Selective Expression of histamine receptors H1R, H2R and H4R, but not H3R, in the human intestinal tract

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ABSTRACT

Histamine is known as a regulator of gastrointestinal (GI) functions such as gastric acid production, intestinal motility and mucosal ion secretion. Most of this knowledge was obtained from animal studies. In contrast, in humans, expression and distribution of histamine receptors (HR) within the human GI tract is unclear. We therefore analysed HR expression in human GI tissue specimens by quantitative RT PCR and immunostaining and found that H1R, H2R and H4R mRNA were expressed throughout the GI tract, while H3R mRNA was absent. No significant differences in distribution of HR were found between different anatomical sites (duodenum, ileum, colon, sigma, rectum) concerning distribution of HR were found. Immunostaining of neurons and nerve fibres revealed that H3R is absent in the human enteric nervous system (ENS); however, H1R and H2R were found on ganglion cells of the myenteric plexus. Epithelial cells also expressed H1R, H2R and, to some extent, H4R. Intestinal fibroblasts exclusively expressed H1R, while the muscular layers of human intestine stained positive for both H1R and H2R. Immune cells expressed mRNA and protein for H1R, H2R, and low levels of H4R. Analysis of endoscopic biopsies from patients with food allergy and irritable bowel syndrome revealed significantly elevated H1R and H2R mRNA levels compared to controls. In conclusion, we demonstrate that H1R, H2R and, to some extent, H4R are expressed in the human GI tract, while H3R is absent, and we found that HR expression is altered in patients with GI diseases.
INTRODUCTION

Histamine is known to be a major mediator of acute anaphylactic reactions. Since its discovery in 1912 [1] numerous other physiological functions have been attributed to this biogenic amine which exerts its biological actions through four different receptors (H1R – H4R) that have been identified so far [2]. In the gastrointestinal (GI) tract, histamine is considered to regulate at least three major functions: 1) enhancement of gastric acid production [2][3], 2) modulation of GI motility [4][5][6][7] and 3) alteration of mucosal ion secretion [8][9]. The data confirming the last two concepts were mainly generated in animal models; whereas attempts to transfer the findings to the human system were often of limited success [10]. Hitherto it is not even clear which of the four HR are expressed in the human GI tract. Especially, the expression and function of H3R, which is subject to a controversial debate [10][11], remains to be examined in human tissue. An inhibitory role of H3R upon neurotransmitter release from intestinal nerves has been repeatedly suggested [12], but not yet documented in the human GI tract.

Recent studies have identified histamine as a potent modulator of immune functions [13]. Considering that the GI tract represents the largest immune organ of the human body hosting high numbers of mucosal MC [14], histamine might be of particular relevance for MC-dependent immune regulation. In the present study we raised three questions: 1) Which HR are expressed in the human intestine at different anatomical sites? 2) Does the enteric nervous system (ENS) express HR? 3) Is the expression pattern of HR altered in patients with functional or immunological GI disorders such as IBS or allergic inflammation? To address these questions, we examined the expression of HR by means of quantitative RT PCR and immunostaining in surgical
specimens of normal intestinal tissue. Furthermore, we measured HR mRNA expression levels in endoscopic tissue samples from patients with IBS or food allergies (FA) and control patients. Different cell populations were isolated from human intestinal mucosa including intestinal mast cells (MC), intestinal fibroblasts (FB) and lamina propria mononuclear cells (LPMC) and analysed for HR mRNA expression.
MATERIAL AND METHODS

Patients. Thirty-three individuals undergoing bowel dissection due to GI cancer were recruited for the HR expression study. Samples from the resectates were taken from macroscopically unaffected tissue both from the mucosa/submucosa and the muscular layer and subjected to quantitative RT PCR analysis, primary cell isolation or histological analysis. HR expression in intestinal mucosa from patients with GI disorders was measured in homogenates of endoscopic biopsies.

Thirty patients with IBS fulfilling the ROME II criteria and an additional history of allergy (37 ± 11 yr.) and fourteen control patients (C; 38 ± 15 yr.) were recruited. In 19 of the IBS patients diagnosis of intestinal FA could be confirmed based on a history of adverse reactions to food combined with provocation tests such as open oral challenges and colonoscopic allergen provocation (COLAP) test, as described previously [15]. The remaining 11 patients were classified as IBS patients according to the ROME II criteria. All medications related to the intestinal and/or allergic symptoms were stopped 10 days before the colonoscopy. Control patients had no history of allergy or food intolerance and underwent colonoscopy for diverse reasons (i.e. surveillance, polyps, blood in stool). Only patients without endoscopic or histological evidence of mucosal inflammation were included in the control group. All patients underwent colonoscopy (videocoloscope, Olympus Optical Company, Hamburg, Germany). Endoscopic biopsies were taken in the terminal ileum (IBS, n= 10; FA, n=7; C, n=5), the ceacum (IBS, n= 11; FA, n= 19; C, n=14) and the rectum (IBS, n= 10; FA, n=7; C, n=8). Biopsies were immediately incubated in RNAlater buffer (Qiagen, Hilden, Germany) and stored for 24h at 4°C and subsequently
homogenised in RLT buffer (Qiagen, Hilden, Germany) supplemented with β-mercaptoethanol.

**RNA preparation; real-time and conventional RT-PCR.** Total RNA was prepared from intestinal specimens (surgical and endoscopical) and subjected to reverse transcription as described [16]. The real-time fluorescence-monitored PCR reactions were performed using SybrGreen® PCR Master Mix (Applied Biosystems, Warrington, UK) and a PE Applied Biosystems model 7700 Sequence Detection System. The following oligonucleotide primers were used for RT PCR reactions: GAPDH (Genebank access number : NM_002046) (5'-CAGCCTCAAGATCATCAGCA-3'), (5'-TTAAGACCACACAGATGGCG-3') fragment size 140bp, H1R (Genebank access number : NM_000861) (5'-GTCTAACACAGGCCTGGATT-3'), (5'-GGATGAAGGCTGCCATGATA-3') fragment size 140bp, H2R (Genebank access number : NM_022304) (5'-ATTAGCTCCTGGAAGGCAGCAG-3'), (5'-CTGGAGCTTCAGGGGTTTCT-3') fragment size 374bp, H3R (Genebank access number : NM_007232) (5'-TCGTGCTCATCAGCTACGAC-3'), (5'-AAG CCGTGATGAGGAAGTAC-3') fragment size 214bp, H4R (Genebank access number : NM_021624) (5'-GGCTCACTACTGACTATCTG-3'), (5'-CCCTCATCCTTCCAGACTC-3') fragment size 197bp. PCR products were loaded on 1% agarose gel to check the specificity of the reactions. The comparison of two different runs with the same primer pair showed a strong reproducibility (correlation coefficient r_s=0.91, p<0.001, n=76 for example with GAPDH primers). Real-time PCR data were expressed as a ratio of GAPDH mRNA expression.
**Western blot analysis.** Tissue samples were homogenised in extraction buffer containing 25 mM Tris-Hcl, pH7.5, 0.5 mM EDTA, 0.5mM EGTA, 0.05 % Triton X-100, supplemented with the protease inhibitor cocktail Complete™ Mini (Roche Diagnostics, Basel, Switzerland). Protein concentration in the homogenates was determined using Bio-Rad protein assay (Bio-Rad, Hercules, CA). The protein extracts (10-20 µg protein each) were separated on a 12 % SDS-polyacrylamide gel and blotted onto a nitrocellulose membrane (Schleicher and Schuell, Einbeck, Germany) in 0.1 % SDS, 20 % methanol, 400 mmol/l glycine, 50 mmol/l tris-HCL pH 8.3, 300 mA by electroblotting using Trans-Blot™ Cell (Bio-Rad, Hercules, CA). Membranes were blocked with 5 % skim milk in PBS containing 0.025 % tween for one hour. Membranes were probed with anti-H1R (Alpha Diagnostic International, San Antonio, TX, rabbit polyclonal antibody, recognises a peptide highly conserved in humans, fish and monkeys, but no other HR), anti-H2R (Alpha Diagnostic International, rabbit polyclonal antibody, recognises a peptide highly conserved in humans and fish, but no other HR), anti-H3R (Acris Antibodies GmbH, Hiddenhausen, Germany, rabbit polyclonal antibody, specific to the human H3R) or with anti-H4R (Alpha Diagnostic International, rabbit polyclonal antibody, specific to the human H4R). The bands were visualized using an electrochemiluminescence detection system as described by the manufacturer (NEN™ Life science, Boston, MA).

### 2.4. Immunohistochemistry and immunofluorescence

Tissue samples of 20 gut resectates (each four of duodenum, ileum, colon, sigma and rectum) were fixed in paraformaldehyde 4%, embedded in paraffin wax, and sectioned at 4 µm. For the immunostaining a Histostain® Plus Broad spectrum (AEC) kit (Zymed Laboratories Inc., San Francisco, CA) was used following the
manufacturer’s instructions. The sections were incubated over night at 4°C with the following primary antibodies (all from Acris Antibodies GmbH, Hiddenhausen, Germany) at the given concentrations:

H1R rabbit polyclonal antibody at 10 μg/ml, H2R rabbit polyclonal antibody at 5 μg/ml, H3R rabbit polyclonal antibody at 10 μg/ml, H4R rabbit polyclonal antibodies at 10 μg/ml; protein gene product 9.5 (PGP 9.5) sheep polyclonal antibody (cross reacts with most mamals) at a dilution of 1:100. In addition, tyrosine hydroxylase mouse monoclonal antibody (Immunostar Inc., Hudson, WC, USA, cross reacts with most mamals) was used at a dilution of 1:1000. Secondary antibodies were supplied with the Histostain® Plus kit and used according to the instructions, except for rabbit anti-sheep biotinylated antibodies (Zymed Laboratories Inc.), which were used at a concentration of 15 μg/ml for 30min. For counter staining sections were exposed to hemalaun for 10 sec.

For immunofluorescence, sections were blocked in 10% horse native serum (HNS) for 30min, the primary antibodies were added at the concentrations given above together with 5% HNS over night at room temperature. The following secondary antibodies were added for 30min at room temperature: Alexa Fluor® goat anti-mouse 488 (3μg/ml), Alexa Fluor® donkey anti-rabbit 594 (3μg/ml), Alexa Fluor® donkey anti-sheep 488 (3μg/ml). Alexa Fluor® streptavidin 594 (3μg/ml) was used for detection of biotinylated rabbit anti-sheep. Confocal fluorescence microscopy was performed using a LSM 510 META microscope (Carl Zeiss AG, Oberkochen, Germany).

**Cells and cell culture.**
Isolation of LPMC. LPMC were isolated from intestinal mucosa of surgical specimen by mechanical and enzymatic digestion as described elsewhere [17]. The cell suspension gathered from the isolation procedure was then separated on Ficoll-Hypaque (Amersham Pharmacia Biotech, Uppsala, Sweden) gradients. The cells were washed in PBS and resuspended in RPMI 1640 containing 10% FCS and 50ng/ml IL-2 at a density of 5x10⁵/ml.

Isolation of human intestinal MC. Human intestinal MC were isolated from surgical tissue specimens. The methods of mechanical and enzymatic tissue dispersion yielding single cell preparations containing 4 ± 2% (mean ± SD) MC have been described in detail elsewhere [16]. After overnight incubation in culture medium (RPMI 1640 supplemented with 10% heat-inactivated fetal calf serum, 25 mM HEPES, 2 mM glutamine, 100 µg/ml streptomycin, 100 µg/ml gentamycin, 100 U/ml penicillin and 0.5 µg/ml amphotericin; all cell culture reagents were from Gibco Invitrogen, Paisley, U.K.), MC were enriched by positive selection of c-kit expressing cells using magnetic cell separation (MACS™ system, Miltenyi Biotech, Bergisch-Gladbach, Germany) and the monoclonal antibody (mAb) YB5.B8 (Pharmingen, Hamburg, Germany) as described [16]. The fraction containing the c-kit-positive cells (mast cell purity 60±25%) was cultured at a density of 2 x 10⁵ MC per ml for up to 28 days in medium supplemented with 50 ng/ml of recombinant human stem cell factor (SCF; Amgen, Thousand Oaks, CA) until purity of 97 - 100% MC was achieved.

Isolation and culture of human intestinal FB. Human intestinal FB were isolated from surgical specimen from patients undergoing bowel resection. Isolation and culture methods have been described in detail recently [18]. FB preparations of at
least 95% purity where used for mRNA extraction and subsequent expression studies.

**Isolation of PBMC.** Blood from five healthy donors was separated on Ficoll-Hypaque gradients. The ring fraction was washed and subjected to mRNA isolation.

**Isolation of HUVEC.** Human umbilical vein endothelial cells were isolated from human cords as described earlier [19]. Cells were harvested when a confluent monolayer was achieved and subjected to RNA isolation.

**Culture of MHH-NB-11.** This human neuroblastoma cell line was purchased from the DSZM (Braunschweig, Germany). Cells were cultured in medium containing 90% RPMI 1640 plus 10% FBS (Biochrom AG, Berlin, Germany) and 2ml L-glutamine + MEM non essential amino acids. The adherent cells were harvested at a density of $10^6$ cells/80 cm$^2$ and RNA was extracted.

**Statistical analysis.** Statistical analysis was performed using unpaired (Mann-Whitney) two-tailed, non-parametric test, P value less than 0.05 was considered significant.
RESULTS

HR mRNA expression in the human intestinal tract

Analysis of surgical specimens of 33 patients revealed that H1R, H2R and H4R are expressed in the human GI tract (Fig.1A,B); H3R was absent in most samples, but could be detected at low levels in only five of the 66 samples (= 8.3%, one mucosa/submucosa and four muscle layer samples, from four different patients, data not shown). HR mRNA proved to be equally distributed between mucosa/submucosa and muscular layer (Fig.1A,B). The overall mRNA expression of HR, displayed as HR-GAPDH ratio, was rather low in the intestinal samples tested compared to e.g. H2R expression in gastric mucosa (n=2, H2R/GAPDH ratio; median 2.4x10^{-2}) or H1R expression in umbilical veins (n=3, H1R/GAPDH ratio; median 1.3x10^{-1}) used as positive controls (data not shown). H1R and H2R were expressed at higher levels than H4R, both in the mucosa and in the muscular layer (P < 0.001). H1R, H2R and H4R expression could be confirmed on the protein level by western blot analysis (Fig.1C). There were no substantial differences in HR mRNA expression at different anatomical sites of the intestine (Fig. 1D). When analysing distinct cell populations of the human intestinal mucosa, namely LPMC, MC and FB, we found differing patterns of HR expression (Fig.2). LPMC, the fraction of cells consisting of lymphocytes, macrophages and dendritic cells mainly, expressed H1R, H2R and H4R while H3R was absent. Mucosal MC expressed H1R, H2R and H4R as well, but not H3R. FB, representing connective tissue cells of the intestine found to interact with MC [18], expressed exclusively H1R at high levels.

Histological analysis of human intestinal tissue
In order to analyse the expression of HR in further detail, immunostaining of paraffin embedded human small and large intestine was performed. The staining pattern of H1R, H2R and H4R corresponded to the mRNA expression. H1R and H2R were both found to be ubiquitously expressed throughout the gut wall. Enterocytes as well as connective tissue cells, immune cells, blood vessels, myocytes and enteric nerves were all shown to express H1R (Fig.3a-d). H2R was found to be expressed on enterocytes as well, although the staining appeared less pronounced; and on immune cells such as lymphocytes in the Payer’s patches (Fig.3e+f). Myenteric ganglia along with smooth muscle cells also stained positive for H2R (Fig.3g+h).

Only a few cells located within the human intestinal tissue expressed H4R (Fig.3m-p). Mainly leukocytes inside small mucosal and submucosal blood vessels were stained positive and exhibited a much stronger reactivity against H4R than did tissue resident leukocytes (Fig.3n+p). Moreover, intraepithelial cells with typical morphological characteristics of neuroendocrine cells (Fig.3n) stained positive for H4R. Finally, enterocytes at the apical end of the crypts of Lieberkühn were found to be H4R positive (Fig.3m).

**Human intestinal tissue in general, and the ENS in particular, is devoid of H3R**

Confirming the mRNA expression data, H3R antibodies did not stain any cellular structures in human intestinal tissue. Protein gene product (PGP) 9.5, a pan-neuron marker, was used to trace nerves within the gut wall, in order to find out if such cells express H3R. Immunofluorescence double staining with anti-H3R and PGP 9.5 Ab’s in the human intestine revealed no co-localisation of the two antigens (Fig.4d,j), indicating that intestinal nerve fibres do not express H3R. Sympathetic nerve terminals, which have been reported to express H3R in guinea pigs were specifically
identified by anti tyrosine hydroxylase staining. However, they were also found to be devoid of H3R (Fig.4e). In comparison, neurons in human brain cortex, included as positive controls, exhibited strong H3R expression (Fig.4f). Notably, H3R antibodies exhibited some cross reactivity to perivascular fibres (Fig.3j); however, employment of high resolution confocal microscopy revealed that H3R staining was nearly absent, once highly autofluorescent structures like elastic fibres in blood vessels were subtracted (Fig.4l). In contrast, H1R and PGP 9.5 double-staining of myenteric ganglia showed a high degree of co-localisation, indicating that enteric nerves indeed express H1R, but not H3R (Fig.4). We therefore conclude that, despite of contradictitious findings from guinea-pig studies, human large and small intestine does not express H3R under normal conditions.

**Elevated HR expression in patients with FA or IBS**

HR expression was examined in biopsies from patients with IBS and FA respectively. Biopsies were taken from the terminal ileum, the caecum and the rectum. Since there were no substantial differences between the different anatomical regions, the results are presented cumulatively. Both patients with IBS and FA exhibited significantly higher levels of mRNA encoding for H1R and H2R in comparison to controls (Fig.5A, B). The levels of H4R mRNA were not significantly changed in biopsies of patients compared to controls (Fig.5C).

H3R mRNA could only be detected in 2 samples (1 IBS and 1 control patient), suggesting that this receptor is not upregulated in these disorders.
DISCUSSION

The present study shows for the first time that H1R, H2R and H4R are expressed in the human GI tract. In contrast, basically no H3R mRNA expression was seen in the normal human intestine, which was further confirmed on the protein level. The expression pattern of HR did not change at different anatomical sites of the intestine. Expression and distribution of HR in the human or animal GI tract has not been examined in detail so far. However, the existence of HR in the GI tract had to be anticipated since functional and pharmacological experiments in Guinea pigs and other species revealed that histamine acting through particular receptors regulates multiple intestinal functions such as mucosal secretion [8][9] and motility [4][6][7]. We show here that H1R and H2R are indeed expressed throughout the human GI tract. To all of our knowledge, the expression of H1R and H2R on ganglion cells of the myenteric plexus has not been shown in humans so far, and functional implications still need to be defined.

The H4R was discovered four years ago, and found to be expressed on leukocytes and in bone marrow [20][21]. High expression was described in spleen and liver [20], while rather low expression in other peripheral organs was reported, which is consistent with the results presented in our study. H4R mRNA levels were significantly lower compared to H1R and H2R mRNA levels. The immunohistochemical approach revealed that leukocytes inside blood vessels were highly positive for H4R, while tissue cells expressed H4R at lower levels. Recent reports have described a chemotactic property of histamine binding to H4R, enhancing leukocyte migration and recruitment from bone marrow [22][23][24][25]. This observation could tempt to speculate that H4R are downregulated after migration of leukocytes from blood stream into the tissue. Interestingly, enterocytes
stained positive for H4R at the apical tip of the crypts of Lieberkühn. According to the epithelial cell dynamics, enterocytes migrate from the base of the crypts to the tip of the villi (or crypts in the colon) where they are fully mature before they undergo apoptosis. Thus, functionality of H4R on these cells has to be questioned. Pronounced staining of cells with typical morphological characteristics of neuroendocrine cells was surprising, and it will be exciting to investigate possible histaminergic effects upon these cells.

The H3R, expressed predominantly on neurons, has been shown to function as an autoreceptor inhibiting synthesis and release of histamine [26] and other neurotransmitters [27][28]. The effects of H3R activation on the ENS, have been subject to numerous studies in Guinea pigs. These experiments revealed an involvement of this receptor in the control of contractile responses of the intestine, mainly by modulating transmitter release from enteric neurons [5][12][29]. Surprisingly, despite the fact that intestinal tissue contains neurons in considerable amounts [30] (Fig.4), we found no H3R mRNA expression in the human intestine. Specific staining of enteric nerves in the tissue revealed that H3R was virtually absent also on the protein level. Since it had been repeatedly proposed that H3R controls the transmitter release from sympathetic nerve terminals, we double-stained for tyrosine hydroxylase and H3R, but found no co-localisation. As mentioned before, cross reactivity of H3R to non-neurogenic fibres occurred within circular structures in the walls of blood vessels. These were totally devoid of PGP9.5 or tyrosine hydroxylase staining and showed a high degree of unspecific autofluorescence, as detected by confocal microscopy. Moreover, immunoblot staining revealed staining of unspecific protein bands in tissue lysates (Fig.1C). Our data are in accordance with previous studies reporting a lack of H3R mRNA and function in human tissues.
Thus, substantial interspecies differences with regard to HR expression in the intestine and pharmacological properties of the different HR have to be considered [11][32][33].

It is well-known that histamine is involved in the regulation of intestinal secretion and motility, processes that, when dysregulated, cause clinical symptoms like diarrhoea and abdominal pain [34]. Therefore, we were interested if HR expression might be altered in patients with GI diseases. We found that mRNA for H1R and H2R was significantly upregulated in patients with IBS with and without FA, which might be related to the IBS-like symptoms these patients are suffering from. Indeed, Jarisch and co-workers have described a spectrum of intestinal symptoms that can be evoked by histamine in selected individuals [35]. This histamine intolerance was attributed to an impaired histamine metabolism [36][37]. Alternatively, such symptoms could be a result of elevated HR expression as found here in patients with IBS and FA, which, similarly to reduced histamine degradation, may cause histamine hypersensitivity. This hypothesis needs to be confirmed by showing that not only FA but also IBS and other GI diseases characterised by secretory and motility disorders improve by treatment with antihistamines or histamine-free diet.

In summary, we show that H1R, H2R and H4R are present in the human GI tract and that the expression of H1R and H2R is upregulated in patients with FA or IBS. In contrast to previous studies performed in guinea pigs, we conclude that H3R are not expressed in the human intestine.
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Conflict of interest

The authors declare that no conflict of interest exists.

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APPENDIX:

Abbreviations used in this paper

FA, food allergy; FB, fibroblasts; GI, gastrointestinal; HR, histamine receptor; IBD, inflammatory bowel disease; IBS, irritable bowel syndrome; LPMC, lamina propria mononuclear cells; MC, mast cells; PBMC, peripheral blood mononuclear cells; SCF, stem cell factor; TNF, tumor necrosis factor
Figure legends

**Fig.1.** Histamine receptor (HR) mRNA expression in 66 human intestinal tissue samples taken from surgical specimen from 33 individuals. Data were generated by quantitative RT PCR and are displayed as GAPDH/HR ratio (P values as indicated). A,B HR expression in the mucosa/submucosa (A) and the muscular layer (B). H3R was not detected in >90% of the samples. C Protein expression of HR in intestinal tissue homogenates shown by immunoblot. Mucosa and muscular layer samples of colonic tissue were probed with H1R, H2R and H4R antibodies. Human umbilical vein endothelial cells, gastric mucosa homogenate, human neuroblastoma cells MHH-NB 11 and purified human intestinal MC were used as positive controls for H1R, H2R, H3R and H4R, respectively. As indicated, H3R antibodies detected an unspecific protein band of approximately 60kD in lysates of human colon, while the specific band migrates at the expected weight of 50kD. D Comparison of HR expression at different intestinal sites. 1= duodenum, 2= colon, 3= sigmoid, 4= rectum.

**Fig.2.** HR mRNA expression in LPMC, MC and FB isolated and purified from surgical specimens of normal intestinal tissue. PBMC expressing H1R, H2R and H4R and the human neuroblastoma cell line MHH-NB-11 expressing H3R were used as controls. One representative experiment out of five is shown.

**Fig.3.** Immunohistochemical staining of human large and small intestine. Bars indicate 20μm, except in i-k bars indicate 100μm. **H1R (a-d)** a epithelium, b cells of the lamina propria, c small submucosal blood vessel, d myenteric nerve plexus located between the circular and the longitudinal muscle layer. **H2R (e-h)** e
epithelium, f Payer’s patch, g smooth muscle in the longitudinal muscle layer, h myenteric nerve plexus. H3R (i-l) i colonic mucosa (epithelium and lamina propria), no positive cells, j submucosa, unspecific staining of fibres in and around the walls of blood vessels (arrow heads), k muscular layers and myenteric plexus (arrow head), l ganglion cells of the myenteric plexus (arrow head). H4R (m-p) m granular staining of H4R in enterocytes at the tip of a crypt of Lieberkühn, n H4R positive cell with morphological characteristics of a neuroendocrine cell (white arrow head), no staining for H4R on a leukocyte in the lamina propria (black arrow head), o mononuclear cells in the lamina propria, probably macrophages (arrow heads), p pronounced staining of intravascular granulocytes (arrow heads), the isotype control is negative (arrow head in the small image) excluding unspecific peroxidase reactions.

**Fig.4.** Immunofluorescence staining of human intestinal tissue and human brain cortex (f) examined by confocal microscopy. a-c Myenteric ganglion cells in human colon stained with PGP9.5 (a, red), tyrosine hydroxylase (b, green), or both (c, merge of a and b). d PGP9.5 (green) and H3R (red) double staining of a myenteric ganglion in human colon, no H3R staining was observed. e tyrosine hydroxylase (green) and H3R (red) double staining a myenteric ganglion in human colon, sympathetic nerve endings did not express H3R. f H3R staining (red) of neurons in human brain cortex. g-i Myenteric ganglion cells in human colon stained with anti-H1R Ab (g, red), PGP9.5 (h, green), or both (i, merge of g and h). Note the prominent co-localisation of H1R and PGP9.5 staining. j PGP9.5 (green) and H3R (red) staining of the circular muscle layer, the nerve fibres (green) are devoid of H3R. k PGP9.5 (green) and H1R (red) staining of the circular muscle layer, H1R was expressed on smooth muscle but not on nerve fibres. l PGP9.5 (green) and H3R (red) staining of a submucosal blood
vessel; after subtraction of unspecific autofluorescence there remains a faint red staining of a circular structure (arrow head) that reminds of the basal lamina.

**Fig.5. A,B,C** H1R mRNA expression (A), H2R mRNA expression (B) and H4R mRNA expression (C) in bioptic samples from patients with IBS tested positive for FA (n= 19), tested negative for FA (n= 11) and from control subjects (n= 14) measured by quantitative RT PCR. H3R mRNA was only detected in two samples. (Methods and data presentation as in Fig.1.)
Fig. 2

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Fig. 5

A  H1R
   P=0.004
   P=0.002
   10^{-7} 10^{-6} 10^{-5} 10^{-4} 10^{-3} 10^{-2} 10^{-1}
   FA  IBS  Control

B  H2R
   P=0.009
   P=0.04
   10^{-7} 10^{-6} 10^{-5} 10^{-4} 10^{-3} 10^{-2} 10^{-1}
   FA  IBS  Control

C  H4R
   n.s.
   n.s.
   10^{-7} 10^{-6} 10^{-5} 10^{-4} 10^{-3} 10^{-2} 10^{-1}
   FA  IBS  Control
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