior to bregma, 0.4 mm lateral to midline, and 7.6 mm below outer surface of the skull) according to the Paxinos and Watson stereotaxic atlas (22) from a glass micropipette of 10- to 15-μm tip diameter.

After injections, the micropipette was left in place for an additional 10 min to minimize leakage and nonspecific labeling. Experiments were performed 1 wk after the injection. Double staining for fluorogold and c-fos was performed as described previously (4, 5, 38). The 20-μm thick brain sections were stained with c-fos antibody as described above, and stained sections were rinsed in PBST and subsequently incubated with fluorogold antiserum (rabbit polyclonal; Fluorochrome, Denver, CO) diluted 1:1,000 in PBST for 24 h at 4°C. Sections were then incubated in biotinylated anti-rabbit IgG (Vector Laboratories, Burlingame, CA) diluted 1:1,000 for 2 h at room temperature, followed by avidin-biotin complex and diaminobenzidine reactions as described above, except that nickel ammonium sulfate was omitted from the diaminobenzidine solution. Fluorogold was visualized as a brown reaction product in the cytoplasm, whereas c-fos was visualized as a purple reaction product in the nuclei.

RESULTS

We first examined the effects of gastric distension on the release of 5-HT from the ex vivo vascularly and luminally perfused rat stomach (Fig. 1). The basal release of 5-HT into the vasculature (5.47 ± 0.44 ng/ml, n = 3) was significantly increased during the mechanical dilatation of the stomach (8.54 ± 0.33 ng/ml, n = 3), whereas the basal release of 5-HT into the gastric lumen (1.48 ± 0.24 ng/ml, n = 3) was not affected by the gastric distension (1.21 ± 0.68 ng/ml, n = 3) (Fig. 1).

To examine the brain nuclei responsive to gastric distension, we studied c-fos expression in various brain nuclei using immunohistochemical staining. Heavy c-fos expression was observed in the nuclei of AP (Fig. 2A), the medial area of the NTS (Fig. 2A), PVN (Fig. 2B), and SON (Fig. 2C) in animals treated with gastric distension. However, in sham-operated or normal animals, faint or reduced c-fos expression was observed in AP (Fig. 2, D and G), NTS (Fig. 2, D and G), PVN (Fig. 2, E and H), and SON (Fig. 2, F and I). The number of c-fos-positive cells in the AP, NTS, PVN, and SON was significantly higher compared with sham-operated animals (Table 1).

Although there are many central connections to induce c-fos expression in specific brain nuclei, we examined the pathways that most likely transmit sensory information from the stomach to the brain. For this purpose, truncal vagotomy was performed in combination with gastric distention and changes in c-fos expression in the brain were examined. Before the evaluation of truncal vagotomy on c-fos expression induced by gastric distension, the effects of truncal vagotomy itself on c-fos expression in the brain nuclei were examined. No significant increase in the number of c-fos-positive cells was observed in the brain nuclei of vagotomized animals (Fig. 3, A–C, Table 2) compared with normal or sham-operated animals (Fig. 2, D–I). In animals treated with gastric distension combined with truncal vagotomy, the number of c-fos-positive cells in AP, NTS, PVN, and SON induced by gastric distension was significantly reduced by truncal vagotomy (Fig. 3, D–F, Table 2). Because truncal vagotomy incises both afferent and efferent pathways of the vagal nerve, this method appears to be insufficient to examine the involvement of vagal afferent pathways in mediating stomach-to-brain signaling. Capsaicin is an alternative method of blocking the vagal afferent pathways. Results showed that capsaicin treatment did not change the number of c-fos-positive cells in any of the brain nuclei examined (Fig. 4, A–C, Table 2), although the number of c-fos-positive cells in AP, NTS, PVN, and SON induced by gastric distension was significantly reduced by capsaicin treatment (Fig. 4, D–F, Table 2).

Involvement of 5-HT3 receptors in mediating the response of specific brain nuclei to gastric distension was examined. To examine the site of action of the 5-HT3 receptor, the 5-HT3 receptor antagonist granisetron was administered intravenously as well as intracerebroventriculatly. Intracerebroventricular injection of granisetron did not affect the number of c-fos-positive cells in brain nuclei induced by gastric distension (Fig. 5, D–F, Table 3), although intravenous injection of granisetron caused significant reduction of the number of c-fos positive cells induced by gastric distension (Fig. 5, A–C, Table 3).

Retrograde tracer experiments were performed to ascertain whether specific c-fos-expressing neurons in the NTS induced by gastric distension project axons to the PVN. Fluorogold was injected in the PVN before the gastric distension experiment. Brain sections were processed for fluorogold as well as c-fos immunohistochemistry. Results showed that fluorogold and
c-fos were overlapped in neurons in the NTS in which fluoro-gold was stained in the cytoplasm, whereas c-fos was stained in the nuclei (Fig. 6).

**DISCUSSION**

In the present study, we examined the specific brain nuclei responsive for mechanical distension of the stomach and investigated whether 5-HT released from the stomach by gastric distension mediates this response, and the precise location of 5-HT activated 5-HT3 receptors in the afferent pathways. We applied mechanical dilatation with a balloon 15–17 mm in diameter (filled with 3 ml of air) for 2 min. In previous studies (31) addressing brain response to gastric distension, several levels of stimulation had been used. For noxious stimulation to the stomach, high pressure of up to 80 mmHg had been applied. Another study applied gastric distension with a balloon inflated with 9–18 ml of water over 10–60 min (34), which mimics the gastric volume of rats given a liquid diet after overnight starvation (7). Therefore, induced gastric distension in these studies mimicked the peak volume of the stomach after the ingestion of a large meal in fasted refed rats. On the other hand, to examine the gastric distension-induced pyloric relaxation, a smaller volume of gastric distension with a balloon 18 mm in diameter inflated with 2 ml of air was given, which mimics the gastric volume after a normal-sized meal (16). Therefore, the level of gastric distension induced in the present study corresponds to the postprandial dilatation of the stomach after a normal-sized meal. Whereas normal ingestion of a meal activates many additional neural and humoral sensory systems, including signals from the oral cavity, the small intestine and postabsorptive sites, balloon distension is a method to selectively activate gastric mechanoreceptors.

**Table 1. The number of c-fos-positive cells in brain nuclei from animals with gastric distension, sham operation, and normal controls**

<table>
<thead>
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<th>NTS</th>
<th>AP</th>
<th>PVN</th>
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<tbody>
<tr>
<td>Gastric distention</td>
<td>78.0±14.5*</td>
<td>84.3±20.8*</td>
<td>199.3±28.0*</td>
<td>88.3±5.4*</td>
</tr>
<tr>
<td>Sham operation</td>
<td>11.7±1.5</td>
<td>10.3±1.2</td>
<td>13.0±1.0</td>
<td>4.3±0.6</td>
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<tr>
<td>Normal</td>
<td>4.0±1.0</td>
<td>6.0±1.0</td>
<td>11.3±1.2</td>
<td>3.3±0.6</td>
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Values are means ± SD from 3 animals. *P < 0.01 compared with values in sham-operated rats. NTS, nucleus of the solitary tract; AP, area postrema; PVN, paraventricular nucleus; SON, supraoptic nucleus.
Results showed that gastric distension caused an increase in 5-HT release from the ex vivo-perfused rat stomach. 5-HT detected in the vasculature was significantly increased, whereas luminal release of 5-HT was not affected by gastric distension. 5-HT detected in the present study possibly originated from EC cells located in the mucosa of the gastric antrum (37), because EC cells are known to play a role as mechanoreceptors in the digestive tract and release 5-HT in response to luminal pressure (8). It has been demonstrated (8) that EC cells release 5-HT both at the apical site as well as at the basal site of the cells, and basally released 5-HT may enter the lamina propria and then enter the bloodstream. The present results suggest that gastric distension stimulates the release of 5-HT from the EC cells into the lamina propria in which released 5-HT may exert paracrine effects on vagal afferent nerve terminals.

In the present study, c-fos expression in various brain nuclei in response to gastric distension was examined in conscious rat models to avoid the suppressive effects of anesthesia on c-Fos protein synthesis (30). Gastric distension induced c-fos expression in the NTS, AP, PVN, and SON, although no positive reaction was observed in these brain nuclei of sham-operated or normal animals. Dense distribution of c-fos-positive neurons was observed at medial levels of NTS subjacent to AP, and in such subnuclei as the medial, dorsomedial, and commissural. These subnuclei of the NTS have been shown to be strongly exposed to gastric afferent pathways as examined by horseradish peroxidase injection in subdiaphragmatic vagal branches (21). The present results are consistent with previous studies that showed heavy c-fos expression in medial levels of NTS subnuclei induced by gastric distension (34) or nutrient infusion in the digestive tract (23).

To examine the pathways mediating c-fos expression in specific brain nuclei induced by gastric distension, the effects of truncal vagotomy as well as cervical perivagal capsaicin treatment on c-fos expression were examined. Perivagal capsaicin treatment provides more selective blockage of vagal afferent pathways than truncal vagotomy (15), because capsaicin is a neurotoxin that impairs the function of sensory c fibers. The c-fos expression in nuclei of NTS, AP, PVN, and SON was completely blocked by capsaicin treatment as well as by truncal vagotomy, suggesting that vagal afferent pathways may mediate gastric distension-induced c-fos expression in specific brain nuclei in the medulla and hypothalamus. It has been reported that mechanoreceptors in the gastrointestinal tract are located in the serosa and/or muscle, whereas chemo-

![Fig. 3. Effects of vagotomy on gastric distension-induced c-fos expression in the brain nuclei. Vagotomy itself does not induce the c-fos expression in the NTS, AP, PVN, and SON (A–C). Vagotomy blocks the gastric distension-induced c-fos expression in the NTS, AP, PVN, and SON (D–F). Bars, 500 μm.](image-url)

Table 2. Effect of truncal vagotomy or perivagal capsaicin treatment on gastric distention-induced c-fos expression in brain nuclei

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<td>84.3 ± 20.8</td>
<td>199.3 ± 28.0</td>
<td>88.3 ± 5.4</td>
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<tr>
<td>Vagotomy</td>
<td>15.3 ± 2.1</td>
<td>12.0 ± 2.6</td>
<td>13.7 ± 1.5</td>
<td>6.3 ± 1.5</td>
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<tr>
<td>Vagotomy + gastric distention</td>
<td>11.7 ± 1.2*</td>
<td>14.7 ± 3.2*</td>
<td>16.9 ± 1.7*</td>
<td>7.0 ± 2.0*</td>
</tr>
<tr>
<td>Capsaicin</td>
<td>12.3 ± 2.5</td>
<td>9.3 ± 1.5</td>
<td>18.3 ± 4.1</td>
<td>6.6 ± 2.1</td>
</tr>
<tr>
<td>Capsaicin + gastric distention</td>
<td>11.3 ± 2.5*</td>
<td>8.6 ± 3.1*</td>
<td>13.0 ± 4.4*</td>
<td>6.6 ± 1.2*</td>
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Values are means ± SD from 3 animals. *P < 0.01 compared with values in animals with gastric distention.
Fig. 4. Effects of perivagal capsaicin treatment on gastric distension-induced \( c_{-}fos \) expression in the brain nuclei. Perivagal capsaicin treatment itself does not induce the \( c_{-}fos \) expression in the NTS, AP, PVN, and SON (A–C). Perivagal capsaicin treatment blocks the gastric distension-induced \( c_{-}fos \) expression in the NTS, AP, PVN, and SON (D–F). Bars, 500 \( \mu m \).

Fig. 5. Effects of granisetron on gastric distension-induced \( c_{-}fos \) expression in the brain nuclei. Intravenous (iv) injection of granisetron blocks the gastric distension-induced \( c_{-}fos \) expression in the NTS, AP, PVN, and SON (A–C). However, intracerebroventricular (icv) injection of granisetron does not affect the \( c_{-}fos \) expression in these nuclei. Bars, 500 \( \mu m \).
receptors are located in the mucosa (12). Vagal afferent fibers that transmit sensory information from the chemoreceptors are capsaicin-sensitive, whereas those from the mechanoreceptors are capsaicin-insensitive (28). Therefore, vagal afferent informations caused by the gastric distension in the present study seems to be mediated by chemoreceptors in the mucosa rather than mechanoreceptors in the serosa and/or muscle. The candidate of the mediator is 5-HT released from epithelial EC cells. The involvement of splanchnic nerves in gastric distension-induced response can be ruled out, because spinal afferents transit signals of noxious levels of distension; however, vagal afferents respond to more physiological levels of distension as shown in the previous study (12).

Vagal afferent pathways terminate in the NTS as well as the AP, and there are synaptic connections between these nuclei (13, 27). In addition to receiving vagal afferent pathways, AP is known as a circumventricular organ that allows circulating hormones to enter the brain (41). Because c-fos expression in the AP was blocked by perivagal capsaicin treatment in the present study, it seems possible that peripheral stimulation activates AP neurons through vagal afferent pathways but not through circulating hormones stimulated by gastric distension. We can therefore rule out the hypothesis that circulating 5-HT released from the stomach by gastric distension entered the brain and directly activated the AP neurons to induce c-fos expression.

In addition to the peripheral localization of 5-HT3 receptors on vagal afferent terminals in the gastric wall, it has been shown that 5-HT3 receptors are distributed in the brain on vagal afferent terminals within the NTS (11, 24). Therefore, in the present study, we examined whether 5-HT released from the stomach acts on peripheral vagal afferent terminals in the gastric wall or central vagal afferent terminals in the medulla. Results showed that intravenous injection of 5-HT3 receptor antagonist completely blocked c-fos expression in all brain nuclei examined, although intracerebroventricular injection of 5-HT3 receptor antagonist did not affect c-fos expression. These results suggest that 5-HT released from the stomach on gastric distension may act on the peripheral 5-HT3 receptors of vagal afferent terminals in the gastric wall but not on central 5-HT3 receptors in the NTS. Previous studies (1) have shown the involvement of multiple 5-HT receptor subtypes in mediating the gastrointestinal input to NTS and subsequently integrating this information to the dorsal motor nucleus of the vagus, in which 5-HT1A, 1B, and 5-HT2 receptors are involved. Therefore, previous and present data indicate that 5-HT1 and -2 receptors may act at the brain site of vagal afferent pathways, whereas the 5-HT3 receptor may act at the peripheral site of vagal afferent pathways. In the brain site of vagal afferent pathways, not only 5-HT but also other neurotransmitters are involved, because previous studies have shown that gastric distension activates the GLP-1/2 (32)- or catecholamine (34)- containing neurons in the NTS.

In the present study, we used a retrograde tracer experiment to examine whether specific c-fos-expressing neurons in the NTS induced by gastric distension project axons to the PVN. Although interaction between medullary nuclei, such as NTS and AP, and hypothalamic nuclei, such as PVN and SON, have been widely investigated (2, 25, 33), no information has been shown that NTS neurons activated by gastric distension distinctively project axons to the PVN. Results showed that c-fos positive cells in the NTS were simultaneously labeled with fluorogold injected in the PVN, suggesting that NTS neurons activated by gastric distension project axons to the PVN. The previous and present findings indicate that c-fos expression in the hypothalamic nuclei of PVN and SON might be a secondary reaction to efferent projections from the NTS and/or AP in the medulla.

In conclusion, the present study shows that gastric distension made by a balloon placed in the gastric corpus, mimicking postprandial gastric dilatation after a normal-sized meal stimulates 5-HT release from EC cells densely distributed in the gastric antrum. Basolaterally released 5-HT may activate 5-HT3 receptors on vagal afferent nerve terminals in the lamina propria of the stomach. Activation of vagal afferent pathways may induce c-fos expression in neurons in the NTS and AP, which in turn activate PVN and SON in the hypothalamus. The activation of these hypothalamic nuclei by gastric distension may play an important role in the autonomic control of food intake.

**Table 3. Effect of 5-HT3 receptor antagonist granisetron on gastric distention-induced c-fos expression in brain nuclei**

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<td>199.3±28.0</td>
<td>88.3±5.4</td>
</tr>
<tr>
<td>Granisetron (iv) + gastric distention</td>
<td>11.3±3.5*</td>
<td>7.7±3.5*</td>
<td>19.9±6.7*</td>
<td>5.0±2.0*</td>
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<tr>
<td>Granisetron (icv) + gastric distention</td>
<td>71.0±17.1</td>
<td>77.0±8.5</td>
<td>167.0±20.4</td>
<td>77.6±2.1</td>
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Values are means ± SD from 3 animals. *P < 0.01 compared with values in animals with gastric distention.

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Fig. 6. Double staining for c-fos and fluorogold in the NTS from rat treated with retrograde tracer fluorogold injected in the PVN as well as gastric distension. The c-fos-positive neurons in the NTS are also labeled with fluorogold (arrow); the former is stained in the nuclei and the latter is stained in the perikaryon of the neuron. **Right**, a higher magnification of **left**. Bars, 100 µm.
REFERENCES


