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Endogenous neuropeptide-Y depresses the afferent signalling of gastric acid challenge to the mouse brainstem via Y2 and Y4 receptors

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Abstract

Vagal afferents signal gastric acid challenge to the nucleus tractus solitarii (NTS) of the rat brainstem. This study investigated whether NTS neurons in the mouse also respond to gastric acid challenge and whether this chemonociceptive input is modified by neuropeptide-Y (NPY) acting via Y2 or Y4 receptors. The gastric mucosa of female mice was exposed to different concentrations of HCl or saline, excitation of neurons in the NTS visualized by c-Fos immunohistochemistry, gastric emptying deduced from the gastric volume recovery, and gastric lesion formation evaluated by planimetry. Relative to saline, intragastric HCl (0.15-0.35 M) increased the number of c-Fos-expressing cells in the NTS in a concentration-dependent manner, inhibited gastric emptying but failed to cause significant haemorrhagic injury in the stomach. Mice in which the NPY Y2 or Y4 receptor gene had been deleted responded to gastric acid challenge with a significantly higher expression of c-Fos in the NTS, the increases amounting to 39 and 31 %, respectively. The HCl-induced inhibition of gastric emptying was not altered by deletion of the Y2 or Y4 receptor gene. BIIE0246 (0.03 mmol/kg subcutaneously), a Y2 receptor antagonist which does not cross the blood-brain barrier, did not modify the c-Fos response to gastric acid challenge. The Y2 receptor agonist peptide YY-(3-36) (0.1 mg/kg intraperitoneally) likewise failed to alter the gastric HCl-evoked expression of c-Fos in the NTS. BIIE0246, however, prevented the effect of peptide YY-(3-36) to inhibit gastric acid secretion as deduced from measurement of intragastric pH. The current data indicate that gastric challenge with acid concentrations that do not induce overt injury but inhibit gastric emptying is signalled to the mouse NTS. Endogenous NPY acting via Y2 and Y4 receptors depresses the afferent input to the NTS by a presumably central site of action.

Keywords

Nucleus of the solitary tract; neuropeptide-Y Y2 receptor gene knockout; Y4 receptor gene knockout; Y2 receptor antagonist BIIE0246; c-Fos expression; gastric emptying

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Introduction

Hydrochloric acid (HCl)-induced nociception in the rat stomach is mediated by vagal afferent neurons, since the visceromotor pain response to intragastric (IG) HCl administration is abolished by vagotomy (Lamb et al., 2003). This finding is consistent with the observation that gastric HCl challenge is signalled to the nucleus tractus solitarii (NTS) of the rat brainstem as shown by expression of the immediate early gene c-fos (Schuligoi et al., 1998; Danzer et al., 2004a). For the treatment of gastric pain it is hence relevant to know which endogenous factors control vagal afferent input to the NTS. It has been found that glutamate and tachykinins participate in the transmission between gastric vagal afferents and NTS neurons (Jocic et al., 2001) and morphine dampens gastric HCl-evoked c-fos transcription in the NTS (Schuligoi et al., 1998). Neuropeptide (NPY) may also modify vagal afferent signalling, because this neuropeptide (McLean et al., 1997; Lawrence et al., 1998; Thiele et al., 2000) and NPY receptors of the Y1, Y2 and Y4 type (Ergene et al., 1993; Gustafson et al., 1997; Larsen and Kristensen, 1997; McLean et al., 1997; Zhang et al., 1997; Dumont et al., 1998; Parker and Herzog, 1999; Glass et al., 2002; Kopp et al., 2002; Koda et al., 2005) are expressed both by certain vagal afferents and NTS neurons.

There is evidence that NPY modulates visceral afferent input to the NTS via activation of presynaptic Y2 receptors (Ergene et al., 1993) and stimulation of NPY receptors in the rat brainstem influences gastric motility, acid secretion and mucosal integrity (Humphreys et al., 1992; Chen et al., 1997; Yang et al., 1998; Yang et al., 1999; Yang, 2002). Since the NPY receptors prevailing in the NTS are of the Y2 and Y4 type (Gustafson et al., 1997; Larsen and Kristensen, 1997; Dumont et al., 1998; Parker and Herzog, 1999), we hypothesized that endogenous NPY acting via Y2 or Y4 receptors controls the gastric HCl-evoked input to the NTS. As this question was addressed with Y2 and Y4 receptor knockout mice, the first specific aim was to establish and characterize the experimental model of NTS activation by gastric HCl challenge in the mouse. The effect of HCl on gastric emptying and injury was also assessed, given that in the rat excess IG HCl blocks gastric emptying (Holzer et al., 2003).

The second specific aim was to compare the effect of gastric HCl challenge on c-Fos expression in the NTS, gastric emptying and gastric lesion formation in control, Y2 and Y4 knockout mice. In addition, we examined whether the neurochemical code of the NTS neurons that respond to gastric HCl challenge with c-Fos expression (Danzer et al., 2004b) differs between control and Y2 receptor knockout mice in terms of the codistribution of c-Fos with NPY and the excitatory amino acid transporter-1 (EAAC1, a marker of glutamatergic neurons). Finally, we investigated whether systemic administration of the Y2 receptor agonist peptide YY-(3-36), abbreviated PYY-(3-36) (Challis et al., 2003), and the Y2 receptor antagonist BIIE0246 modify the gastric HCl-evoked c-Fos response in the NTS. The activity of BIIE0246 as a Y2 receptor antagonist was tested by its ability to prevent the inhibitory effect of PYY-(3-36) on gastric acid secretion.

Experimental procedures

Experimental animals

This study was carried out with female mice which were housed in groups of 3-4 per cage under controlled temperature (21 °C) and a 12 h light/dark cycle (lights on at 6:00 AM, lights off at 6:00 PM). All experiments were approved by an ethical committee at the Federal Ministry of Education, Science and Culture of the Republic of Austria and conducted according to the Directive of the European Communities Council of 24 November 1986 (86/609/EEC). The experiments were designed in such a way that the number of animals used and their suffering was minimized.

Studies 1, 2, 3 and 7 and part of study 6 were performed with outbred mice (strain Him:OF1, Abteilung Labortierkunde und -genetik, Medical University of Vienna, Himberg, Austria) weighing 17-23 g. For studies 4 and 5 and part of study 6, germline Y2 and Y4 receptor knockout (Y2-/- and Y4-/-) mice and non-induced conditional Y2 and Y4 receptor knockout (FY2 and FY4) mice (Department of Pharmacology, Medical University of Innsbruck, Austria) weighing 16-24 g were used. The generation of Y2-/-, Y4-/-, FY2 and FY4 mice has been described previously (Sainsbury et al., 2002a; Sainsbury et al., 2002b). Germline Y2-/- and Y4-/- mice were generated from the same founders on the same mixed C57BL/6–129SvJ background as the conditional FY2 and FY4 knockout mice. Germline Y2-/- and Y4-/- knockout mice were obtained by crossing chimeric mice carrying a Y2 floxed gene ($Y2^{lox/lox}$) or a Y4 floxed gene ($Y4^{lox/lox}$), respectively, with oozyte-specific Cre recombinase-expressing C57BL/6 mice (Sainsbury et al., 2002a; Sainsbury et al., 2002b). Non-induced conditional FY2 and FY4 knockout mice were used as controls in all experiments and termed control mice throughout the paper. As demonstrated before, these non-induced conditional $Y2^{lox/lox}$ and $Y4^{lox/lox}$ mice do not differ from wild-type mice, as the level of expression of Y2 and Y4 receptors is not influenced by the introduction of the loxP sites (Sainsbury et al., 2002a; Sainsbury et al., 2002b). The deletion or presence of Y2 and Y4 receptors in the germline and non-induced conditional knockout mice was verified by receptor autoradiography using [^{125}I]PYY_{3-36} and [^{125}I]PP, respectively, and in situ hybridization (Tschenett et al., 2003) as well as by polymerase chain reaction using oligonucleotide primers recognizing DNA sequences adjacent to the loxP sites flanking the deleted or residing Y2 and Y4 receptor gene (Sainsbury et al., 2002a; Sainsbury et al., 2002b).

Experimental protocols

In all studies, the mice were fasted for 20 h to ensure that the stomach was empty, but had free access to water, before HCl or physiological saline (0.15 M NaCl) was administered IG at a volume of 0.01 or 0.02 ml/g through a soft infant feeding tube (outer diameter 1.5 mm; Rüsch, Montevideo, Uruguay). After the IG treatment the animals were no longer allowed to drink until tissue collection 0.5, 1 or 2 h later. For this purpose, the mice were euthanized by intraperitoneal injection of an overdose of pentobarbital (100 mg/kg).

Seven specific studies were carried out. The aim of study 1 which involved 26 Him:OF1 mice was to investigate how the c-Fos expression in the NTS after IG treatment with HCl

depends on the time of examination post-treatment and on the volume of the IG HCl bolus. To this end, two groups of mice were treated IG with HCl (0.25 M; 0.02 ml/g) and the expression of c-Fos in the NTS examined 1 and 2 h later, respectively. In two other groups of Him:OF1 mice, HCl (0.25 M) was administered IG at volumes of 0.01 ml /g and 0.02 ml/g, respectively, and c-Fos in the NTS visualized 2 h post-treatment. In study 2, which was carried out with 24 Him:OF1 mice, the relationship between the IG concentration of HCl and the c-Fos expression in the NTS was investigated. For this purpose, the gastric mucosa was exposed to saline (0.15 M NaCl; 0.02 ml/g) or different concentrations of HCl (0.15, 0.25, 0.35 M; 0.02 ml/g) and the expression of c-Fos in the NTS determined 2 h post-treatment. Study 3 involved 66 Him:OF1 mice and addressed the relationship between IG concentration of HCl, duration of exposure to HCl, IG pH, gastric volume recovery (an indirect measure of gastric emptying) and gastric mucosal lesion formation. Specifically, mice were treated IG with saline (0.15 M NaCl; 0.02 ml/g) or different concentrations of HCl (0.15, 0.25, 0.35 M; 0.02 ml/g) 30, 60 or 120 min before IG pH, gastric volume recovery and gastric injury were quantified.

Study 4 was carried out with 59 mice to test whether the c-Fos expression in the NTS evoked by IG HCl treatment differs in FY2, FY4, Y2-/- and Y4-/- mice. For this purpose, saline (0.15 M NaCl; 0.02 ml/g) or HCl (0.25 M; 0.02 ml/g) was administered IG 2 h before the expression of c-Fos was determined in the NTS. In study 5, which involved 27 mice, analogous experiments were carried out to examine gastric volume recovery and gastric lesion formation in FY2, FY4, Y2-/- and Y4-/- mice 30 min after IG treatment with HCl (0.25 M; 0.02 ml/g).

Study 6 was performed with 24 Him:OF1 mice and 17 control (FY2 and FY4) mice, in which the effect of the Y2 receptor agonist PYY-(3-36) (Bachem, Basel, Switzerland) and the Y2 receptor antagonist BIIE0246 (Boehringer Ingelheim, Biberach, Germany) on the gastric HCl-evoked expression of c-Fos in the NTS was investigated. In the Him:OF1 mice BIIE0246 (0.03 mmol/kg; Chu et al., 2003) or its vehicle (30 % polyethylene glycol 200 in distilled water) was injected subcutaneously 10 min before IG treatment with HCl (0.25 M; 0.02 ml/g). PYY-(3-36) (0.1 mg/kg; Challis et al., 2003) or its vehicle (saline) was injected intraperitoneally 5 min before IG treatment with HCl. The expression of c-Fos in the NTS was analyzed 60 min post-HCl. The control (FY2 and FY4) mice were injected subcutaneously with BIIE0246 (0.03mmol/kg) or its vehicle 10 min before IG treatment with HCl (0.25 M; 0.02 ml/g), and the NTS was subjected to immunohistochemistry 2 h later. In study 7, 24 Him:OF1 mice were used to examine the effect of PYY-(3-36) on IG pH and gastric volume recovery in the absence and presence of BIIE0246. To this end, BIIE0246 (0.03 mmol/kg) or its vehicle was injected subcutaneously 10 min before IG treatment with saline (0.02 ml/g), while PYY-(3-36) (0.1 mg/kg) or its vehicle was injected intraperitoneally 5 min before IG treatment with saline. IG pH and gastric volume recovery were determined 30 min post-saline.

Immunohistochemistry

Following euthanasia with pentobarbital, the mice were transcardially perfused with 0.1 M phosphate-buffered saline (PBS) of pH 7.4 (25 ml), followed by 4 % buffered

paraformaldehyde (45 ml). The brainstems 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. Serial coronal sections of 40 μm thickness were cut from the brainstem over the whole length of the area postrema (Paxinos and Franklin, 2001) with a cryostat. Only every second section was used. Immunohistochemistry was performed with free-floating sections which first were washed three times in 0.1 M PBS and then incubated in 0.3 % H_2O_2 for 30 min. After three washes (each for 10 min in PBS + 0.3 % Triton X 100), tissues were 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, Santa Cruz Biotech, Santa Cruz, California, USA) for 48 h at 4 °C. Sections were washed again three times in PBS + 0.3 % Triton X 100 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, California, USA). After three washes in PBS, they were incubated for 1 h in avidin-biotin complex (Vectastain Elite Kit). Tissues were rinsed afterwards and developed with 3,3-diaminobenzidine (DAB) substrate (Vectastain Elite Kit) intensified with nickel sulphate for 135 s. Tissues were subsequently mounted on gelatin-covered slides, air-dried and dehydrated through an alcohol series (50 - 70 - 100 %). Slides were coverslipped with Entellan (Merck, Darmstadt, Germany). To control for the specificity of the anti-c-Fos antibody signal, a c-Fos blocking peptide (Santa Cruz Biotech) was added to the primary antibody dilution.

For double-labelling immunohistochemistry, the sections were first processed for c-Fos immunohistochemistry as described above and, after their development in nickel sulphate, washed for 1 h in PBS. Following three other washes (each for 10 min in PBS + 0.3 % Triton X 100) the 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 second primary antibody for 48 h at 4 °C. The primary antibodies used were rabbit polyclonal anti-NPY (1:4,000, Pensinsula Laboratories, San Carlos, California, USA) and rabbit polyclonal anti-EAAC1 (1:1,000, Biotrend, Cologne, Germany). After this second labelling the sections were washed three times in PBS + 0.3 % Triton X 100 and then incubated for 1.5 h in a solution containing the biotinylated secondary antibody (goat anti-rabbit IgG; Vectastain Elite Kit). Following three washes in PBS, the tissues were incubated for 1 h in avidin-biotin complex, rinsed afterwards and developed with either DAB substrate (Vectastain Elite Kit) or 3-amino-9-ethylcarbazole (AEC, AEC Peroxidase Substrate Kit, Vector Laboratories). The DAB substrate (development time: 2 min) was used for visualizing EAAC1, whereas AEC (development time: 2 min) was used to visualize NPY. DAB-treated sections were mounted on gelatin-covered slides, air-dried, dehydrated through an alcohol series (50 - 70 - 100%) and coverslipped with Entellan. AEC-treated sections were mounted on gelatin-covered slides and coverslipped with AquaMount. Negative controls were obtained by processing the sections without the primary anti-EAAC1 or anti-NPY antibody.

The immunohistochemically processed brainstem sections were examined with a light microscope (Axiophot, Zeiss, Oberkochen, Germany) coupled to a computerized image analysis system (MCID-M2, version 3.0, Rev 1.1, Imaging Research Inc., Brock University,

St. Catharines, Ontario, Canada). The sections were coded such that the examiner did not know which treatment group or genotype they came from. Eight sections per animal were analyzed and all c-Fos-positive cells counted on one side of the NTS. In order to avoid that the same cells were counted twice, only every second section was taken for analysis. All counts in each section of each animal were averaged to give the number of c-Fos-positive cells in the NTS of that animal. These average values from each animal were then used to calculate the mean number of c-Fos-positive cells in the NTS of each experimental group. In the double-labelling experiments the number of c-Fos-positive neurons counted unilaterally in the NTS was set as 100 %. All cells double-stained for another antigen were expressed as a percentage of the c-Fos-positive cell group.

Gastric fluid recovery, IG pH and gastric lesion formation

After the animals had been euthanized with an overdose of pentobarbital, the abdomen was quickly opened by a midline incision, and the stomach clamped at the lower oesophageal sphincter and pylorus. Then the fluid-filled stomach was weighed, opened along the greater curvature, blotted dry on tissue paper and re-weighed to determine the volume of the fluid present in the stomach. Gastric fluid recovery was calculated by expressing the weight of the fluid present in the stomach as a percentage of the weight of the fluid administered into the stomach. The pH of the gastric contents was determined with a pH meter that was fitted with a Micro Line pH electrode (ThermoOrion, New Hyde Park, NY, USA) and calibrated with standard buffers of pH 1, 4 and 7. To determine gastric lesion formation, the stomach was pinned flat on a silicon elastomer-coated plate and covered with PBS. The stomach was photographed, the picture transferred to a personal computer, and gross gastric injury assessed by computerized planimetry. The mucosal area covered by visible haemorrhagic damage was expressed as a percentage of the total area of the glandular mucosa.

Statistics

Statistical evaluation of the results was performed on SigmaStat (SPSS, Chicago, Illinois, USA) with Student's *t*-test or one way analysis of variance (ANOVA) followed by the Student-Neumann-Keuls test. All data are presented as means ± SEM, *n* referring to the number of mice in the respective group. Probability values of $P < 0.05$ were regarded as significant.

Results

Relationship between duration of gastric HCl exposure, gastric HCl volume, c-Fos expression in the NTS, gastric fluid recovery, IG pH and gastric damage in Him:OF1 mice (studies 1 and 3)

Compared with saline (0.15 M NaCl), IG administration of HCl (0.25 M; 0.02 ml/g) caused many neurons in the NTS and some neurons in the area postrema to express c-Fos (Figure 1A and B). The number of c-Fos-positive cells counted in the unilateral NTS 1 and 2 h after IG treatment with HCl (0.25 M; 0.02 ml/g) was 111±9.3 (n=7) and 103±6.8 (n=7), respectively. These counts did not statistically differ from each other. Unlike the exposure time, the volume of the IG administered HCl bolus had a significant influence on the expression of c-Fos in the NTS as determined 2 h post-treatment. When HCl (0.25 M) was

administered in a volume of 0.01 ml/g, only 75±7.4 (n=6) c-Fos-positive cells were counted in the unilateral NTS, compared with 103±6.8 (n=6) cells expressing c-Fos after administration of HCl (0.25 M) in a volume of 0.02 ml/g ($P < 0.05$).

The gastric fluid recovery and gastric damage measured after IG administration of HCl did not significantly depend on the gastric HCl exposure time and the volume of the IG administered HCl bolus. While 30 and 120 min after IG administration of saline (0.02 ml/g) only 24 and 21 % of the volume administered IG were recovered from the stomach, 30, 60 and 120 min after IG administration of HCl (0.25 M) 105, 98 and 91 % of the administered fluid were regained, respectively (Table 1). These rates of gastric volume recovery did not significantly differ from each other. There was likewise no significant difference when HCl (0.25 M) was administered IG in a volume of 0.01 or 0.02 ml/g, as the gastric fluid recovery 30 min post-treatment was 120±17.1 % (n=4) and 102±12.1 % (n=4), respectively.

Before IG administration, the pH of saline (0.15 M) was 5.07 and the pH of 0.25 M HCl was 0.70. Following administration of saline into the stomach, IG pH fell over time to less than 1.5 as recorded 120 min post-NaCl (Table 1). In contrast, following administration of HCl to the stomach, IG pH gradually rose to a value beyond 1.2 as measured 120 min post-HCl (Table 1).

The macroscopic haemorrhagic injury in the stomach following IG administration of saline or HCl was minor as it covered 0.52 % or less of the area of the glandular mucosa and consisted of petechiae and some small streaks of haemorrhage. As can be seen in Table 1, the damage induced by IG administration of HCl (0.25 M; 0.02 ml/g) was nominally largest 30 min post-treatment and subsequently diminished so that 120 min post-HCl the damage was less than that seen 30 min post-saline. However, at none of these time points was there any significant difference in the extent of damage induced by saline or HCl. In view of these observations, gastric volume recovery and lesion formation was determined 30 min post-treatment in all following experiments.

Relationship between gastric HCl concentration, c-Fos expression in the NTS, gastric volume recovery and gastric damage in Him:OF1 mice (studies 2 and 3)

Relative to saline (0.02 ml/g), IG administration of increasing concentrations of HCl (0.15, 0.25, 0.35 M; 0.02 ml/g) enhanced the number of c-Fos--expressing cells in the NTS in a HCl concentration-dependent manner (Figure 2A) as determined 2 h later. The lowest concentration of HCl tested (0.15 M) augmented the number of c-Fos-positive cells by a factor of 1.7 ($P < 0.05$), while 0.25 M HCl elevated it by a factor of 2.7 ($P < 0.05$), and the highest concentration of HCl tested (0.35 M) enhanced it by a factor of 4.1 ($P < 0.01$).

After IG administration of saline (0.02 ml/g), only 15 % of the IG administered fluid volume was recovered from the stomach 30 min post-treatment, whereas after IG administration of 0.15 M HCl (0.02 ml/g) the gastric volume recovery rate was 104 % (Figure 2B). IG administration of 0.25 M and 0.35 M HCl (0.02 ml/g) further increased the gastric volume recovery rate to 117 % and 135 %, respectively (Figure 2B). When the gastric mucosa was examined for haemorrhagic lesions 30 min post-treatment, there was a trend towards increased damage as the concentration of IG administered HCl was increased from 0.15 to

0.35 M (Figure 2C). However, even the extent of damage caused by 0.35 M HCl (0.4 %) was not significantly different from that seen in mice treated with IG saline (Figure 2C).

Gastric HCl-induced c-Fos expression in the NTS, gastric volume recovery and gastric damage in control, Y2-/- and Y4-/- mice (studies 4 and 5)

Having established the experimental model in the first part of this study, we addressed the implication of Y2 and Y4 receptors in the HCl-evoked stomach-NTS communication in the second part of the study. The gastric mucosa of control, Y2-/- and Y4-/- mice was exposed to saline or 0.25 M HCl (0.02 ml/g). Two hours later, neurons in the NTS staining for c-Fos, NPY and EAAC1 were counted. Following IG treatment with saline there was no significant difference in the number of c-Fos-positive neurons in the NTS of control (FY2 and FY4), Y2-/- and Y4-/- mice (Figure 3A). IG challenge with HCl increased the number of c-Fos-positive cells in the NTS of FY2 and FY4 mice to an identical degree so that the two groups were combined into one control group (Figure 3A). Y2-/- and Y4-/- mice responded to the IG HCl stimulus with a significantly larger expression of c-Fos in the NTS than control mice, the increases in the number of c-Fos-positive cells amounting to 39 and 31 %, respectively (Figures 1C,D and 3A).

The co-distribution of c-Fos with NPY and EAAC1 in NTS neurons of Y_2 -/- mice was not different from that in control mice. As can be seen in Figure 4A, only about 4 neurons in the unilateral NTS of control mice contained NPY whereas some 46 neurons contained EAAC1, and this distribution was similar in Y2-/- mice. When expressed relative to the c-Fos-positive cells, some 60 % of the HCl-activated cells in the NTS stained for EAAC1 while only about 2 % of the neurons were co-labelled for c-Fos and NPY (Figure 4B).

The gastric fluid recovery and formation of gastric haemorrhagic damage as examined 30 min after IG treatment with HCl (0.25 M, 0.02 ml/g) did not differ between Y2-/-, Y4-/- and their respective control mice (Figure 3B,C). We noted, however, that the gastric volume recovery in Y4-/- mice and their respective control (FY4) mice was higher than in Y2-/- and their respective control (F2Y) mice (Figure 3B).

Effect of PYY-(3-36) and BIIE0246 on the gastric HCl-induced c-Fos expression in the NTS of Him:OF1 and control mice (study 6)

Him:OF1 mice and control (FY2) mice were pretreated with the Y2 receptor antagonist BIIE0246 (0.03 mmol/kg subcutaneously), the Y2 receptor agonist PYY-(3-36) (0.1 mg/kg intraperitoneally) or the respective vehicle 10 and 5 min, respectively, before the gastric mucosa was exposed to 0.25 M HCl (0.02 ml/g). One hour (Him:OF1 mice) or two hours (control mice) later the c-Fos expression in the NTS was quantified by immunocytochemistry. Neither PYY-(3-36) nor BIIE0246, alone or in combination, had any significant effect on the gastric HCl-evoked c-Fos expression in the NTS of Him:OF1 mice (Table 2). Similarly, BIIE0246 failed to alter the effect of gastric HCl challenge to induce c-Fos in the NTS of control (FY2 and FY4) mice (Table 2).

Effect of PYY-(3-36) and BIIE0246 on gastric volume recovery and IG pH in Him:OF1 mice (study 7)

BIIE0246 (0.03 mmol/kg subcutaneously), PYY-(3-36) (0.1 mg/kg intraperitoneally) or the respective vehicle was administered 10 and 5 min, respectively, before the gastric mucosa was exposed to 0.15 M NaCl (0.02 ml/g). Gastric volume recovery and IG pH were determined 30 min after IG treatment with saline. As shown in Figure 5A, none of the treatments had any significant influence on the gastric volume recovery. In contrast, PYY-(3-36) inhibited gastric acid secretion as revealed by a marked rise of IG pH (Figure 5B). This effect of PYY-(3-36) was prevented by BIIE0246 which per se had no influence on IG pH (Figure 5B).

Discussion

The current study has shown that, firstly, challenge of the mouse gastric mucosa with excess HCl signals NTS neurons to express c-Fos and, secondly, endogenous NPY dampens the afferent input from the acid-threatened stomach to the NTS because knockout of the Y2 or Y4 receptor gene enhances the c-Fos response to IG HCl. Excitation of the NTS was visualized by immunocytochemistry for the protein product of the immediate-early gene c-fos, which has been established as a standard tool in functional neuroanatomy to delineate the stimulus-evoked activation of neurons (Hughes and Dragunow, 1995; Herdegen and Leah, 1998). Since in the rat the NTS is the major central projection area of vagal afferents (Norgren and Smith, 1988; Altschuler et al., 1989), it is inferred that this population of sensory neurons likewise carries HCl-evoked signals from the mouse stomach to the brainstem. The effect of gastric HCl challenge to evoke c-fos gene transcription in the rat NTS is in fact abrogated by chronic bilateral vagotomy (Schuligoi et al., 1998; Michl et al., 2001).

In order to appropriately evaluate the effects of receptor gene knockouts and receptor antagonists on the stomach-NTS axis, we first analyzed how activation of the mouse NTS is related to IG HCl concentration, IG HCl volume, time of gastric exposure to HCl, gastric emptying and gastric lesion formation. As the formation of c-Fos protein reaches its maximum between 1 and 3 h post-stimulus (Traub et al., 1996; Kovacs, 1998), the induction of c-Fos in the rat NTS is typically measured 2 h after gastric stimulation (Traub et al., 1996; Danzer et al., 2004a). Because the metabolic activity of the mouse is higher than that of the rat, we reasoned that the HCl-induced expression of c-Fos in the mouse NTS may peak after a shorter latency than in the rat NTS. Although the number of c-Fos-positive cells appeared to reach a maximum 1 h after gastric acid challenge, the induction of c-Fos was not significantly smaller 2 h post-stimulus, which validated our approach to visualize c-Fos immunoreactivity 2 h after gastric HCl challenge in all other experiments.

The magnitude of the c-Fos response in the NTS depended both on the volume and concentration of the IG HCl bolus. Thus, the expression of c-Fos in the NTS increased as the concentration of IG administered HCl was raised to 0.15, 0.25 and 0.35 M. The observation that the volume of 0.02 ml/g HCl (0.25 M) caused more NTS neurons to express c-Fos than the volume of 0.01 ml/g is most probably due to multiple factors. We assume that, apart from the concentration of HCl, also the total load of HCl and distension of the stomach

(Monroe et al., 2004) determine the afferent input to the NTS. The relationship between IG HCl concentration and c-Fos induction in the mouse NTS compares well with that seen in the rat NTS (Danzer et al., 2004a), whereas the absolute number of c-Fos-expressing cells in the mouse NTS is higher than in the rat NTS. Further analysis of this species difference, which was seen both after IG administration of NaCl and HCl, was beyond the scope of the study. One explanation could be that the mouse NTS is more susceptible to the stress of gastric intubation than the rat NTS. Another explanation could be related to the high rate of basal acid secretion in the mouse stomach, given that an IG pH of 1.4 is reached 120 min after IG administration of saline (pH=5.07). As previously found in the rat, basal acid secretion makes some contribution to the expression of c-Fos in the NTS (Danzer et al., 2004a).

The IG HCl concentrations that induced c-Fos in the NTS were supraphysiological (0.15 M and more) but did not cause appreciable macroscopic injury of the mouse gastric mucosa. It has previously been found that the extent of HCl-induced macrosopic injury correlates with the degree of histological surface ablation in the rat stomach (Schuligoi et al., 1998). In analogy with studies in the rat (Schuligoi et al., 1998; Danzer et al., 2004a) we conclude, therefore, that the magnitude of the c-Fos expression in the NTS is determined by the IG HCl concentration rather than by the extent of gastric mucosal damage. It is unlikely that the hyperosmolarity of the HCl stimulus is a contributory factor, given that the number of c-fos mRNA-positive cells in the rat NTS is the same after IG administration of 0.15 and 0.5 M NaCl (Michl et al., 2001). It follows that an increase of the H^+ gradient across the gastric mucosal barrier is sufficient to drive enough H^+ ions from the lumen into the lamina propria of the gastric mucosa where they can excite vagal afferents either directly (Clarke and Davidson, 1978; Hillsley and Grundy, 1998) or indirectly via neuroactive factors released in the tissue. Our experimental paradigm could thus reflect a pathophysiological model in which backdiffusion of luminal H^+ ions excites sensory nerve fibres in the gastric mucosa (Danzer et al., 2004a).

The afferent signalling of HCl challenge is determined not only by the concentration of the noxious chemical but also by the duration of its presence in the gastric lumen. As shown before in the rat (Holzer et al., 2003; Danzer et al., 2004a), 90 % or more of the IG administered volume of HCl (0.15 - 0.35 M) was recovered from the mouse stomach 30 - 120 min later, compared with a 15 - 24 % recovery rate when NaCl was administered IG (Table 1, Figure 2B). This finding indicates that HCl caused a sustained inhibition of gastric emptying (Osinski et al., 2002; Holzer et al., 2003). The underlying motor effects are mediated by neural reflexes that are initiated both in the stomach and duodenum (Cervero and McRitchie, 1982; Forster et al., 1990; Raybould and Hölzer, 1993; Lu and Owyang, 1999; Holzer et al., 2003). Since the IG retention of HCl persisted for 120 min, the time of tissue collection for c-Fos immunocytochemistry, we infer that IG HCl induces c-Fos in the NTS primarily by excitation of vagal afferents in the stomach rather than in the proximal small intestine. The observation that the gastric volume recovery following IG administration of 0.25 - 0.35 M HCl sometimes exceeded 120 % (Figure 2B) is indicative of HCl-induced fluid secretion (Holzer et al., 2003), a protective mechanism whereby noxious chemicals in the gastric lumen are diluted (Hatakeyama et al., 1995). This dilution process is also reflected by the course of IG pH which after IG administration of HCl (pH=0.7) rose to

1.23 within 2 h. Although the distension caused by fluid secretion in the presence of a closed pylorus may boost the HCl-evoked afferent input to the NTS, particularly if administered in a volume of 0.02 ml/g, we hypothesize that acid backdiffusion, and not distension, is primarily responsible for the HCl-induced expression of c-Fos in the NTS. In particular, we think that the initial difference in the pH of the HCl and NaCl solutions administered into the stomach determines the HCl-evoked induction of c-Fos in the brainstem.

The second aim of the present study was to investigate whether endogenous NPY modifies the chemonociceptive stomach-brainstem axis via Y_2 and Y_4 receptors which are the NPY receptors prevailing in the rat NTS (Gustafson et al., 1997; Larsen and Kristensen, 1997; Dumont et al., 1998; Parker and Herzog, 1999). It was revealed that deletion of the Y2 or Y4 receptor gene led to a significant increase in the IG HCl-induced expression of c-Fos in the NTS. In interpreting these findings it needs to be considered that NPY as well as Y1, Y2 and Y4 receptors are expressed by some vagal afferents and NTS neurons of the rat (Barraco et al., 1991; Kummer et al., 1993; Ghilardi et al., 1994; Gustafson et al., 1997; Larsen and Kristensen, 1997; McLean et al., 1997; Zhang et al., 1997; Dumont et al., 1998; Lawrence et al., 1998; Parker and Herzog, 1999; Thiele et al., 2000; Glass et al., 2002; Kopp et al., 2002; Koda et al., 2005), which implies that NPY may be both a transmitter and modulator of the communication between vagal afferents and their projection neurons in the NTS.

The HCl-evoked inhibition of gastric emptying and formation of haemorrhagic gastric injury was not altered by Y2 or Y4 receptor gene knockout. It is also unlikely that the acidity of the gastric juice following IG administration of HCl (pH= 0.70) was altered by the deletion of the Y2 and Y4 receptor genes. Although NPY and related peptides are known to modify gastric acid output (Humphreys et al., 1992; Yang et al., 1998; Yang, 2002), gastric acid secretion is completely suppressed at an intraluminal pH of 0.70 by negative feedback mechanisms that have been characterized in rats and humans (Walsh et al., 1975; Lippe et al., 1989; Holzer et al., 1991; Manela et al., 1995). Similar mechanisms appear to operate in the murine stomach, given that 2 h after IG administration of HCl (pH=0.70) and NaCl (pH=5.07) IG pH converged at a range of 1.23 - 1.41 (Table 1).

In view of these considerations we conclude that the increased c-Fos response in the NTS of Y2-/- and Y4-/- mice is due to a functional change in the brainstem. As regards Y2 receptors, this inference is supported by the failure of BIIE0246, a Y2 receptor antagonist which does not cross the blood-brain barrier (H.N. Doods, personal communication), to reproduce the effect of Y2 receptor gene deletion on the stomach-NTS axis. We consider this conclusion valid because the dose of BIIE0246 used here (0.03 mmol/kg) was reported to be effective in blocking peripheral Y2 receptors (Chu et al., 2003) and in the present study was found to prevent the Y2 receptor agonist PYY-(3-36) from inhibiting gastric acid secretion (Figure 5B). We hypothesize, therefore, that the IG HCl-evoked expression of c-Fos in the NTS is enhanced because deletion of Y2 or Y4 receptors removes either a presynaptic inhibitory action of endogenous NPY on the central terminals of gastric vagal afferents or a postsynaptic inhibitory action on the c-Fos-expressing neurons in the NTS. As a consequence, the NTS response to input from the acid-threatened stomach is exaggerated.

If Y2 receptor deletion boosts gastric HCl-evoked input to the NTS, we reasoned that Y2 receptor stimulation should have the opposite effect. This conjecture was tested with the Y2 receptor agonist PYY-(3-36) which has been found to affect food intake in rats and mice by a peripheral and central site of action (Batterham et al., 2002; Challis et al., 2003; Nonaka et al., 2003; Koda et al., 2005). At an intraperitoneal dose (0.1 mg/kg) shown to suppress food intake (Challis et al., 2003) and gastric acid secretion (present study), PYY-(3-36) failed to alter the c-Fos response of the NTS to gastric acid challenge. We interprete this finding as indicative of the failure of peripherally administered PYY-(3-36) to access the NTS at a concentration sufficient to modify the HCl-evoked expression of c-Fos. Although PYY-(3-36) has been reported to permeate the blood-brain barrier to a certain extent (Nonaka et al., 2003), it has recently been suggested that it influences brain functions rather by a peripheral site of action (Koda et al., 2005). We concur with this opinion and conclude that our negative finding with PYY-(3-36) indicates that the Y2 receptors suppressing gastric HCl-induced excitation of NTS neurons are located within the brainstem.

Although the current data do not allow to differentiate between a pre- or postsynaptic inhibitory action of endogenous NPY in the NTS, we hypothesize that the Y2 receptor-mediated effect of NPY arises from a presynaptic site of action. Y2 receptors are expressed by vagal afferent neurons (Zhang et al., 1997; Koda et al., 2005), and there is analogous evidence that NPY inhibits cardiorespiratory and cardiovascular regulation by depressing visceral afferent input through activation of presynaptic Y2 receptors in the subpostremal NTS of the rat (Barraco et al., 1991; Ergene et al., 1993; Smith-White et al., 2002a). Furthermore, NPY has been found to inhibit excitatory transmission in other brain regions including the dorsal motor nucleus of the vagus (Browning and Travagli, 2003), substantia gelatinosa of the spinal cord (Moran et al., 2004) and hippocampus (El-Bahh et al., 2002) via a presynaptic site of action involving primarily Y2 receptors. Thirdly, the chemical coding of the c-Fos-expressing neurons in the NTS did not change in Y2-/- mice inasmuch as the relative coexpression of EAAC1, a marker of glutamatergic neurons, and NPY remained unaltered. This finding suggests that no compensatory or reactive alterations in the neurochemistry of the postsynaptic NTS neurons receiving input from the acid-threatened stomach occurred in response to Y2 receptor gene knockout, which may be taken as indirect evidence for a presynaptic location of the Y2 receptors under study. The relative abundance of EAAC1 is consistent with glutamate being the major excitatory transmitter of NTS neurons (Lawrence and Jarrott, 1996; Maley, 1996).

The finding that Y4 receptor gene knockout enhanced the gastric HCl-induced stimulation of NTS neurons points to a novel role of this NPY receptor type in the control of stomach-brainstem communication. Being highly expressed in the NTS (Larsen and Kristensen, 1997; Parker and Herzog, 1999), Y4 receptors are thought to be of relevance to autonomic nervous system balance and cardiovascular function (Smith-White et al., 2002b). The current data may hence reflect either a direct interference of Y4 receptor activation in the NTS with chemonociceptive afferent input from the stomach or an indirect consequence of the implication of Y4 receptors in autonomic regulation.

In summary, the present findings indicate that HCl challenge of the mouse stomach excites vagal afferent neurons that signal to the NTS. Besides glutamatergic neurons, this afferent

input stimulates central NPY neurons which have terminals in the NTS (Lawrence and Jarrott, 1996; Maley, 1996). Endogenous NPY released from these neurons inhibits transmission between vagal afferents and NTS neurons most likely by activation of presynaptic Y2 receptors located on the central endings of vagal sensory neurons and via Y4 receptors whose location awaits to be determined. This inhibitory action of endogenous NPY on vagal afferent signalling may be of relevance both to the understanding and treatment of gastric chemonociception.

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Abbreviations

AEC	3-amino-9-ethylcarbazole
ANOVA	analysis of variance
BIIE0246	(S)-N^2-[[1-[2-[4-[(R,S)-5,11-dihydro-6(6H)-oxodibenz[b,e] azepin-11-yl]-1-piperazinyl]-2-oxoethyl]cyclopentyl] acetyl]-N-[2-[1,2-dihydro-3,5 (4H)-dioxo-1,2-diphenyl-3H-1,2,4-triazol-4-yl]ethyl]-argininamide
DAB	3,3-diaminobenzidine
EAAC1	excitatory amino acid transporter-1
HCl	hydrochloric acid
IG	intragastric
NPY	neuropeptide Y
NTS	nucleus tractus solitarii
PBS	phosphate-buffered saline
PYY	peptide YY
Y2 receptor	neuropeptide Y receptor of type Y2
Y4 receptor	neuropeptide Y receptor of type Y4

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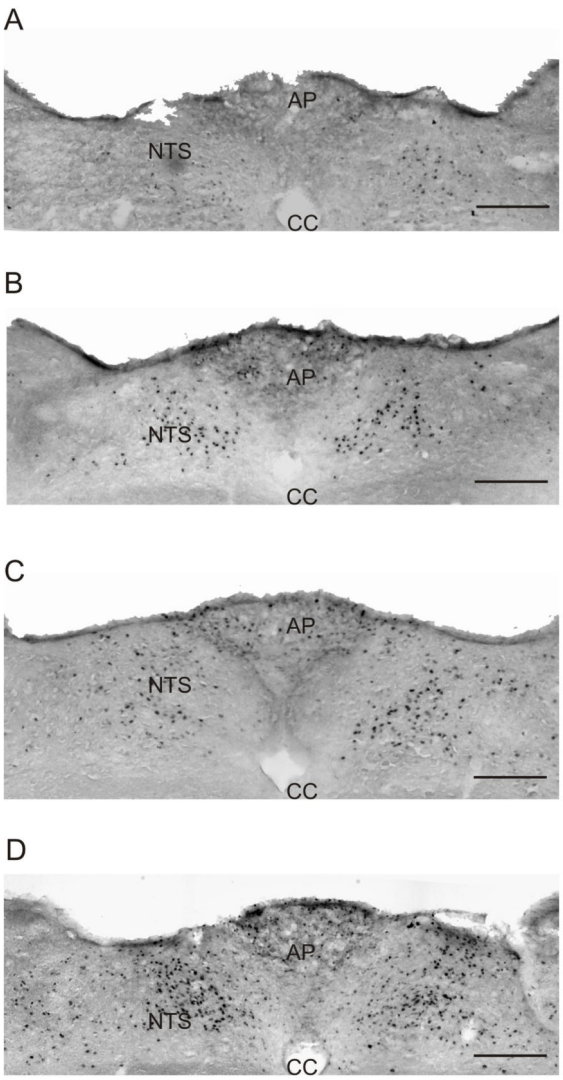

Figure 1.

Photomicrographs of the nucleus tractus solitarii (NTS) and area postrema (AP) taken from (A) a Him:OF1 mouse treated IG with 0.15 M NaCl (0.02 ml/g) as well as from (B) a Him:OF1 mouse, (C) a control (FY2) mouse and (D) a Y2-/- mouse treated IG with 0.25 M HCl (0.02 ml/g) 2 h before immunohistochemical visualization of c-Fos-positive cells in the brainstem. Relative to NaCl, HCl induced many cells in the medial and subpostremal nuclei of the NTS and some cells in the AP to express c-Fos. Coordinates according to Paxinos and Franklin (2001): interaural −3.68 mm, bregma −7.48 mm. CC, central canal. Calibration bar: 0.1 mm.

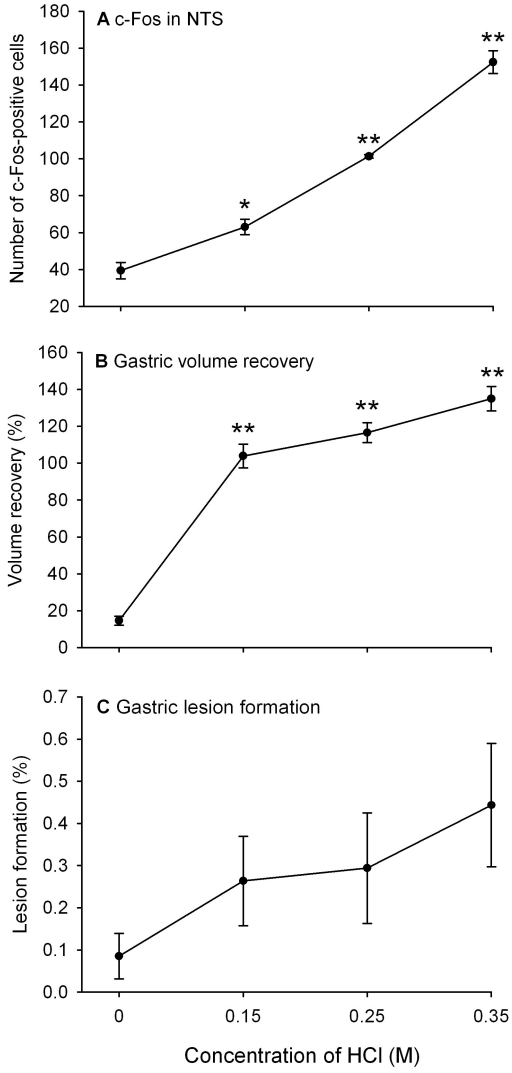

Figure 2.

Concentration-related effect of IG administered HCl on (A) expression of c-Fos in the unilateral nucleus tractus solitarii (NTS), (B) gastric volume recovery and (C) haemorrhagic lesion formation in the glandular gastric mucosa of Him:OF1 mice. NaCl (0.15 M; depicted as 0 M HCl) and HCl (0.15, 0.25 and 0.35 M) were administered IG at a volume of 0.02 ml/g (A) 2 h before immunohistochemistry and (B,C) 30 min before determination of gastric volume recovery and gastric lesion formation. The values represent means ± SEM, n = 6. * P<0.05, ** P<0.01 versus 0 M HCl (ANOVA).

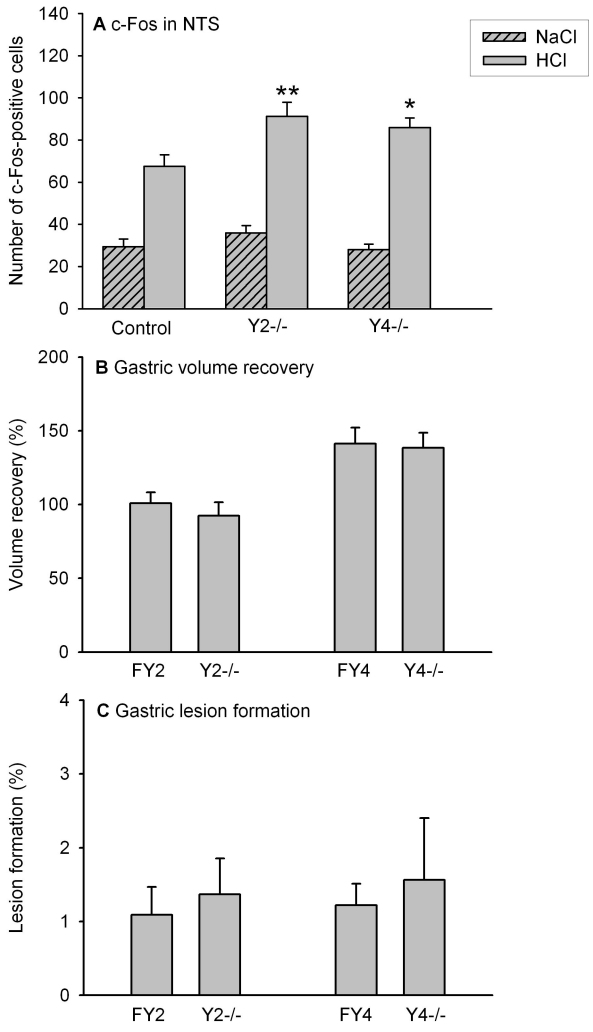

Figure 3.

Effect of IG administered NaCl and HCl on (A) expression of c-Fos in the unilateral nucleus tractus solitarii (NTS), (B) gastric volume recovery and (C) haemorrhagic gastric lesion formation in control (FY2, FY4), Y_2 receptor knockout (Y2-/-) and Y_4 receptor knockout (Y4-/-) mice. NaCl (0.15 M) and HCl (0.25 M) were administered IG at a volume of 0.02 ml/g (A) 2 h before immunohistochemistry and (B,C) 30 min before determination of gastric volume recovery and gastric lesion formation. The values represent means ± SEM, n = 7-16 (A), 6-9 (B), 4-8 (C). * $P<0.05$, ** $P<0.01$ versus control mice treated with HCl (ANOVA).

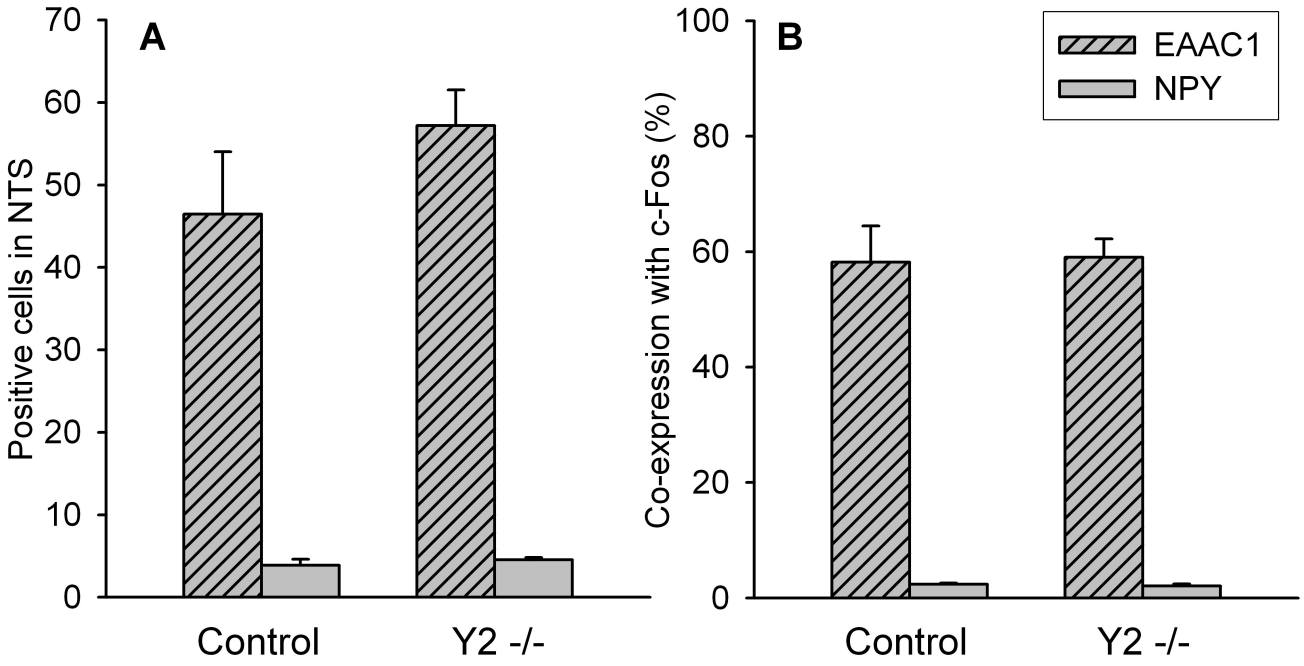

Figure 4.

Colocalization of c-Fos with the excitatory amino acid carrier 1 (EAAC1) and neuropeptide Y (NPY) in the nucleus tractus solitarii (NTS) of control (FY2) and Y_2 receptor knockout (Y2-/-) mice as examined 2 h after IG treatment with HCl (0.25 M, 0.02 ml/g). (A) Absolute number of c-Fos-positive cells that are also immunoreactive for EAAC1 and NPY. (B) Percentage of the total number of c-Fos-positive cells that coexpress EAAC1 and NPY. The values represent means ± SEM, n=5.

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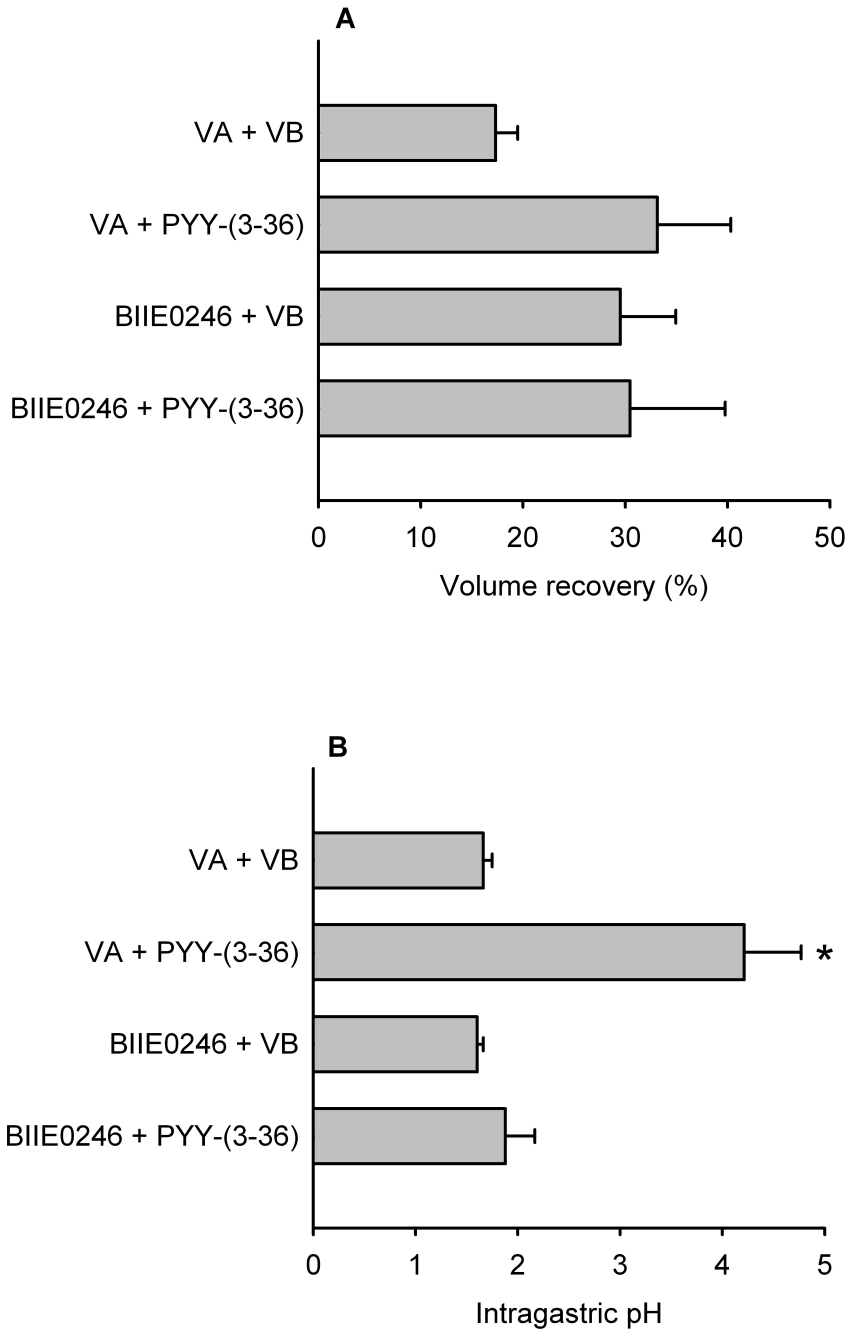

Figure 5.

Effect of PYY-(3-36) and BIIE0246 on (A) gastric volume recovery and (B) IG pH in Him:OF1 mice. BIIE0246 (0.03 mmol/kg subcutaneously) or its vehicle (VA) and PYY-(3-36) (0.1 mg/kg intraperitoneally) or its vehicle (VB) were administered 10 and 5 min, respectively, before the gastric mucosa was exposed to 0.15 M NaCl (0.02 ml/g). Gastric volume recovery and IG pH were determined 30 min later. The values represent means ± SEM, n=4-6. * P<0.05 versus all other IG pH values (ANOVA).

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Table 1

Gastric volume recovery, IG pH and gastric haemorrhagic injury following IG administration of saline and HCl

Test parameter	30 min post-NaCl	30 min post-HCl	60 min post-HCl	120 min post-NaCl	120 min post-HCl
Volume recovery (%)	24.4 ± 4.7 (12)	105 ± 6.1 (20)	98 ± 6.8 (4)	21.1 ± 3.5 (6)	91 ± 17.5 (8)
IG pH	2.21 ± 0.21 (6)	1.14 ± 0.07 (5)	N.D.	1.41 ± 0.05 (6)	1.23 ± 0.11 (5)
Mucosal injury (%)	0.25 ± 0.08 (12)	0.52 ± 0.16 (9)	0.35 ± 0.14 (4)	0.05 ± 0.05 (5)	0.22 ± 0.09 (10)

Him:OF1 mice were treated IG with saline (0.15 M NaCl; 0.02 ml/g) or different concentrations of HCl (0.25 M; 0.02 ml/g) 30, 60 or 120 min before gastric volume recovery, IG pH and gastric injury were quantified. The pH of saline and HCl before IG administration was 5.07 and 0.70, respectively. Gastric fluid recovery was calculated by expressing the weight of the fluid present in the stomach as a percentage of the weight of the fluid administered into the stomach. The mucosal area covered by visible haemorrhagic damage was expressed as a percentage of the total area of the glandular mucosa. Means ± SEM, n as indicated in brackets. N.D., not determined.

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Table 2

Effect of PYY-(3-36) and BIIE0246 on gastric HCl-induced c-Fos expression in the NTS of Him:OF1 and control mice

Pretreatment	Number of c-Fos-positive cells in the unilateral NTS
Him:OF1 mice Vehicle A + Vehicle B	83 ± 6.0 (6)
Him:OF1 mice Vehicle A + PYY-(3-36)	80 ± 15.5 (6)
Him:OF1 mice BIIE0246 + Vehicle B	77 ± 7.7 (6)
Him:OF1 mice BIIE0246 + PYY-(3-36)	76 ± 6.7 (6)
Control mice Vehicle A	86 ± 2.8 (9)
Control mice BIIE0246	94 ± 11.8 (8)

Him:OF1 mice and control (FY2) mice were pretreated with BIIE0246 (0.03 mmol/kg subcutaneously) or its vehicle (vehicle A) and PYY-(3-36) (0.1 mg/kg intraperitoneally) or its vehicle (vehicle B) 10 and 5 min, respectively, before the gastric mucosa was exposed to 0.25 M HCl (0.02 ml/g). One hour (Him:OF1 mice) or two hours (control mice) later the c-Fos expression in the NTS was quantified by immunohistochemistry. Means ± SEM, n as indicated in brackets.