Pathophysiology of stress-induced visceral hypersensitivity

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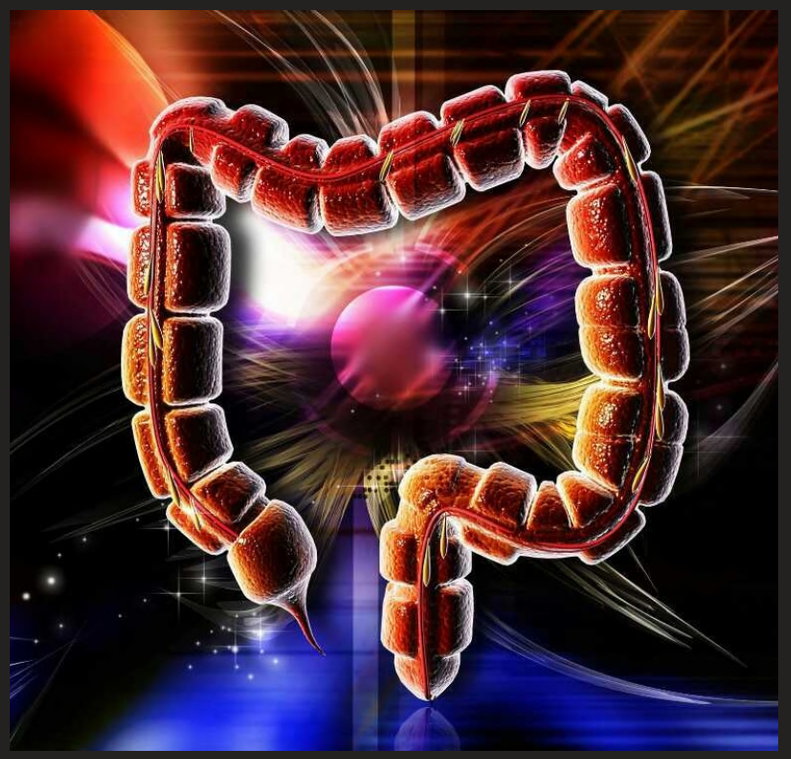

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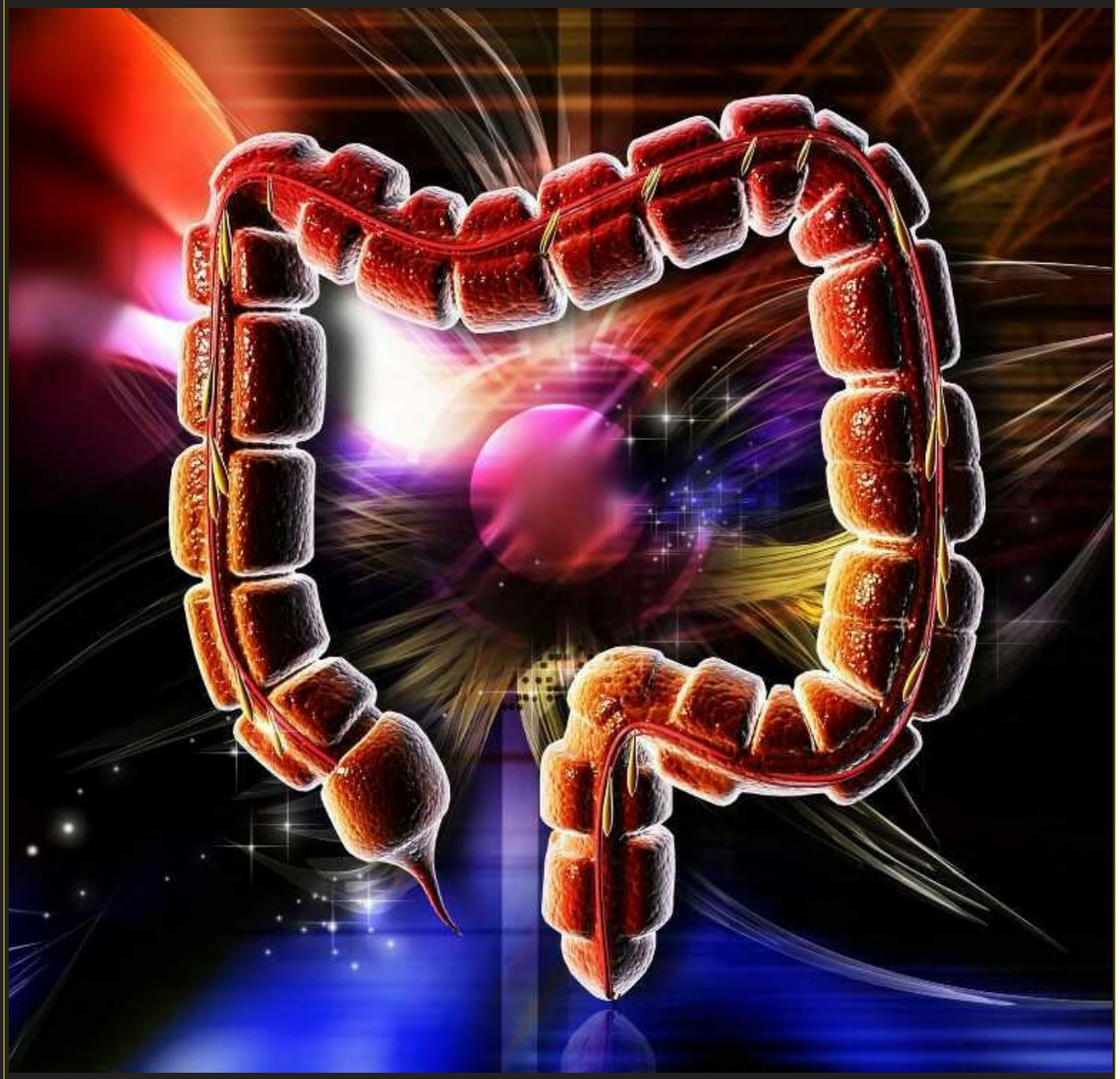

Oana Ingrid Stănişor

Pathophysiology of stress-induced visceral hypersensitivity

ACADEMISCH PROEFSCHRIFT

ter verkrijging van de graad van doctor
aan de Universiteit van Amsterdam
op gezag van de Rector Magnificus
prof. dr. D.C. van den Boom
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Faculteit der Geneeskunde

“Everybody is a genius.

But if you judge a fish by its ability to climb a tree, it will live its whole life believing that it is stupid.”

Albert Einstein, 1879-1955

"Toți suntem genii.

Însă dacă judecăm un pește după abilitatea sa de a se căţăra în copaci, acesta va trăi toată viața cu impresia că este prost."

Albert Einstein, 1879-1955

Dedic această carte

soțului meu Liviu şi familiei mele

Contents

Chapter 1

Introduction

HYPOTHESIS

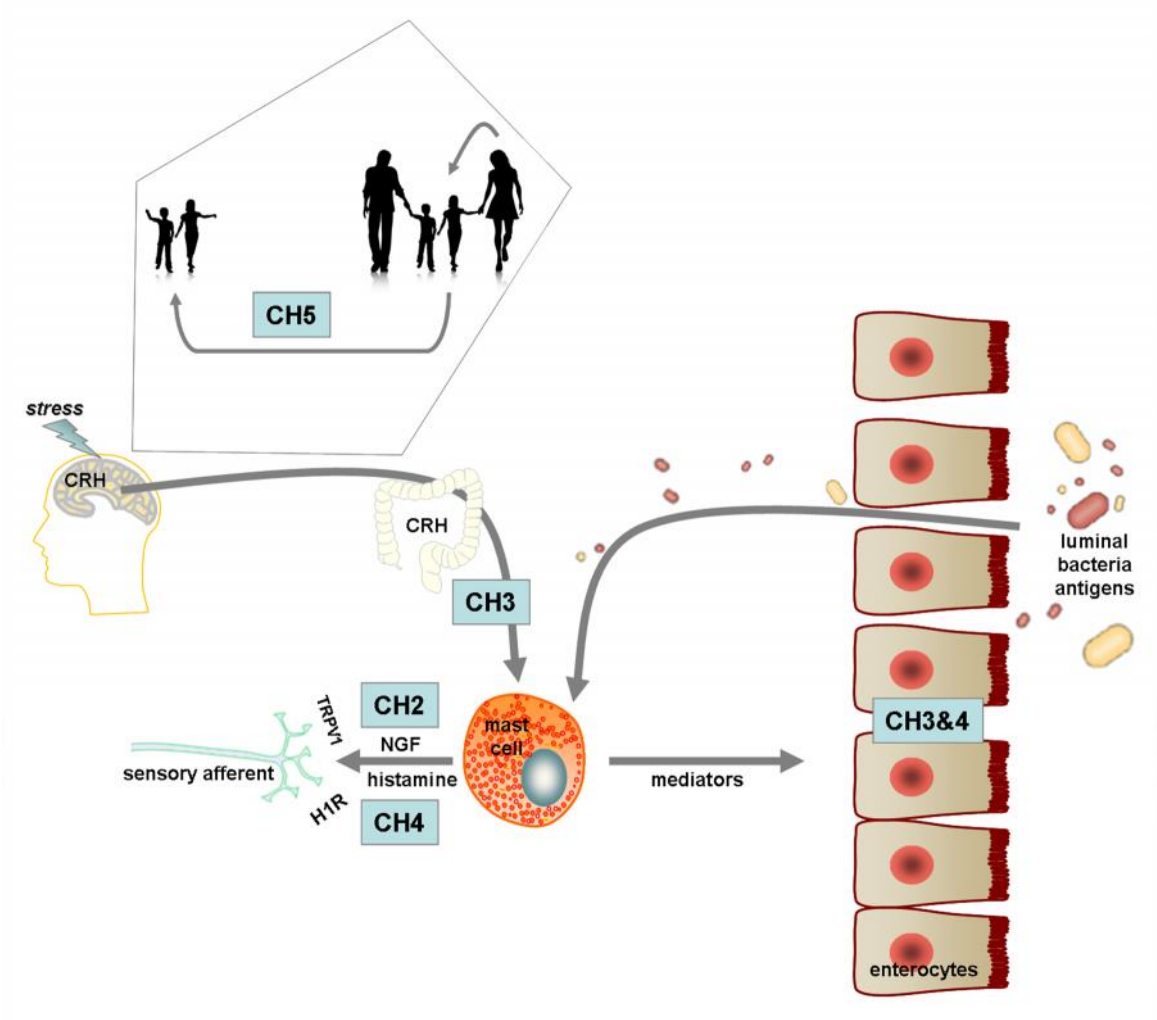

Figure 1. Schematic representation of the working hypothesis addressed in the different chapters (CH) of this thesis. Stress is a major trigger for visceral hypersensitivity because it leads to central and peripheral release of corticotrophin releasing hormone (CRH). Peripheral CRH induces the release of mast cell mediators like histamine, that modulate (e.g. via the histamine 1 receptor (H1R) afferent expressed transient receptor ion channel 1 (TRPV1). Next to TRPV1 mediated afferent activation, mast cell mediators induce gut barrier dysfunction. The subsequent influx of antigens may explain prolonged post-stress mast cell dependent visceral hypersensitivity. Finally, we hypothesize that susceptibility to stress induced visceral hypersensitivity can be transferred across generations via so called 'soft inheritance'.

INTRODUCTION

Irritable bowel syndrome (IBS) is the most common functional gastrointestinal disorder. It affects 10 to 20% of the general population in Western countries and involves chronic abdominal pain or discomfort and alterations in bowel movements in the absence of an organic explanation.[1] Next to restrictions in daily life it also leads to high medical costs. Despite the prevalence and socioeconomic impact of IBS the pipeline for novel drugs is limited. One of the main reasons for this lack of therapeutic possibilities is the poor understanding of mechanisms relevant to this disorder. Nevertheless, enhanced sensitivity to colon or recto-sigmoid distension can be diagnosed in an approximate 30%-60% of patients. This so called visceral hypersensitivity is considered a pathophysiological mechanism that may explain abdominal pain complaints. In patients stress is a known trigger for visceral hypersensitivity[2, 3] and indications are that mast cells may be involved in the occurrence of the post-stress phenotype.[4, 5] However, the exact mechanisms are still poorly understood. Therefore, the aim of the work described in this thesis was to obtain a better understanding of the stress related pathophysiology of visceral hypersensitivity. Investigations were carried out in the rat model of maternal separation. Early life stressors are known to contribute to IBS in adults[6, 7] and maternal separation in rats is often used to mimic such predisposing factor.[8] In maternal separated rats, the adverse early life experience pre-disposes for complaints like visceral hypersensitivity and barrier dysfunction in adult animals. In contrast to others who reported differences in baseline responsiveness to distension between nonhandled and maternally-separated rats,[9, 10] previously separated Long Evans rats need an acute stress at adult age to bring out the hypersensitive phenotype.[11] Since this feature mimics observations in IBS patients we used Long Evans rats true out our investigations. The possible role of mast cells, their mediators and triggers for their activation are an important focus of this thesis (figure 1).

Because others already indicated that mast cells may be involved in post stress visceral hypersensitivity, we first set out to confirm these observations in our animal model. These experiments are described in **chapter 2**. Next to pre-stress administration of the mast cell stabilizer doxantrazole we investigated the role of nerve growth factor (NGF) by administering anti-NGF antibodies. NGF was evaluated because, in addition to histamine, it is one of the mast cell mediators known to modulate transient receptor ion channel 1 (TRPV1).[12] This non-selective ligand-gated cation channel is essential for selective modalities of pain sensation.[13] Investigations by Akbar et al. showed that TRPV1 expression was up regulated in recto-sigmoid biopsies of IBS patients and that increased expression correlated with the

degree of abdominal pain.[14] Therefore, we also used two different TRPV1 antagonists to evaluate the functional role of TRPV1 in post stress visceral hypersensitivity. In addition, we compared TRPV1 expression levels in DRG neurons of nonhandled and maternal separated rats.

We next focused on the possible role of corticotrophin releasing hormone (CRH). Others already indicated that central expression of this stress hormone is highly relevant in the occurrence of post-stress IBS like features in animal models. Later it was shown that stress-induced colonic mast cell degranulation depends on peripheral CRH.[15] Despite these evidences, two large clinical trials with CRH-receptor antagonists failed.[16, 17] In **chapter 3** we attempted to clarify these contrasting findings. In relation with this, most investigations concerning a role for peripheral CRH only evaluated pre-stress administration of CRH-receptor antagonists. Clearly such results elucidate the role of CRH in an acute stress setting but extrapolating these data to post stress time points may not be appropriate; continued post-stress mast cell activation may depend on factors other than CRH. Here, we first evaluated whether there is such a thing as prolonged post-stress mast cell dependent visceral hypersensitivity in maternally separated rats. Subsequently, we used the CRH-receptor antagonist α-helical CRF (9-41) to compare the possible role of CRH in pre- and post-stress intervention protocols.

In a clinical trial with the supposed mast cell stabilizer ketotifen this compound decreased visceral hypersensitivity and improved intestinal symptoms in IBS patients.[18] However, when pre- and post-therapy rectal biopsies were compared for release of histamine and tryptase, results showed no signs of ketotifen induced mast cell stabilization. Since ketotifen is also a histamine-1-receptor (H1R) antagonist, these data suggested that the observed therapeutic effect depended on the blocking of this receptor. This may open up new possibilities for therapy because a long list of H1R antagonists is available for use in the treatment of allergic rhinitis and urticaria.[19] Importantly, in contrast to ketotifen, these second generation H1-antihistamines do not cross the blood-brain barrier; they are safe, effective and well tolerated. Therefore, in **chapter 4**, we tested 2 of these peripherally restricted H1R-antagonists (ebastine and fexofenadine) for their capacity to reverse post-stress visceral hypersensitivity.

IBS clusters in families, therefore an important line of international research is directed towards the identification of relevant genetic factors.[20] Although twin-studies confirmed that there is a genetic component in IBS, they also indicated that environmental factors have equal or perhaps even greater influence.[21-25] Similar conclusions can be drawn from studies showing an increased frequency of IBS in first degree relatives of IBS patients[26-30] genetic and intra-familial environmental factors may both play a role in the observed familial aggregation. Thus, IBS transfer across generations may largely depend on environmental factors. This is, however, difficult to establish in the human setting. Therefore, in **chapter 5**, we used our animal model to investigate whether susceptibility to stress

induced visceral hypersensitivity in maternal separated Long Evans rats can be transferred across generations without further separation protocols and, if so, whether this depends on maternal care. Finally, the possible role of mast cells in the post stress phenotype of these second generation animals was investigated by the use of the mast cell stabilizer doxantrazole.

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Chapter 2

Essential role for TRPV1 in stress-induced (mast cell-dependent) colonic hypersensitivity in maternally separated rats

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ABSTRACT

Irritable bowel syndrome is in part characterized by an increased sensitivity to colonic distension. Stress is an important trigger factor for symptom generation. We hypothesized that stress induces visceral hypersensitivity via mast cell degranulation and transient receptor ion channel 1 (TRPV1) modulation. We used the rat model of neonatal maternal separation (MS) to investigate this hypothesis. The visceromotor response to colonic distension was assessed in adult MS and non-handled (NH) rats before and after acute water avoidance (WA) stress. We evaluated the effect of the mast cell stabilizer doxantrazole, neutralizing antiserum against the mast cell mediator nerve growth factor (NGF) and two different TRPV1 antagonists; capsazepine (non-specific) and SB-705498 (TRPV1-specific). Immunohistochemistry was used to assess post-WA TRPV1 expression in dorsal root ganglia and the presence of immunocytes in proximal and distal colon. Retrograde labelled and micro dissected dorsal root ganglia sensory neurons were used to evaluate TRPV1 gene transcription. Results showed that acute stress induces colonic hypersensitivity in MS but not in NH rats. Hypersensitivity was prevented by prestress administration of doxantrazole and anti-NGF. Capsazepine inhibited and SB-705498 reversed poststress hypersensitivity. In MS rats, acutestress induced a slight increase in colonic mast cell numbers without further signs of inflammation. Post-WA TRPV1 transcription and expression was not higher in MS than NH rats. In conclusion, the present data on stress-induced visceral hypersensitivity confirm earlier reports on the essential role of mast cells and NGF. Moreover, the results also suggest that TRPV1 modulation (in the absence of overt inflammation) is involved in this response. Thus, mast cells and TRPV1 are potential targets to treat stress-induced visceral hypersensitivity.

INTRODUCTION

Irritable bowel syndrome (IBS) affects approximately 15% of the population. It is characterized by abdominal pain or discomfort associated with defecation or a change in bowel habit.[1] In 50–70% of patients the distal gastrointestinal tract has an increased sensitivity to distension, contributing to abnormal perception of pain and discomfort. This visceral hypersensitivity is regarded as an important pathophysiologal mechanism in IBS and indications are that mucosal mast cells may play an important role. Several studies indicated increased mast cell numbers in intestinal patient biopsies and mast cells are located closer to nerve fibres, a finding which correlates with the intensity of pain reported.[2] The supernatant of patient biopsies contains more mast cell mediators such as tryptase and histamine and stimulates calcium mobilization of cultured murine dorsal root ganglia (DRG) neurons. In mice, the intracolonic administration of these supernatants increased the in vivo response to colonic distension,[3] and ex vivo supernatant injection into mesenteric arteries supplying rat jejunum lead to enhanced firing of the mesenteric nerves.[4] Although these findings suggest an important role for mast cell mediators in the activation of sensory neurons in IBS, the trigger(s) leading to their release and the subsequent molecular mechanisms leading to neuronal excitation are yet to be identified. Others showed that stress can cause mast cell degranulation, subsequent gut barrier dysfunction[5] and hypersensitivity to colonic distension in rats.[6] Acute stress was also shown to induce peripheral release of mast cell mediators in humans[7] and is known to induce enhanced visceral perception in IBS patients.[8–10]

A potential mechanism via mast cell mediators induce visceral hypersensitivity could be the activation or modulation of transient receptor ion channel1 (TRPV1). This is a non-selective ligand-gated cation channel essential for selective modalities of pain sensation. TRPV1 is predominantly expressed on peripheral sensory neurons and can be activated by capsaicin, noxious temperature, extracellular low pH and high concentration of the endogenous cannabinoid anandamide.[11] In addition, several mast cell mediators capable of inducing enhanced sensitivity to colonic distension [e.g. serotonin,[12] tryptase[13] and nerve growth factor[14–17] (NGF)] are known to modulate TRPV1-mediated responses. Animal experiments suggest that TRPV1 is involved in mechanosensation[18,19] and visceral hypersensitivity induced by neonatal irritation of the colon[20] and hypersensitivity after intracolonic administration of zymosan.[21] Recently, increased TRPV1 expression on nerve fibres in recto-sigmoid biopsies of IBS patients was shown to correlate with the degree of abdominal pain.[22] These accumulative data suggest that stress-induced mast cell degranulation and subsequent TRPV1 modulation are crucial steps in the development of stress-related visceral hypersensitivity in IBS. We

investigated this hypothesis in the rat maternal separation (MS) model in which early life experience leads to stress-induced complaints later in life.[23–25]

MATERIAL AND METHODS

Animals

Long-Evans rats (Harlan, Horst, The Netherlands) were housed at the animal facility of the AMC (Amsterdam, The Netherlands) under conditions of controlled light (06:00–18:00 hours), temperature (20–22 °C) and humidity (45%) and kept in standard macralon cages with a layer of wood shavings. Water and food (SDS; Technilab BMI, Someren, The Netherlands) were available ad libitum. Non-handled (NH) and MS animals were bred in our own animal facilities.

Maternal separation

Primiparous pregnant rats reared NH male pups; second time pregnant dams reared male pups that were subjected to the MS protocol. MS dams were separated from the nest from postnatal day (PND) 2 to 14 for 3 h per day as described earlier.
Separation was achieved by placing the dams in another cage in a separate room. During the separation period the litter was placed under infrared light (27–30 °C). Weaning was performed on PND 22, rats were then raised in pairs of two, NH pups were nursed normally.

Measurement of the visceromotor response to colonic distension and data analysis

Colorectal distension in rats leads to reproducible contractions of abdominal musculature, the so called visceromotor response (VMR). The quantification of these contractions by electromyography (EMG) was often used to assess visceral pain responses in rodents. Here we used radiotelemetric transmitters to record the abdominal EMG signals in freely moving rats (described before in Welting et al.25). In short, the transmitter (Physiotel Implant TA10AE-F20; Data Sciences International, St Paul, MN, USA) was positioned in the abdominal cavity, the two connected electrodes were placed in the

abdominal muscles. During distension protocols, animals were placed in a standard macralon cage (exact size of the receiver) that was positioned on top of a receiver (Data Sciences International). The receiver was linked to a Biopac MP100 data acquisition system (Biopac Systems Inc., Santa Barbara, CA, USA) and a personal computer via a raw data analog converter (Data Sciences International). Data were acquired with AcqKnowledge software (Biopac Systems Inc.) and analysed as described before.[25] Briefly, each 20-s distension period and its preceding 20-s of baseline recording were extracted from the original raw EMG data file. After correction for movement and breathing, data were rectified and integrated. Absolute datasets were then obtained by subtracting the 20-s baseline recording from the 20-s distension result. Similar to other publications [23, 25] the final results are given as normalized data sets, which were calculated from the absolute data by setting the 2 mL value of the first (prestress) distension at 100%.

Colonic distension protocol, water avoidance and tissue collection

Colonic distensions were performed with a latex balloon (Ultracover 8F; International Medical Products, Zutphen, The Netherlands) and carried out as described before.[25] Insertion of the catheter was performed under brief isoflurane anesthesia. Distensions started after a 20-min recovery period. They were performed at the minimum age of 3 months and achieved by inflation of graded volumes of water (1.0, 1.5 and 2.0 mL). Length and diameter of the balloon during a 2-mL maximum volume distension were 18 and 15 mm respectively. After each 20-s distension period, water was quickly removed and an 80-s resting period was exercised. In order to anticipate possible pharmacological effects on compliance the pressure–volume relationship was determined in a subset (n = 5) of separated rats. As published earlier,[25] we used a polyethylene balloon connected to a slightly adjusted sphygmomanometer. Measurements were carried out before and after administration of the investigated compounds. The time span between measurements correlated with the experimental protocols described below.

In our earlier investigations we showed that acute water avoidance (WA)-stress induced enhanced sensitivity to colonic distension in MS rats and not in NH rats. Stress- induced hypersensitivity was still present at 24 h post-WA and was not induced by sham WA stress.[25] Therefore, whenever acute stress was applied in the present investigations, distensions and concurrent EMG recordings were performed just before and 24 h after 1 h WA stress during which rats were positioned on a pedestal surrounded by water. Directly after the last distension protocol rats were sacrificed and the

transmitter was removed. Because prevailing evidence indicates that spinal afferents are the intestinal nociceptors[26] and TRPV1+ nerve fibres in the gastrointestinal tract are mainly spinal in origin,[27] DRG T13-L2 [28,29] were dissected for further evaluation of TRPV1 expression.

VMR to colonic distension in MS and NH rats before and after WA (in vivo pharmacological interventions)

All protocols were approved by the Ethical Animal Research Committee of the University of Amsterdam. The mast cell stabilizer doxantrazole (gift of Agne` s Francois, Institut Gustave Roussy, Villejuif, France) was dissolved in 0.5% NaHCO3/0.9% saline, pH 7.5, and administered intraperitoneally (i.p., 10 mg kg-1) 30 min prior to the pre-WA distension protocol.
Separate groups of rats received vehicle alone. NGF neutralization was achieved by pre- and post-WA administration (T = -20 h, -30 min, +5 min and +23.5 h) of saline-diluted anti-NGF; 1 mL of rabbit polyclonal anti-NGF 2.5S (Sigma-Aldrich, Zwijndrecht, The Netherlands) was i.p. administered in a 1/2000 dilution.[30] Control experiments were carried out by administering 1 mL of control rabbit IgG (Sigma-Aldrich) to separate groups of NH and MS rats. The non-selective TRPV1 antagonist capsazepine (10 mg kg-1, Sigma-Aldrich) or vehicle alone [5% Tween 80, 5% ethanol, 20% dimethyl sulfoxide (DMSO), 70% phosphate-buffered saline] were administered (i.p.) 30 min prior to the last (24 h time point) distension protocol. The selective TRPV1 antagonist SB-705498 (Glaxo Smith Kline, Stevenage, UK) was evaluated in a different manner. After measuring the pre-WA and 24 h post-WA response to distension in MS rats, SB-705498 (3 and 30 mg kg-1, Glaxo- SmithKline or vehicle alone (DMSO) was administered (i.p.) 30 min prior to an additional 25 h post-WA distension protocol.

Immunohistochemistry

Staining protocols The following antibodies were used in this study: mouse anti-rat CD3 (pan T-cell marker, 1/30; Serotec, Oxford, UK), mouse anti-rat ED1 (pan-macrophage marker, 1/100; Pharmingen, Alphen a/d Rijn, The Netherlands), mouse anti-rat RMCP2 (mucosal mast cells, 1/750; Moredun Scientific, Pinicuik, Scotland), rabbit anti-TRPV1 (1/1000; Abcam, Cambridge, UK). Paraffin (RMCP2) and freeze sections (CD3/ ED1) were cut from proximal and distal colon,

processed for staining and incubated with monoclonal antibody, horseradish peroxidase-labelled (Po) goat-anti mouse (1/200; Dako, Glostrup, Denmark) and swine anti-goat-Po (1/200; Biosource, Camarillo, CA,USA) respectively. Snap-frozen tissue of DRG was cut, processed for staining and incubated with polyclonal anti- TRPV1, biotin-labelled goat-anti-rabbit (1/200; Dako) and avidin–biotin complex (ABC kit, Dako). Po activity was visualized with 3-amino-9-ethyl-carbazole (Sigma-Aldrich, St Louis, MO, USA) and sections were counterstained with Mayer's haematoxylin before being mounted in glycerine–gelatine (Dako). Serial sections of frozen DRG tissue were also stained by classical Nissl staining (staining all DRG neurons).

Assessment of per cent mast cells, T-cells and macrophages in colonic mucosa of NH and MS rats Proximal and distal colonic tissue used for these stainings was obtained from rats that were evaluated earlier for their development of stress-induced visceral hypersensitivity in time.[25] Shortly, NH and MS rats were subjected to either WA or sham-WA (empty tank), and 10 rats per group were evaluated. In these experiments WA-treated MS rats became hypersensitive to colon distension, sham-WA had no effect on the VMR. Tissue samples were taken 24 h after WA or sham-WA and from each sample three non-serial sections were evaluated. Earlier we validated a digital image analysis (DIA) system for the counting of immunocytes in rat colonic tissues (article in preparation) and T-cell and macrophage stainings were fully evaluated by this system. The operator of the DIA system (DvdC) was blinded to the experimental groups. Technical details and usage of the DIA system were described before.[31] In case of T-cells, cell numbers were expressed as % of CD3+ cells. Because ED1 staining was more disperse, the integrated optical density (IOD, which is proportional to the total amount of protein staining[32]) was determined for this antigen. The validation study showed variable staining intensities for RMCP2+ mast cells, which made accurate fully automated counting impossible. Therefore, we used a combination of DIA and manual counting to evaluate mast cell numbers. DIA was used to automatically count the total number of haematoxylin+ cells per field, whereas the number of RMCP2+ cells was counted manually by evaluating the exact same photographs that were also used by the DIA system. In addition, these pictures were used to evaluate IOD (acquired with DIA) of RMCP2 expression. Division of IOD by the number of RMCP2+ cells resulted in semi-quantitative data for the average level of RMCP2 per mast cell. In all stainings only cells present in the mucosal layer were evaluated.

Assessment of per cent of TRPV1+ neurons in DRG For TRPV1 stainings we used sections obtained from DRG of the mast cell stabilization experiment (collected 24 h post-WA) and evaluated NH and MS rats treated with either vehicle or doxantrazole (n = 7 per group). After selection of identical areas, serial sections stained for either TRPV1 or by Nissl were photographed and

counted by manual tag point counting (Image-pro plus software; Media Cybernetics, Siver Spring, MD, USA). A minimum of 100 neurons was evaluated for each rat (average 145 Nissl+ neurons/rat) and the percentage of TRPV1+ neurons was calculated.

Quantification of TRPV1 gene transcription in laser captured DRG (T13-L2) sensory neurons

Retrograde labelling of sensory afferents Surgical interventions needed for intramural injection of tracer may lead to unwanted manipulation of the gut, inducing low grade inflammation and alterations in gene expression.[33,34] We recently showed that all neurons labelled through subserosal administration of retrograde tracer were also labelled by i.p. injection.[35] Moreover, the validity of i.p. tracer administration to identify visceral afferents was also shown by others.[36, 37] Thus, we preferred i.p. injections with the fluorescent tracer Fluoro-Gold (1.0 mg kg-1); Fluorochrome, Den ver, CO, USA). Rats were injected with tracer at day 0, subjected to WA at day 2 and sacrificed on day 3 post Fluoro-Gold application (optimal time for labelling was determined in pilot experiment). DRGs T13-L2 were dissected, quickly frozen in tissue freezing medium and stored in -80°C. Cryostat sections of 12 lM were cut and fluorescent cells were laser microdissected (PALM Micro- laser Technologies, Bernried, Germany). Cells were catapulted into 100 lL RNeasy lysis thiocyanate/b-mercaptoethanol buffer (Qiagen, Hilden, Germany) and processed for real time quantitative polymerase chain reaction (RTQ-PCR).

RNA purification and amplification

After mixing with one volume of 70% ethanol, lysates were immediately transferred onto RNeasy microcolumns (Qiagen). Total RNA was extracted as described by the provider (without DNase treatment). Next, purified RNA samples were used for two rounds of amplification using the Affymetrix two-round Amplification Kit (Affymetrix, Santa Clara, CA, USA). The yield of amplified RNA was measured by Nanodrop (Nanodrop Technologies, Rockland, DE, USA) and quality assessed on the Agilent Bioanalyzer (Agilent Technologies, Palo Alto, CA, USA). Sufficient high quality RNA for subsequent RTQ-PCR was obtained for seven MS and NH samples.

cDNA synthesis and Real-time quantitative PCR for TRPV1 and TrkA

Four micrograms of amplified RNA served as template for cDNA synthesis using random hexamer primers and Superscript III reverse transcriptase in a volume of 20 lL for 1 h at 50°C. The enzyme was subsequently inactivated at 70°C (for 15 min), according to the recommendations of the manufacturer (Invitrogen, Carlsbad, CA, USA). Quantitative PCR was performed on an ABI Prism 7900-HT Sequence Detection System (Applied Biosystems, Foster City, CA, USA) using a qPCR core kit w/o dUTP (Eurogentec, Seraing, Belgium). Thermal cycling conditions were 10 min at 95°C, followed by 40 cycles of 15 s at 95°C and 1 min at 60°C. Validated predesigned Taqman Gene Expression Assays (Applied Biosystems), corresponding to the housekeeping genes Ppib (Rn00574762_m1), Hmbs (Rn00565886_m1) and Pgk1 (Rn00821429_g1), and the genes of interest Trpv1 (Rn01460299_m1 and Rn00583117_m1) and TrkA (Ntrk1, Rn00572130_m1) were used to generate standard curves on serial dilutions of cDNA. Next, the relative standard curve method was used to calculate the expression values, and GeNorm software (http://genomebiology.com/2002/3/7/research/0034/) was applied to identify the most stably expressed housekeeping gene (Ppib, Hmbs, or Pgk1). Based on this analysis, the relative expression values for genes of interest (Trpv1, Ntrk1) were calculated by normalization for Ppib.

Western blotting for NGF

Proximal and distal colon obtained from control rabbit-IgG-treatedNH and MS rats was evaluated for NGF expression (tissue collected 24 h post-WA). Equal amounts of tissue were homogenized in lysis buffer (Cell Signaling, Danvers, MA, USA). After samples were spun down, supernatants were taken up in mercaptoethanol containing sample buffer. Equal volumes were then loaded on a 12.5% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (with prestained molecular weight marker in separate lane), separated and transferred to Immobilon polyvinylidene difluoride membrane (Millipore, Amsterdam, and The Netherlands). After blocking with 5% milk solution, membranes were cut between 30 and 40 kDa and incubated with rabbit anti-NGF (Santa Cruz biotechnology, Santa Cruz, CA, USA, 1/100) or rabbit-anti-actin (Santa Cruz, 1/20000). Upon washing, peroxidase labelled secondary antibody (goat anti-rabbit, 1/100; Dako) was added for 1 h. Excessive antibody was again removed by washing and bands were visualized with Lumi-light plus (Roche Diagnostics, Almere, The Netherlands). Densitometric analyses were carried out with the image processing program ImageJ (http://rsb.info.nih.gov/ij/) and results expressed as NGF/ actin pixel density.

Statistical analysis

Statistical calculations were performed using SPSS for windows (version 11.5.2; SPSS Inc., Chicago, IL, USA). VMR data were analysed with the Wilcoxon-signed ranks test which was applied for the area under the curve (AUC) of the relative response (normalized data) to colonic distension.[25] Statistical differences in immunohistochemical, western and RTQ-PCR evaluations were assessed by Mann–Whitney U-test.

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RESULTS

In vivo mast cell stabilization

I.p. administration of vehicle alone or the mast cell stabilizer doxantrazole 30 min prior to WA did not affect the post-WA sensitivity measurements in NH rats (Fig. 1A). When MS rats were treated with vehicle alone we observed an enhanced VMR to distension after WA (Fig. 1B, *P = 0.018). In contrast, pre-WA administration of doxantrazole prevented the increased response over baseline in the MS rats (Fig. 1B). Doxantrazole did not lead to compliance changes in treated animals (data not shown).

In situ mast cell numbers and RMCP2 expression levels

The percentage RMCP2+ cells in proximal and distal colon is graphically depicted in Fig. 1C. At baseline (-WA), there were no significant differences between NH and MS rats. WA induced a significant increase in the percent of RMCP2+ cells in the proximal colon of separated animals [3.84 ± 0.43 vs 5.27 ± 0.42 (mean ± - SEM), **P = 0.019]. The average expression level of RMCP2/cell (as determined by image analysis) is given in Fig. 1D. At baseline (-WA), proximal colon mast cells of MS rats expressed higher levels RMCP2/cell than those of NH rats [IOD/cell: 146 ± 29 vs 67

± 9 (SEM), #*P = 0.036]. WA induced a significant decrease in RMCP2 expression in proximal colon of MS rats (146 ± 29 vs 67 ± 7, #P = 0.015). Example stainings illustrating high vs low RMCP2 expressing cells are given in Fig. 6A, B.

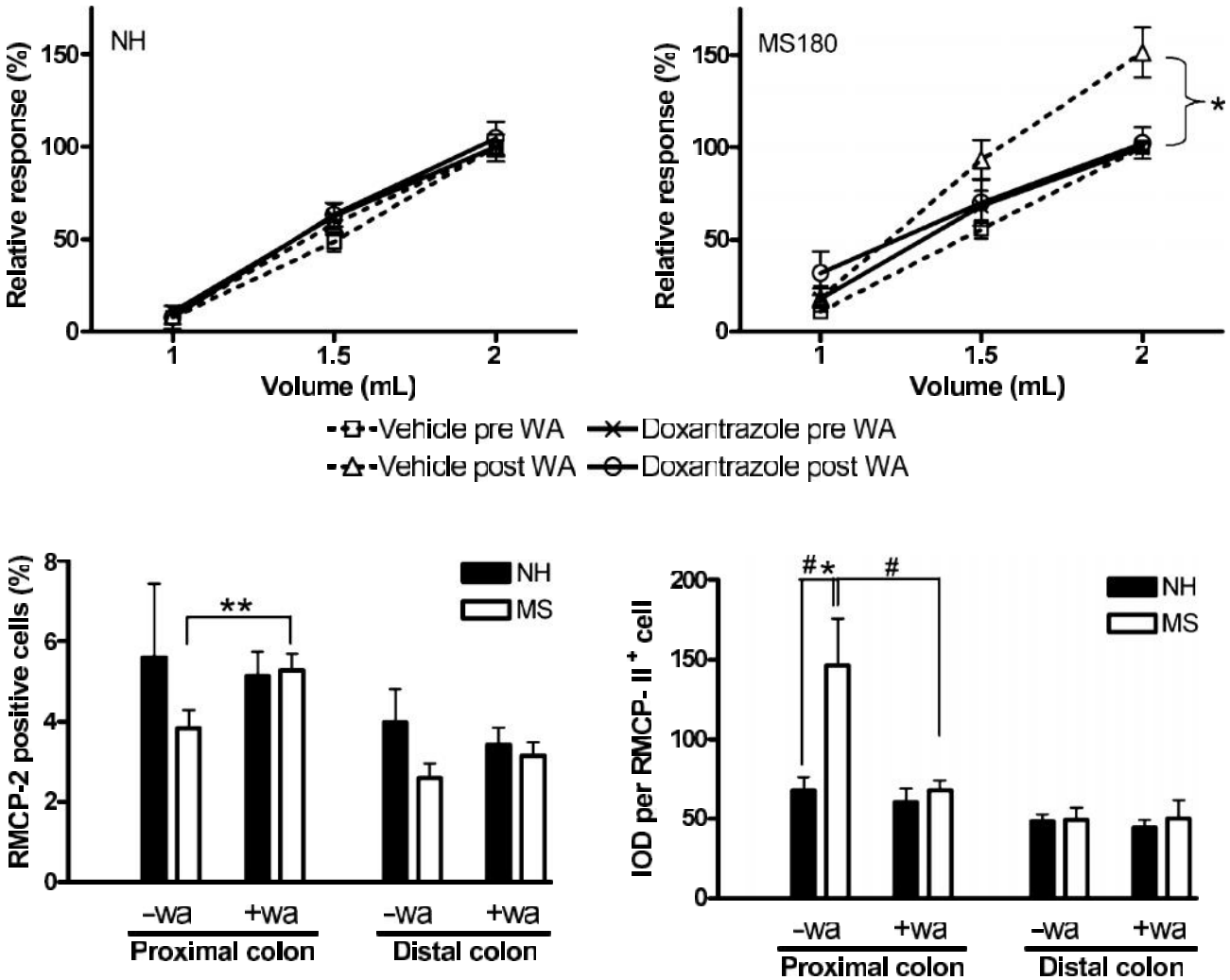

Figure 1 Visceromotor response (VMR) before and after acute water avoidance (WA) stress in non-handled (NH) and maternally separated (MS) rats. n = 7 per group except for doxantrazole-treated NH rats; n = 10. Values are mean ± SEM, differences in area under curve were evaluated for statistical significance. Doxantrazole was administered 30 min before WA. NH rats remained normo-sensitive upon WA irrespective of vehicle or doxantrazole treatment (A). In MS rats a significant increase over baseline AUC was observed with vehicle alone (1B, dotted lines, *P = 0.018), this increase in VMR was absent when rats were pretreated with doxantrazole. Immunohistochemical evaluation of RMCP2 in stressed (+WA) and sham-stressed (-WA) rats is summarized in histograms (C and D; n = 10 per group, data depicted as mean + SEM). Histogram C shows the % of mucosal RMCP2+ mast cells; baseline numbers (-WA) are not significantly different between NH and MS rats. WA

induced a statistically significant influx of mast cells in the proximal colon of MS rats (**P = 0.019). D shows enhanced RMCP2 expression per mast cell in proximal colon of MS rats (#*P = 0.036). WA induces a significant decrease in this expression level (#P = 0.015) IOD, integrated optical density.

In vivo NGF neutralization

I.p. administration of neutralizing rabbit anti-NGF serum or control rabbit IgG had no effect on post-WA sensitivity in NH rats (Fig. 2A). When MS rats were pretreated with control IgG, WA induced an enhanced response to colonic distension (Fig. 2B, *P = 0.042). In contrast, when MS rats were pretreated with anti-NGF serum, WA was no longer capable of inducing an enhanced response to distension (Fig. 2B). The use of anti-NGF serum did not induce compliance changes (data not shown).

NGF expression levels (western blotting)

Figure 2C shows different anti-NGF staining intensities for homogenized distal colon of NH and MS rats. The quantification of NGF/actin pixel density for proximal and distal colon is given in Fig. 2D. Post-WA NGF expression in distal colon of MS rats was significantly lower than that of NH rats (NH vs MS; 1.51 ± 0.07 vs 0.86 ± 0.13, P = 0.002), no differences were observed in proximal colon.

In vivo TRPV1 inhibition with capsazepine

The non-selective TRPV1 antagonist capsazepine was administered just prior to the last (24 h post-WA) distension protocol. Capsazepine and vehicle-treated NH rats remained normosensitive to colonic distension after WA stress. In MS rats, capsazepine treatment was able to inhibit the stress-induced increase in VMR to colonic distension. An enhanced response could still be observed in the vehicle-treated MS rats [please refer to supporting information for graphs (S1) on capsazepine treatment]. No changes in compliance were observed when comparing pressure– volume curves before and after capsazepine administration (data not shown).

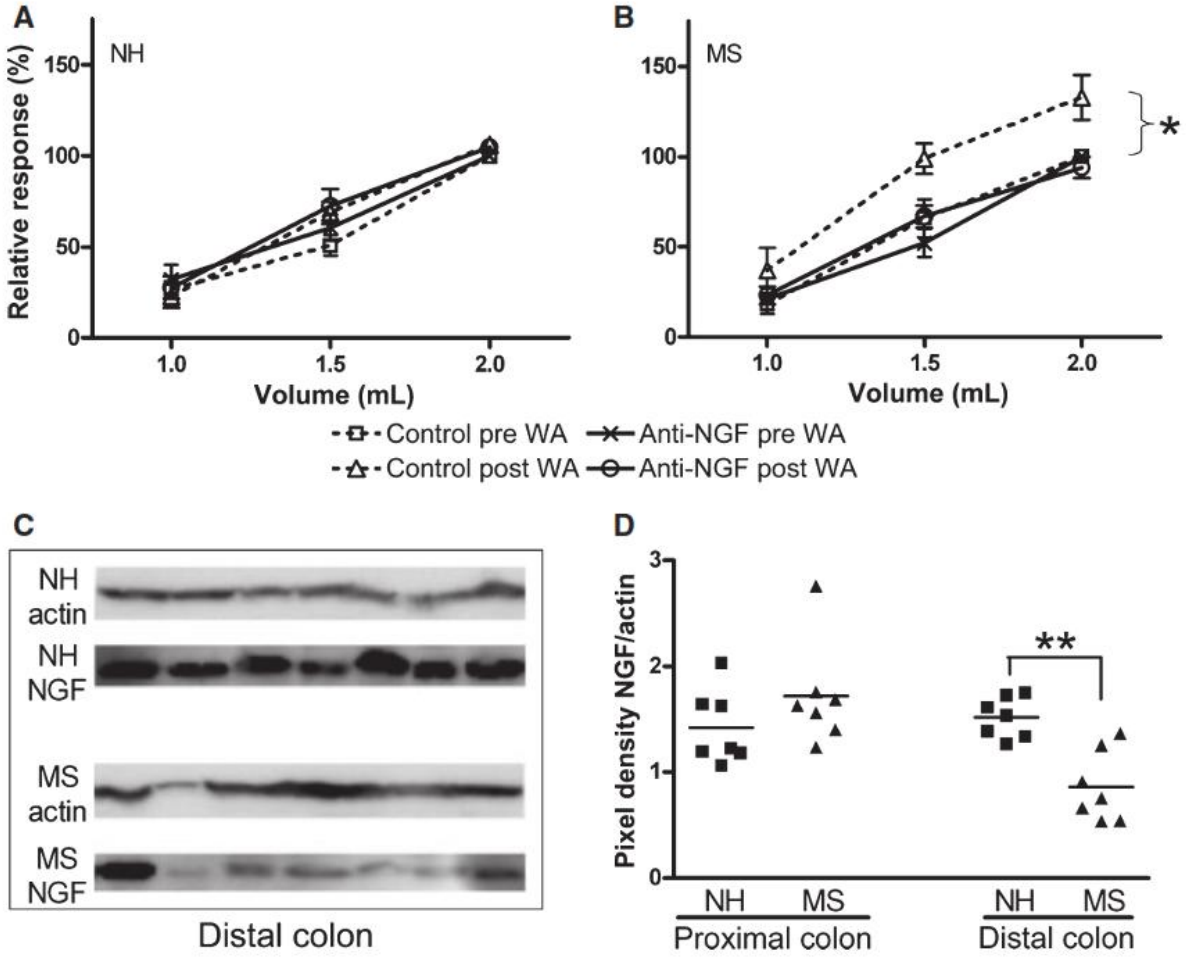

Figure 2 Control serum and anti-nerve growth factor (NGF) serum did not affect the non-handled (NH) groups, these rats remained normo-sensitive upon water avoidance (WA) (A). Maternally separated (MS) rats treated with control serum became hypersensitive upon WA (B, C, D, dotted lines, *P = 0.042) whereas anti-NGF treatment prevented the enhanced response to distension (straight lines, B). Tissue of control serum-treated rats was collected after the last distension protocol and prepared for western blotting with anti-NGF. Staining results for distal colon are shown in C and results of densitometric analysis (distal and proximal colon) are summarized in D. Post- WA NGF expression in distal colon of MS rats is lower than that of NH rats (**P = 0.002).

In vivo TRPV1 inhibition with SB-705498

An enhanced VMR to distension was observed in MS rats at the 24 h post-WA time point (Fig. 3A, B, C; pre-WA vs 24 h post-WA, *P = 0.008 in all three cases). To reverse the observed hypersensitivity, rats were then treated with either vehicle alone or SB-705498 and distensions were repeated 25 h post-WA. Rats treated with vehicle alone (Fig. 3A) or 3 mg SB-705498 per kg (Fig. 3B) remained hypersensitive to distension (pre-WA vs 25 h post-WA; #P = 0.039 and XP = 0.016 respectively).

In contrast, 30 mg SB-705498 per kg reversed the response to the pre-WA level (Fig. 3C, 24 h post-WA vs 30 min post-treatment; P = 0.008). Fig. 3D summarizes the data obtained with compound SB-705498, area under the curve was depicted instead of relative response.

Immunohistochemical evaluation of TRPV1$^+$neurons in (post-WA) DRG

No statistical differences were observed when comparing the percentage of TRPV1+ neurons in T13-L2 DRG sections of vehicle-treated NH and MS rats [43.6 ± 3.6 vs 37.4 ± 3.5 (SEM) respectively] or doxantrazole- treated rats (43.6 ± 1.4 vs 38.6 ± 2.1). There were also no significant differences when comparing vehicle- and doxantrazole-treated animals within one group (NH or MS). Results are summarized in Fig. 4A and an example TRPV1 staining is given in Fig. 6C.

Quantification of post-WA TRPV1 and TrkA gene transcription in laser captured sensory neurons

Fig. 4B, C show results of RTQ-PCR experiments conducted for TRPV1 (Rn00583117_m1, Fig. 4B) and TrkA (Fig. 4C). No significant differences were observed when comparing relative expression values (normalized for the Ppib housekeeping gene) in retrograde-labelled DRG sensory neurons form NH and MS rats. Results obtained with TRPV1 gene expression assay Rn01460299_m1 (results not shown) were similar to those depicted in Fig. 4B; we observed no significant difference between NH and MS rats

Immunohistochemical evaluation of colonic macrophage and T-cell numbers

Fig. 5A shows that there is no difference in the baseline number of macrophages between MS and NH rats. Similarly, WA stress did not lead to increased numbers of macrophages. The evaluation of T-cell stainings is depicted in Fig. 5B; the proximal and distal colon of sham stressed NH rats contained significantly more CD3+ cells than those of MS rats [10.3 ± 2.1 vs 5.7 ± 2.4 and 2.8 ± 0.5 vs 1.9 ± 0.4 (SEM), *P = 0.023 and **P = 0.044 respectively]. No further differences were observed in these stainings.

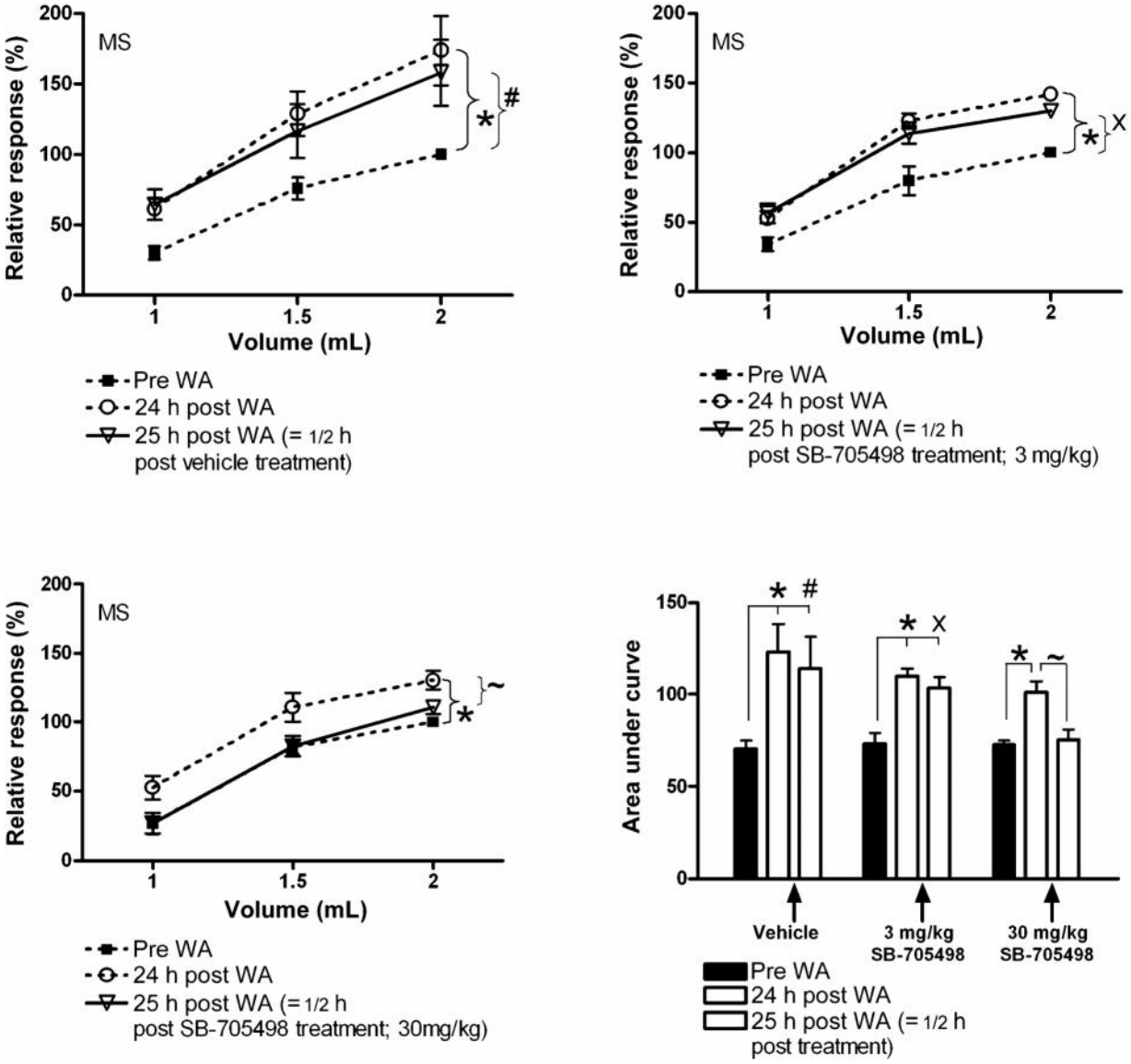

Figure 3 Acute water avoidance (WA) stress in maternally separated (MS) rats (n = 8 per group); intraperitoneal SB-705498 was administered 30 min after the first post-WA distension protocol which was performed at T = 24 h. Post-treatment measurements were performed at T = 25 h. Values are mean ± SEM, differences in area under curve were evaluated for statistical significance. WA induced an enhanced response to distension at the 24 h time point in all three treatment groups (4A, B and C, dotted lines, *P = 0.008 for all groups). Treatment with vehicle alone or 3 mg SB-705498 per kg was unable to reverse the enhanced visceromotor response (VMR) at the 25 h time point (4A and B, P = 0.039 and xP=0.016 respectively). Administration of 30 mg SB-705498 per kg completely reversed the enhanced VMR to the pre-WA level (4C; 24 h post-WA vs 25 h post-WA, P = 0.008). Changes in area under the curve for the different treatment protocols are depicted in 4D (*P = 0.008, #P = 0.039, xP = 0.016 and P = 0.008).

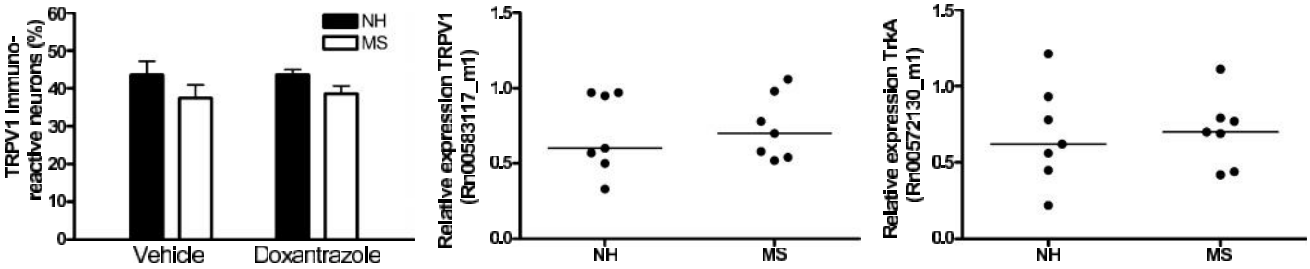

Figure 4 Summary of immunohistochemical evaluations for transient receptor ion channel 1 (TRPV1) on dorsal root ganglions obtained from the mast cell stabilization experiment. Tissue collected 24 h post-WA, n = 7 in each group (no retrograde labelling was performed). No significant differences were observed when comparing vehicle-treated non-handled (NH) and maternally separated (MS) rats or doxantrazole-treated NH and MS rats (5A). Graphs shown in 5B and 5C depict relative expression values for genes Ntrk1 (TrkA) and TRPV1 (both normalized for Ppib) in samples of retrograde labelled and microdissected sensory neurons. Tissue was collected 24 h post-WA. There are no significant differences between NH and MS rats.

DISCUSSION

Hypersensitivity to visceral distension can be demonstrated in the majority of IBS patients. Enhanced sensitivity may arise due to aberrant peripheral or central mechanisms, or a combination of both. Indeed, acute stress leads to visceral hypersensitivity in human and in animal models[6, 8–10] and indications are that peripheral stress-induced degranulation of mast cells may be involved.[5–7] Early life stressors are known to contribute to IBS in adults[38] and the rat neonatal MS model is one of the different animal models used to investigate the role of early life trauma.[39] Therefore, we used this model to further demonstrate the role of mast cells and to establish the possible role of the TRPV1 ion channel in stress-induced visceral hypersensitivity.

Earlier studies have shown that MS in Long-Evans rats led to stress-induced visceral hypersensitivity and motility changes at adult age.[23, 25] Indeed, in the present investigations, a 1-hour WA stress induced an enhanced VMR to distension in adult MS and not in NH rats. Others have shown that during stress responses, peripheral mast cells may be the cellular link between brain and gut[5–7, 40] and several studies suggested that increased intestinal mast cell numbers were relevant to the pathophysiology of IBS.[2] Here, the use of the mast cell stabilizer doxantrazole confirmed their role in stress-induced hypersensitivity to distension, but baseline mast cell numbers did not seem to be enhanced in MS rats. Because colonic tissue was collected directly after the last

distension protocol, distension-induced degranulation might have rendered these cells undetectable by RMCP2 staining, hereby explaining the equal mast cell numbers in distal colon. On the other hand, when Cenac et al.3 compared mast cell numbers in IBS and normal control biopsies, there were also no differences. Nevertheless, their results suggested an imported role for these cells in IBS; mast cell mediator release from IBS biopsies was not only higher but also sufficient to induce visceral hyperalgesia in mice upon intracolonic administration of supernatants.[3] Our immunohistochemical evaluations of RMCP2 staining intensities also suggested that elevated mediator content and release might play a role. Average RMCP2 content per mast cell was more than doubled in (non-distended) proximal colon of MS rats and significantly reduced upon WA. These data suggest that for mast cells to be relevant in inducing visceral hypersensitivity their numbers need not necessarily be enhanced. Importantly, similar conclusions could be drawn from results obtained in the postinflammatory IBS model that was used by La et al.41 Despite these considerations, we did observe a slight WA-mediated influx of mast cells in the proximal colon of MS rats, but post-WA mast cell numbers still equalled those of NH rats. The observed influx may be an epiphenomenon related to the local stress-induced release of NGF which is a known mast cell chemoattractant.[42]

Which peripheral mediator is the actual trigger for the degranulation of colonic mast cells was not addressed here. However, based on earlier studies, corticotropin releasing hormone (CRH) is a likely candidate. Larauche et al.43 showed that stress-induced visceral hypersensitivity in rats could be inhibited by subcutaneous administration of the peripherally acting CRH antagonist, astressin. Similarly, changes in colonic barrier function induced by stress and subsequent mast cell degranulation were prevented by the peripheral use of the non-selective CRH-antagonist, α-helical CRH.[9, 41, 44, 45] Recent ex vivo investigations in human colonic biopsies confirmed that peripheral CRH was capable of inducing colonic mast cell degranulation.[46]

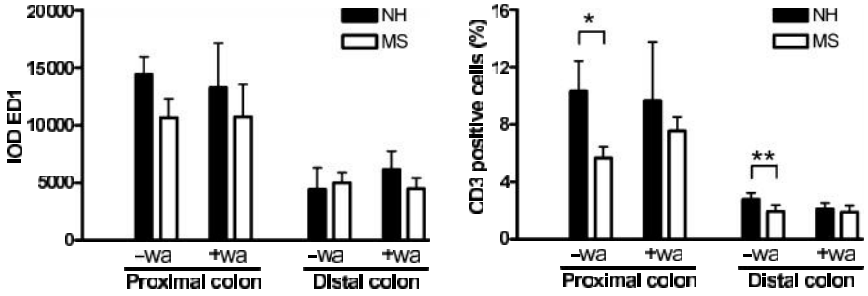

Figure 5 Immunohistochemical evaluations for ED1 (pan-macrophage) and CD3 (pan-T cell), n = 10 per group. Integrated optical density (IOD) for macrophage-expressed ED1; at baseline no statistical differences between maternally separated (MS) and non-handled (NH) rats, water avoidance (WA) did not change ED1 expression (6A). Per cent of CD3+ T cells compared with MS rats, baseline numbers were higher in proximal and distal colon of NH rats (*P = 0.023 and **P = 0.044 respectively). WA did not induce a significant increase of CD3+ T cells in NH and MS animals.

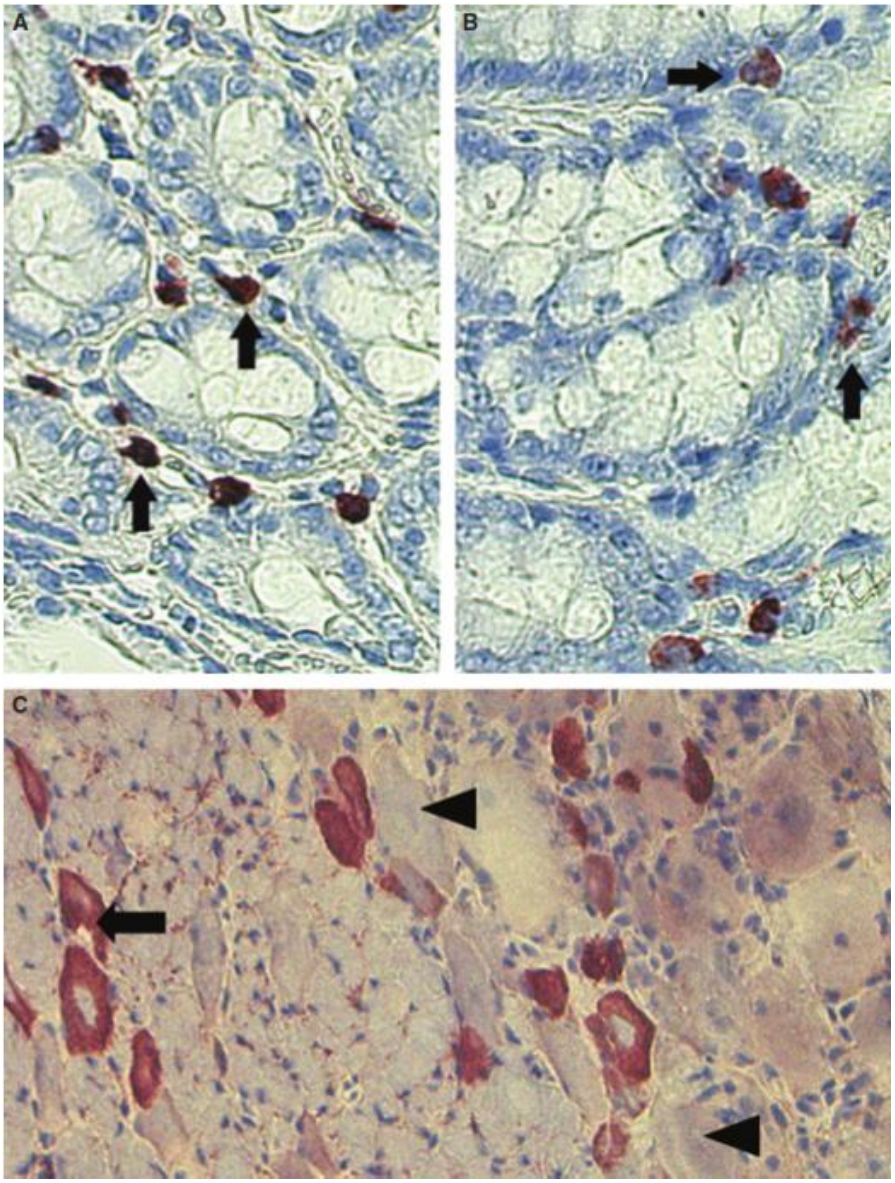

Figure 6

Representative examples of immunohistochemical stainings. Arrows indicate RMCP2-positive cells in colonic tissue obtained 24 h post-sham water avoidance (WA; 6A and B). Note the difference in staining intensity between maternally separated rats (A) and non-handled rats (B). Transient receptor ion channel 1 (TRPV1) staining in dorsal root ganglion obtained 24 h post-WA (C). Arrowheads indicate TRPV1-negative neurons and the arrow shows an example TRPV1 positive neuron. Neurons were defined by Nissl staining in a serial section (not shown).

During degranulation, mast cells release mediators (e.g. serotonin, tryptase and NGF) known to induce enhanced sensitivity to colonic distension.[2, 4] Here, we observed that anti-NGF treatment of adult MS Long-Evans rats resulted in complete inhibition of the post-WA hypersensitivity response. Post-WA NGF expression levels were then evaluated in control IgG- treated NH and MS animals. Results showed equal levels in homogenized samples of proximal colon but lower levels in distal colon of MS rats. These data might reflect release (and subsequent breakdown) of NGF by degranulating mast cells. Why this affects distal and not proximal colon is unclear, especially in regards to the RMCP2 data. This discrepancy could also indicate that NGF was not released by mast cells themselves, but functions as a mast cell degranulator instead. However, data obtained by Barreau et al.[40, 47] who also investigated the role of NGF in a MS model, suggested that NGF was mast cell derived indeed. Interestingly, their observations were carried out in Wistar rats in which it was also shown that upon MS, there was a closer association between mast cells and nerves.[48] Although this could also contribute to increased sensitivity to mast cell degranulation, we did not investigate this possibility in our Long-Evans rats.

When we assessed post-WA expression levels of NGF receptor TrkA in sensory neurons, we observed no differences between NH and MS rats. Earlier, similar data were obtained in MS Wistar rats.[40]

Although this suggests that aberrant levels of expression are not relevant, possible changes on the level of TrkA signalling cannot be excluded on the basis of these data. Recent investigations indicated that the neurotrophin receptor homolog (NRH2) is able to form a receptor complex with TrkA. This not only leads to high-affinity NGF binding sites but also influences TrkA signalling by enhanced activation of the Ras-Raf-Erk mitogen-activated protein kinase pathway.[49] It was shown that blocking of this pathway abrogated NGF-dependent capsaicin sensitivity[50] and heat hyperalgesia.[16] As these phenomena were TRPV1-dependant, enhanced formation of TrkA/NRH2 complexes in MS rats might play a role in TRPV1-dependant visceral hypersensitivity described in the current investigation. Here, TRPV1 dependency of stress-induced visceral hypersensitivity was first investigated with the frequently used antagonist capsazepine. It was administered just prior to the second (post-WA) distension protocol and inhibited the enhanced response to colonic distension. Because capsazepine is not selective for TRPV1,[51, 52] additional experiments were then carried out with the potent and selective antagonist SB-705498.[53, 54] This compound is known to block in vitro TRPV1 activation by capsaicin, low pH and temperature. Moreover, in an experimental somatic pain model in healthy volunteers it alleviated heat-evoked pain and capsaicin-induced skin sensitization.[55] This recent report was the first to show pharmacological effects of a

TRPV1 antagonist in humans. In the present study, SB-705498 was able to reverse in vivo stress-induced visceral hypersensitivity in MS rats.

Although these data show that TRPV1 plays an important role in stress-induced hypersensitivity to distension, the present investigations did not clarify the exact molecular mechanisms involved. In relation to this, it is known that NGF-mediated modulation of TRPV1 can be achieved via (i) the induction of enhanced numbers of TRPV1-expressing neurons[17] increased TRPV1-expression in individual neurons[15], (ii) via TRPV1 phosphorylation[56] or (iii) via enhanced TRPV1 trafficking to the cell membrane.[56] Here we evaluated the first possibility. Retrograde-labelling experiments performed by others have indicated that in rats, in contrast to mice, afferents of the distal colon projected mostly to T13-L2.[28, 29] Our immunohistochemical evaluation of T13-L2 DRGs did not reveal enhanced numbers of TRPV1+ neurons in MS rats. It should, however, be noted that our methodology cannot distinguish visceral from somatic projections and this may be relevant. On the other hand, when Miranda et al.57 used the same approach in a colitis model they observed an increased percentage of TRPV1+ neurons which was associated with visceral hypersensitivity and colonic inflammation. The absence of inflammation in our model might thus explain why the number of TRPV1+ neurons was not increased in MS rats. Importantly, evaluation of TRPV1 mRNA expression levels in retrograde labelled and laser microdissected DRG sensory neurons also failed to show differences in TRPV1 transcription. Similar results have been reported in a combined postinfectious/stress model where vagal instead of DRG afferent neurons showed notable changes in TRPV1 gene expression.[58] Thus, we speculate that stress-induced and TRPV1-dependent hypersensitivity to colonic dis- tension in the separation model may be due to enhanced TRPV1 phosphorylation[56, 59] and/or trafficking to the surface membrane[56] of spinal sensory neurons. Obviously, the exact TRPV1 modulatory mechanisms have to be explored in further investigations.

In a recent publication, Winston et al. 20 showed that a mild chemical irritation (diluted acetic acid) of neonatal colon will also lead to TRPV1-dependent hypersensitivity to distension at adult age. Additionally, TRPV1 antagonism in the neonatal period prevented the development of enhanced sensitivity in adults. At present it is unknown whether neonatal TRPV1 modulation is also essential in the MS model. However, it was shown earlier that daily neonatal NGF treatment of male Wistar rats mimicked MS-induced alterations (hypersensitivity and barrier dysfunction) in adult animals. In contrast, neonatal anti-NGF treatment abolished separation-induced effects.[40] Thus, it is possible that neonatal NGF- mediated TRPV1 modulation is also relevant in MS. In the aforementioned neonatal irritation model it was also shown that hypersensitivity was associated with increased TRPV1

mRNA and enhanced protein expression in whole DRG extracts and increased numbers of TRPV1-expressing DRG neurons.[20] As discussed earlier, such enhanced TRPV1 gene expression was not observed in MS Long-Evans rats. This suggests that the TRPV1 ion channel plays a central role in both hypersensitivity models but most likely via different ways of TRPV1 modulation. Further, in both investigations TRPV1 antagonism did not alter the baseline sensitivity of control rats. These in vivo data suggests that TRPV1 only plays a role in mechanical sensitization of sensory neurons under pathological conditions.

The results of Winston et al.20 also showed that TRPV1 activation was not necessarily associated with inflammation. Our data on mucosal CD3+ T-cell and macrophage numbers corroborated this observation. In fact, baseline T-cell numbers were low in proximal colon of MS rats. Earlier, MS in monkeys also led to reduced peripheral T-cell numbers associated with long-term suppression of cell-mediated immune responses.[60] From the current results it does not become clear whether relevant qualitative differences (e.g. cytokine expression profiles) exist between T cells of MS and NH rats. Nevertheless, the present data do indicate that at least in this mast cell-dependent animal model, augmented T-cell numbers are not a prerequisite for TRPV1-dependent visceral hypersensitivity.

In conclusion, this study supports the hypothesis that stress-induced visceral hypersensitivity in MS Long-Evans rats occurs in the absence of overt inflammation and dependents on mast cell degranulation and subsequent TRPV1 activation. These findings underline the importance of bidirectional brain–gut interactions in colonic hypersensitivity and identify mast cells and TRPV1 as potential targets for future therapeutical intervention strategies in IBS.

SUPPORTING INFORMATION

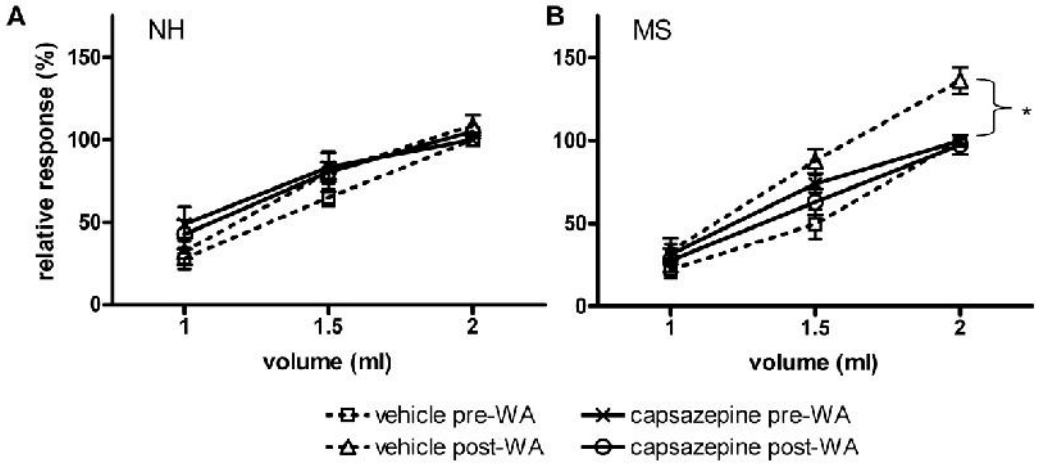

Figure S1. Visceromotor response (VMR) before and after acute water avoidance (WA)-stress in non-handled (NH) and maternally separated (MS) rats (n = 7/group); i.p. capsazepine was administered 30 minutes prior to the 2nd distension protocol. Values are mean +/- SEM, differences in area under curve were evaluated for statistical significance. NH rats remained normo-sensitive upon WA, irrespective of vehicle or capsazepine treatment (A). MS rats were hypersensitive to post-WA colonic distension when pretreated with vehicle alone (B, dotted lines, *P = 0.043), capsazepine treated rats became normo-sensitive (B, straight lines).

ACKNOWLEDGMENTS

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Chapter 3

Peripheral α-helical CRF (9-41) does not reverse stress-induced mast cell dependent visceral hypersensitivity in maternally-separated rats

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ABSTRACT

BACKGROUND:

Acute stress-induced hypersensitivity to colorectal distension was shown to depend on corticotropin releasing factor (CRF)-induced mast cell degranulation. At present it is unclear whether CRF also induces chronic post-stress activation of these cells. Accordingly, the objective of this work was to compare pre- and post-stress CRF-receptor antagonist treatment protocols for their ability to, respectively, prevent and reverse mast cell dependent visceral hypersensitivity in a rat model of neonatal maternal separation.

METHODS:

The visceromotor response to colonic distension was assessed in adult maternally-separated and non-handled rats before and at different time points after 1 hour of water avoidance (WA). Rats were treated with the mast cell stabilizer doxantrazole and the CRF receptor-antagonist α-helical-CRF(9-41). Western blotting was used to assess mucosal protein levels of the mast cell protease RMCP-2 and the tight junction protein occludin.

KEY RESULTS:

In maternally-separated, but not in non-handled rats, WA induced chronic hypersensitivity (up to 30 days) to colorectal distension. Visceral hypersensitivity was prevented but could not be reversed by administration of α-helical-CRF (9-41). In contrast however, the mast cell stabilizer doxantrazole reversed visceral hypersensitivity. Compared to vehicle-treated rats, pre-WA α-helical-CRF(9-41) treated animals displayed higher mucosal RMCP-2 and occludin levels.

CONCLUSIONS & INFERENCES:

WA-stress leads to persistent mast cell dependent visceral hypersensitivity in maternally-separated rats, which can be prevented but not reversed by blockade of peripheral CRF-receptors. We conclude that persistent post-stress mast cell activation and subsequent visceral hypersensitivity are not targeted by CRF-receptor antagonists.

INTRODUCTION

The irritable bowel syndrome (IBS) is a functional bowel disorder characterized by abdominal pain or discomfort associated with defecation or change in bowel habit.[1] Stress plays an important role in the onset and modulation of IBS. It induces increased perception of gastro-intestinal stimuli, so called visceral hypersensitivity, which is thought to be an important pathophysiological mechanism in this disorder.[2, 3] In animal models, stress not only leads to visceral hypersensitivity but also induces intestinal permeability changes.[4, 5] *Ex vivo* studies in patients also showed intestinal barrier dysfunction and related changes in expression of tight junction proteins (zonula occludens (ZO)-1 and occludin).[4, 6-9] Animal experiments indicated that stress-induced barrier and sensitivity changes may be caused by activation of intestinal mucosal mast cells.[4, 5, 10] Investigations performed with supernatants of submerged intestinal biopsies from IBS patients and normal controls confirmed that these cells may indeed be relevant.[11, 12] Recently, a possible *in vivo* role for mast cells was corroborated in a double-blind placebo-controlled patient trial conducted in our own laboratory. The mast cell stabilizer and H_1-receptor antagonist ketotifen reduced threshold of discomfort and IBS-symptoms and improved health related quality of life.[13] Next to the use of mast cell stabilizers, targeting of degranulation triggers may also be a treatment option for IBS.

In pre-clinical investigations it was shown that stress-induced IBS-like phenotypical changes (visceral hypersensitivity and barrier dysfunction) are mediated by CRF[4, 5, 14] and, consequently, the possible role of CRF-mediated mast cell degranulation was investigated. Hypersensitivity to distension, induced by partial restraint stress, was mimicked by intracerebroventricular CRF-administration and prevented when rats were pre-treated with central CRF-receptor antagonist or peripheral mast cell stabilizer.[15] Importantly, chronic subcutaneous CRF administration in normal (+/+) and mast cell deficient (Ws/Ws)-rats indicated that**,** next to central, also peripheral CRF may be relevant: CRF treatment resulted in barrier dysfunction in +/+ but not Ws/Ws rats.[16] These results were confirmed when CRF was added *ex vivo* in Ussing-chamber experiments with mast cell-sufficient and -deficient colonic rat tissue.[17] Finally, Ussing-experiments performed by Wallon *et al.* indicated that human colonic mast cells are also susceptible to CRF-mediated mast cell degranulation.[18] Although this cumulative evidence suggests that CRF-receptors are an attractive therapeutical target in IBS, it is important to note that most studies were aimed at prevention of stress-induced phenotypical changes[4, 5, 14] whereas reversal may be more relevant to patients. At present it is unclear whether post-stress receptor-antagonist treatment is able to reverse mast cell dependent visceral hypersensitivity or, alternatively, other triggers induce chronic post-stress mast cell activation. Accordingly, the objective

of this work was to compare pre- and post-stress CRF-receptor antagonist treatment protocols for their ability to, respectively, prevent and reverse mast cell dependent visceral hypersensitivity. These investigations were carried out in the maternal separation model for rats in which stress-induced mast cell mediated IBS-like phenotypical changes are well described characteristics.[10, 19]

MATERIAL AND METHODS

All protocols were approved by the Ethical Animal Research Committee of the University of Amsterdam.

Animals. Long-Evans rats (Harlan, Horst, the Netherlands) were kept in standard macralon cages with a layer of wood shavings and housed at the animal facility of the AMC (Amsterdam, The Netherlands) under conditions of controlled light (06:00-18.00), temperature (20-22 ^{0}C) and humidity (45%). Water and food (SDS; Technilab BMI, Someren, The Netherlands) were available *ad libitum*. Nonhandled as well as maternally-separated animals were bred in our own animal facilities.

Maternal separation protocol. Primiparous pregnant rats reared nonhandled male pups; second time pregnant dams reared male pups that were subjected to the maternal separation protocol. During separation, dams were placed in another cage in a separate room for 3 hours/day from postnatal day 2 to 14. Meanwhile, litter was not removed from the nest but left undisturbed except for placing of infrared light (27-30 ^{0}C). Nonhandled pups were nursed normally. Maternally-separated and nonhandled pups were weaned on postnatal day 22 and housed in pairs of two.

Measurement of the visceromotor response to colorectal distension and data analysis. Distension of the colon induces contractions of abdominal musculature: the so called visceromotor response. In rodents, its quantification by means of electromyography (EMG) is often used as a surrogate measure for visceral sensitivity. We previously validated a radio-telemetry technique in freely moving rats to record these signals.[20] This technique does not require restraint during EMG measurements, herewith limiting unwanted stress responses that may obscure pre- and post-WA data sets. Further details on techniques and data analysis system have been published extensively.[19-21] Similar to other publications[20, 22] final results are evaluated from normalized data sets, which were calculated form the absolute data by setting the 2 mL value of the first (pre-WA) distensions (1.0, 1.5 and 2.0 ml) of each rat at 100%. These relative response data were used to evaluate possible changes on a per volume basis.

In addition, area under the curve (AUC) of relative responses was calculated for individual rats and also used to show possible changes in visceromotor response within treatment groups.

Colonic distension protocol and water avoidance. Colonic distensions were performed with a latex balloon (Ultracover 8F, International Medical Products, Zutphen, The Netherlands) and carried out as described before.[20] Distensions were performed at the minimum age of 3 months by inflation of graded volumes of water (1.0, 1.5 and 2.0 mL) and started 20 minutes after the catheter was inserted under brief isoflurane anaesthesia. Length and diameter of the balloon during maximum volume distension were 18 and 15 mm respectively. After each 20-s distension episode water was quickly removed and an 80-s resting period was exercised. Possible pharmacological effects on compliance were assessed by determining the pressure-volume relationship in a subset of separated rats (carried out as described in our previous publications[19, 21]). For acute stress at adult age we used 1 hour of water avoidance (WA), during which rats are positioned on a pedestal surrounded by water. It suffices to induce enhanced sensitivity to colonic distension in maternally-separated rats which is not observed in the absence of water.[20]

Experimental design of pharmacological intervention studies. All animal experiments were performed by the same investigator (OW) who was blinded to administration of drug or vehicle alone (disclosed after evaluation of all tracings). Figure 1 A-C provides a schematic representation of all pharmacological intervention protocols described below.

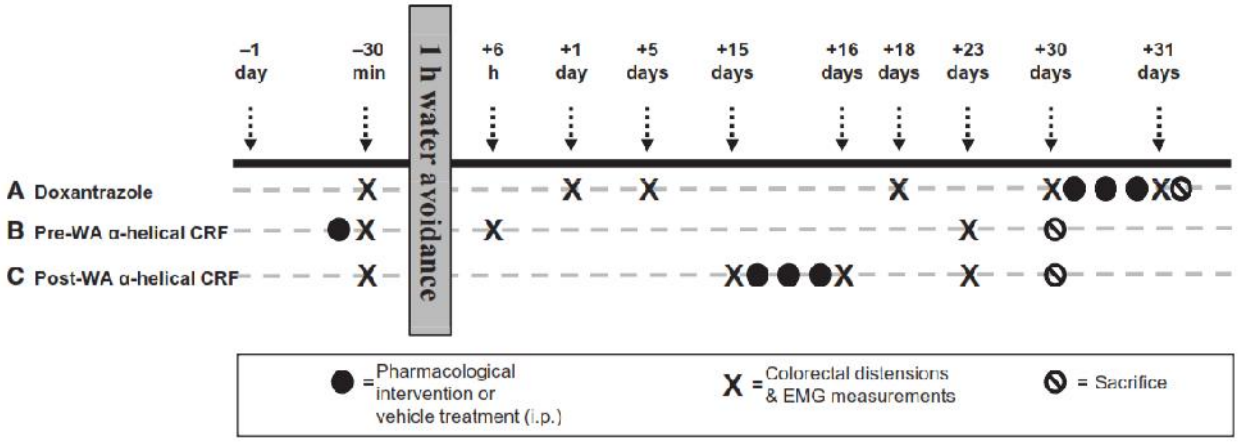


Figure 1. *Schematic representation of pharmacological intervention protocols.* Detailed description in Material and Methods paragraph on 'Experimental design of pharmacological intervention studies' A-C.

A) Reversal of chronic visceral hypersensitivity by mast cell stabilizer doxantrazole: treatment between post-WA day 30 and 31. After measuring their baseline sensitivity to distension, 4 groups

of rats (2 nonhandled and 2 maternally-separated, n=10/group) were subjected to WA and subsequent distension protocols at post-WA day 1, 5, 18 and 30. Directly after distensions at day 30 (09.00 AM) the different groups were treated (intraperitoneally) with either the mast cell stabilizer doxantrazole (10 mg/kg, gift of Agnès Francois, Institut Gustave Roussy, Villejuif, France) which was dissolved in 0.5% $NaHCO_3$/0.9% saline pH 7.5, or vehicle alone. I.p. treatments were repeated at 06.00 PM (day 30) and 09.00 AM of day 31. 30 minutes later rats were subjected to the last distension protocol and sacrificed directly after.

B) Prevention of acute (and chronic) visceral hypersensitivity with CRF-receptor antagonist α-helical-CRF(9-41): pre-WA treatment. The non-selective CRF-receptor antagonist α-helical-CRF(9-41) does not cross the blood brain barrier (BBB). We administered 250 microg/kg[23] (Tocris, Bristol, U.K.) or vehicle (saline) alone (i.p.) to maternally-separated rats (n=9/group) 30 minutes before the pre-WA distension protocol at 08.30 AM. Post-WA distensions were carried out at T+6 hours and T+23 days. Colonic tissue was collected after sacrifice at T+30 days.

C) Reversal of chronic visceral hypersensitivity with α-helical-CRF(9-41): treatment between post-WA day 15 and 16. Baseline sensitivity to distension was measured in 2 groups of maternally-separated rats (n=9/group) which were then subjected to WA. Post-WA distensions were performed at T+15, T+16 and T+23 days (at 09.00 AM). The CRF-receptor-antagonist (or vehicle alone) was administered 3 times (250 microg/kg per i.p. injection) in between distensions at T+15 days (09.30 AM & 18.00 PM) and T+16 days (08.30 AM). Colonic tissue was collected after sacrifice at T+30 days.

Western blotting. Stripped mucosa was obtained from distal colon, homogenized in lysis buffer (Cell Signaling, Danvers, MA, USA) and assessed by SDS-polyacrylamide gel electrophoresis and Western blotting. Blots were cut at appropriate kD and evaluated for expression of the rat chymase analogue RMCP-2 (polyclonal anti-RMCP-2, Moredun Scientific, Penicuik, Scotland), the tight junction protein occludin (rabbit-anti-occludin, Zymed, San Francisco (CA), USA) and GAPDH (mouse-anti-GAPDH, Millipore, Amsterdam, The Netherlands). Peroxidase-labeled secondary antibody was visualized with Lumi-light plus (Roche Diagnostics, Almere, The Netherlands) and densitometric analyses were carried out with the image processing program ImageJ (http://rsb.info.nih.gov/ij/).

Statistical analysis. Statistical calculations were performed using SPSS for windows (version 16.0.1, Chicago, Il, USA). Visceromotor response data within treatment groups were always compared to the previous point in time (e.g. response at day 0 with day 1, day 1 with day 5 etc). Data were analysed with the Wilcoxon signed ranks test which was applied for the AUC of the relative response

(normalized data) to colonic distension as well as for individual distension volumes. Statistical differences in Western-blot evaluations were assessed by Mann-Whitney test. *P* values < 0.05 were considered statistically significant in all tests.

RESULTS

Acute-stress induced persistent mast cell dependent visceral hypersensitivity. We investigated whether WA-induced visceral hypersensitivity is long-lasting and can be reversed by mast cell stabilization. In maternally-separated rats, WA induced a significantly enhanced response to distension at day 1 (increased AUC in Figures 2A and 2B) which remained elevated at post-WA days 5, 18 and 30. Doxantrazole (Figure 2B) administered on day 30 but not vehicle alone (Figure 2A) reversed the observed hypersensitivity. In nonhandled rats WA induced a slight but significant increase in sensitivity in one group only (Figure 2D), which was resolved on day 5 post WA. Vehicle (Figure 2C) and doxantrazole (Figure 2D) treatment on day 30 did not lead to significant changes in nonhandled rats. Supplementary Figure 1 depicts the same set of data but now given as relative response to distension. Statistic evaluation indicated enhanced post-WA response (pre-WA vs day 1) in maternally-separated rats for all 3 distension volumes (Supplementary Figure S1 A and B). A significantly changed response (for all 3 volumes) was also observed when maternally-separated rats were treated with doxantrazole between day 30 and day 31.

Application of α-helical-CRF(9-41) in maternally-separated rats: prevention vs reversal of stress-induced visceral hypersensitivity. A single 1 hour WA-stress leads to long-term (at least 30 days) post-WA visceral hypersensitivity, suggesting that prolonged post-WA mast cell activation may depend on factors other than CRF and, consequently, CRF-receptor antagonism may not suffice to reverse existing visceral hypersensitivity. We assessed possible differences between antagonist-driven prevention and reversal by, respectively, administering the CRF receptor-antagonist α-helical-CRF(9-41) in maternally-separated rats during the acute (i.e. pre-WA administration) and chronic (i.e. post-WA administration) hypersensitivity phase.

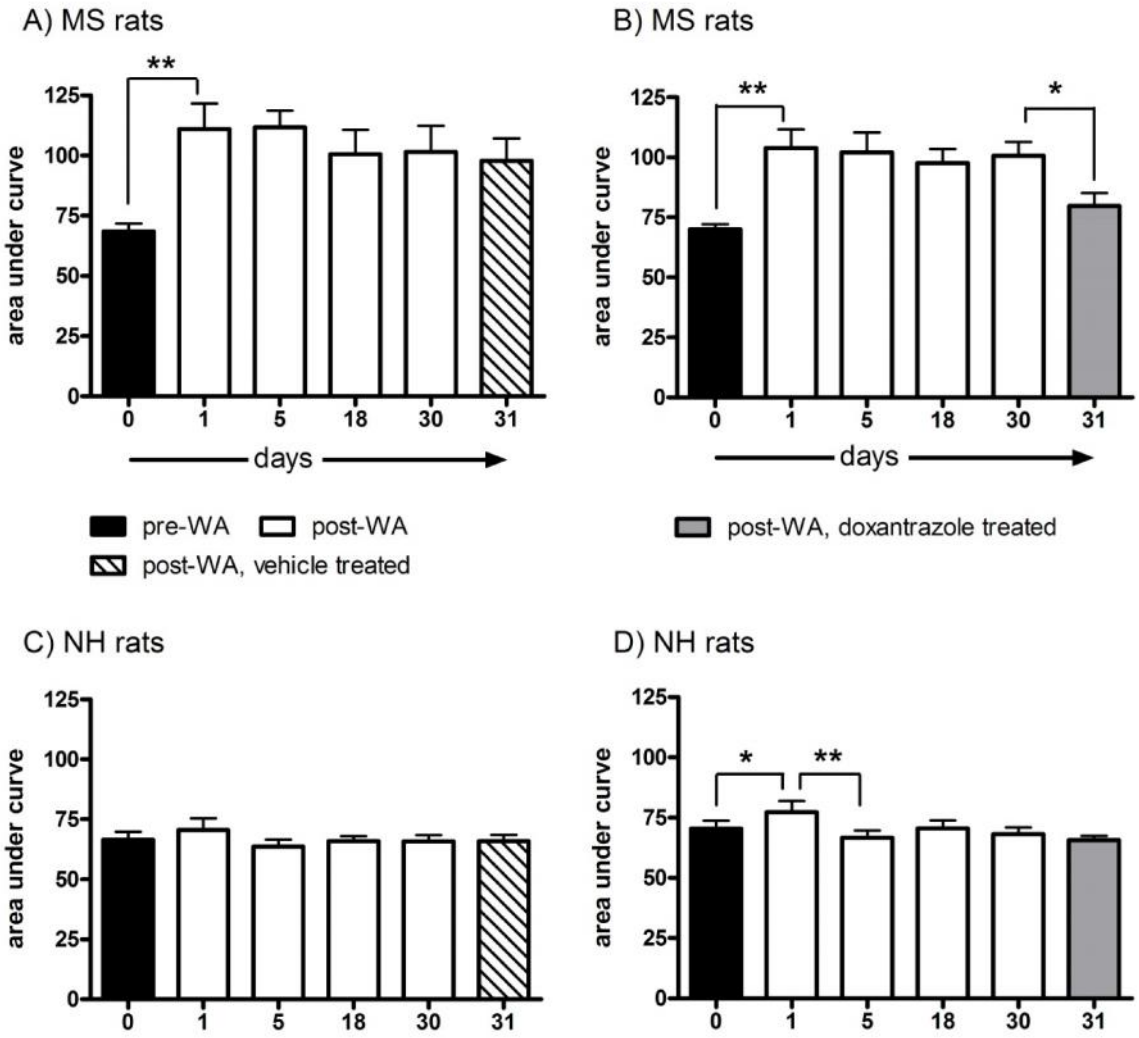
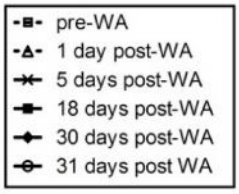
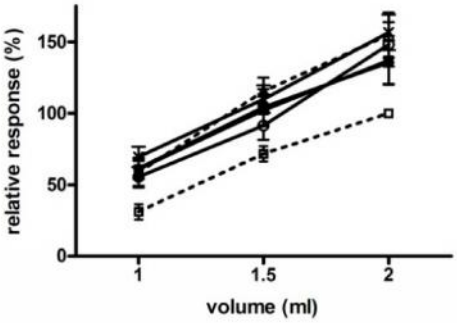


Figure 2. *Post-WA hypersensitivity to distension in maternally-separated rats is long-lasting and can be reversed by mast cell stabilization.* In maternally-separated rats *(panels A and B)*, WA induced long-lasting (30 days) hypersensitivity to distension which was reversed by i.p. doxantrazole treatment (grey bar, *B*) but not by vehicle alone (hatched bar, *A*). Except for temporarily enhanced post-WA sensitivity on day 1 *(panel D)*, we observed no sensitivity changes in nonhandled rats (panels *C* and *D*). Data are shown as average AUC±SEM. Significant differences: **P*<0.05 and ***P*<0.01.



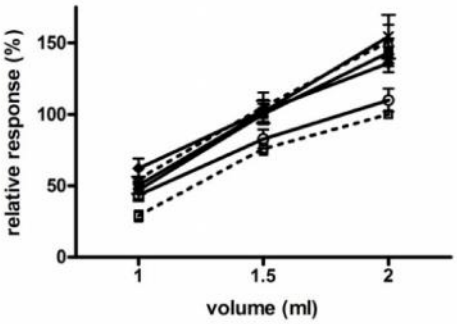
Supplement Figure S1.
vehicle or doxantrazole treatment between day 30 and 31. Left side: relative response to distension. Right side: statistics for individual volumes.

A) vehicle treated MS rats



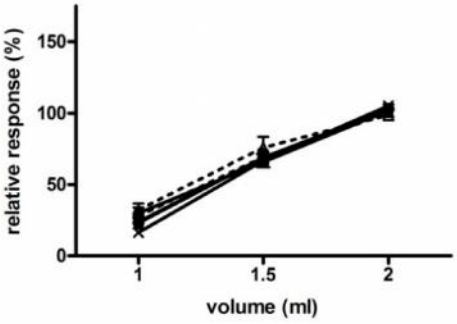
*P<0.05 **P<0.01 ***P<0.001 ns=not significant	volume		
	1.0 ml	1.5 ml	2.0 ml
pre-WA vs. day 1	*	**	**
day 1 vs. 5	ns	ns	ns
day 5 vs. 18	ns	ns	ns
day 18 vs. 30	ns	ns	ns
day 30 vs. 31	ns	ns	ns

B) doxantrazole treated MS rats



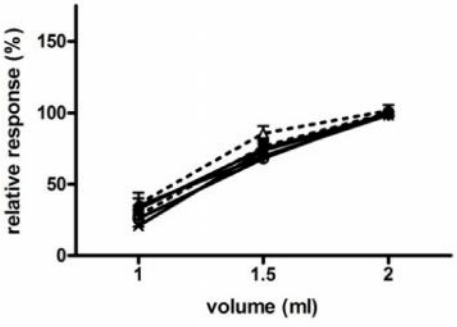
*P<0.05 **P<0.01 ***P<0.001 ns=not significant	volume		
	1.0 ml	1.5 ml	2.0 ml
pre-WA vs. day 1	**	*	**
day 1 vs. 5	ns	ns	ns
day 5 vs. 18	ns	ns	ns
day 18 vs. 30	ns	ns	ns
day 30 vs. 31	*	*	**

C) vehicle treated NH rats



*P<0.05 **P<0.01 ***P<0.001 ns=not significant	volume		
	1.0 ml	1.5 ml	2.0 ml
pre-WA vs. day 1	ns	ns	ns
day 1 vs. 5	*	ns	ns
day 5 vs. 18	*	ns	ns
day 18 vs. 30	ns	ns	ns
day 30 vs. 31	ns	ns	ns

D) doxantrazole treated NH rats



*P<0.05 **P<0.01 ***P<0.001 ns=not significant	volume		
	1.0 ml	1.5 ml	2.0 ml
pre-WA vs. day 1	ns	ns	ns
day 1 vs. 5	*	*	ns
day 5 vs. 18	ns	ns	ns
day 18 vs. 30	ns	ns	ns
day 30 vs. 31	ns	ns	ns

Supplement Figure S1. *Vehicle or doxantrazole treatment between day 30 and 31.* Data correspond to those of Figure 2 but are depicted in a different fashion. Left side: relative response to distension. Right side: statistics for individual volumes. A) vehicle treated maternally-separated rats, B) doxantrazole treated maternally-separated rats, C) vehicle treated nonhandled rats and D) doxantrazole treated nonhandled rats. WA induces enhanced sensitivity to distension in maternally-separated but not in nonhandled rats (pre-WA vs day 1). Hypersensitivity in maternally-separated rats is reversed by doxantrazole but not by vehicle treatment (day 30 vs day 31).

Sensitivity to colonic distension. Antagonist-treatment did not lead to changes in compliance (as assessed by pressure-volume curves, data not shown). In maternally-separated rats, pre-WA administration of vehicle alone led to increased AUC at 6 hours and 23 days post-WA (Figure 3A). In contrast, pre-WA administration of α-helical-CRF(9-41) prevented stress-induced hypersensitivity to distension at the 6 hour time point and AUC remained low 23 days post-WA (Figure 3B). Results from the post-WA treatment groups showed WA-induced increase in AUC at day 15. Administration of vehicle alone (Figure 3C) or α-helical-CRF(9-41) (Figure 3D) between measurements at day 15 and 16 was unable to reverse the observed increase in visceral sensitivity.

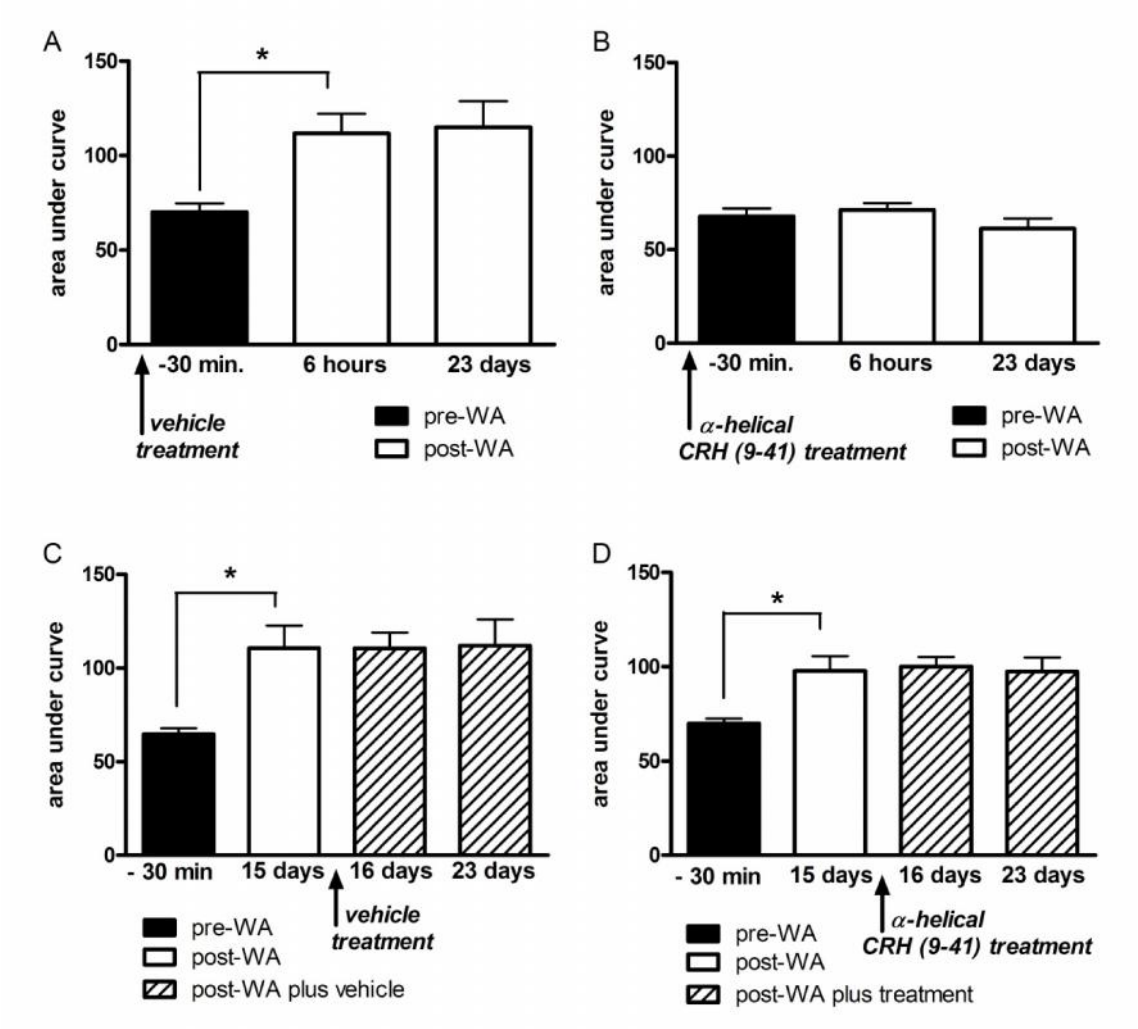
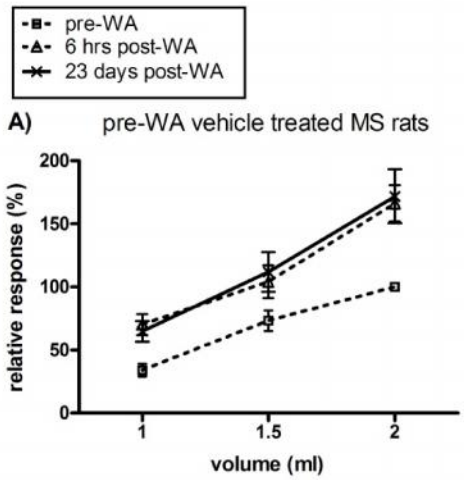


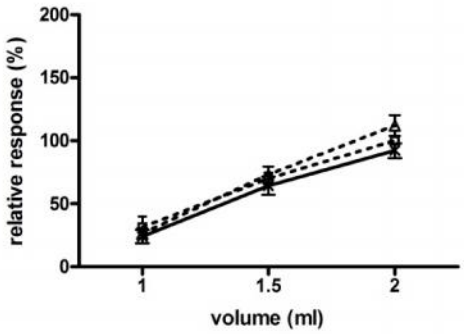
Figure 3. *Pre-WA α helical-CRF(9-41) administration prevents, but post-WA administration does not reverse, hypersensitivity to distension.* Pre-WA administration *(panel A)* of vehicle alone (i.p.) lead to increased AUC at 6 hours post-WA whereas administration of α-helical-CRF(9-41) inhibited stress-induced hypersensitivity to distension *(panel B)*. *Panels C and D* show increased post-WA AUC (T=15 days) in both groups. This response was not reversed by post-WA treatment (between days 15 and 16) with vehicle alone (*panel C*) or antagonist (*panel D*). Data are shown as average AUC±SEM. Significant differences: *$P<0.05$.

Statistic evaluation of the relative response data (Supplementary Figure S2) showed enhanced post-WA response for all 3 distension volumes when rats were pretreated with vehicle alone (pre-WA vs 6 hrs, Figure S2 A) but not upon pre-treatment with α-helical-CRF(9-41) (Figure S2 B). In the post-WA treatment groups (Figures S2 C and D), WA induced enhanced response to distension for all volumes except 2ml in the α-helical-CRF(9-41) treatment group (Figure S2 D). Importantly, α-helical-CRF(9-41) treatment was unable to reverse (day 15 vs day 16) increased post-WA response for any of the 3 investigated distension volumes.

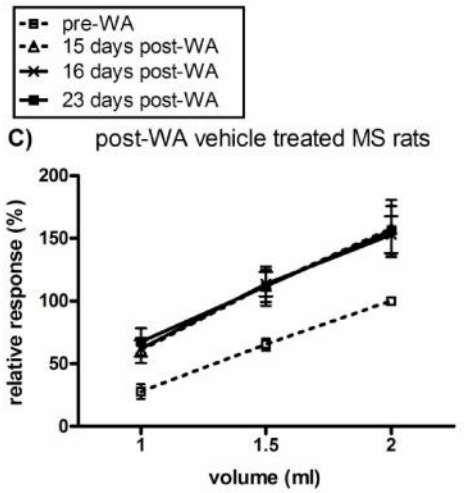


Supplement Figure S2.
pre-WA (A& B) and post-WA (C&D) treatment with vehicle or α-helical CRF.
Left side: relative response to distension.
Right side: statistics for individual volumes.

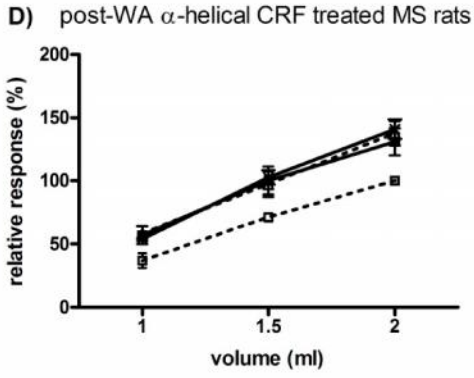
*P<0.05 **P<0.01 ***P<0.001 ns=not significant	volume		
	1.0 ml	1.5 ml	2.0 ml
pre-WA vs. 6 hrs	**	*	**
6 hrs vs. day 23	ns	ns	ns



*P<0.05 **P<0.01 ***P<0.001 ns=not significant	volume		
	1.0 ml	1.5 ml	2.0 ml
pre-WA vs. 6 hrs	ns	ns	ns
6 hrs vs. day 23	ns	ns	ns



*P<0.05 **P<0.01 ***P<0.001 ns=not significant	volume		
	1.0 ml	1.5 ml	2.0 ml
pre-WA vs. day 15	*	*	*
day 15 vs. 16	ns	ns	ns
day 16 vs. 23	ns	ns	ns



*P<0.05 **P<0.01 ***P<0.001 ns=not significant	volume		
	1.0 ml	1.5 ml	2.0 ml
pre-WA vs. day 15	*	*	ns
day 15 vs. 16	ns	ns	ns
day 16 vs. 23	ns	ns	ns

Supplement Figure S2. *Pre-WA and post-WA treatment with vehicle or α-helical-CRF(9-41).*Data correspond to those of Figure 3 but are depicted in a different fashion. Left side: relative response to distension. Right side: statistics for individual volumes. All 4 panels concern maternally-separated rats: A) pre-WA treatment with vehicle alone, B) pre-WA treatment with α-helical-CRF(9-41), C) post-WA treatment with vehicle alone and D) post-WA treatment with α-helical-CRF(9-41). α-helical-CRF(9-41) can prevent but not reverse post-WA visceral hypersensitivity.

RMCP-2 expression in distal colon. The combined doxantrazole and CRF receptor-antagonist data suggest that pre-WA α-helical-CRF(9-41) administration prevented mast cell activation while activation was unaffected in the post-WA treatment protocol. We evaluated total RMCP-2 expression in stripped and homogenized mucosa of distal colon. Protein expression was assessed by densitometric analysis of RMCP-2/GAPDH as analyzed on Western blots (original RMCP-2 Western blots in top panels of Figure 4E). Rats pre-treated with α-helical-CRF(9-41) showed higher relative RMCP-2 tissue levels than those pre-treated with vehicle alone (Figure 4A). In contrast, in the post-WA treatment protocol, colonic RMCP-2 levels did not differ between α-helical-CRF(9-41) and vehicle treatment groups (Figure 4B) indicating that CRF-receptor antagonism affects mast cell activation to a lesser extend in this setting.

Occludin expression in distal colon. When maternally-separated rats were pre-treated with α-helical-CRF(9-41), average post-WA occludin levels in colonic mucosa were higher then those of vehicle pre-treated rats (Figure 4C). Such differences were not observed in the post-WA treatment protocol (Figure 4D). Original Western blots in bottom panels of Figure 4E.

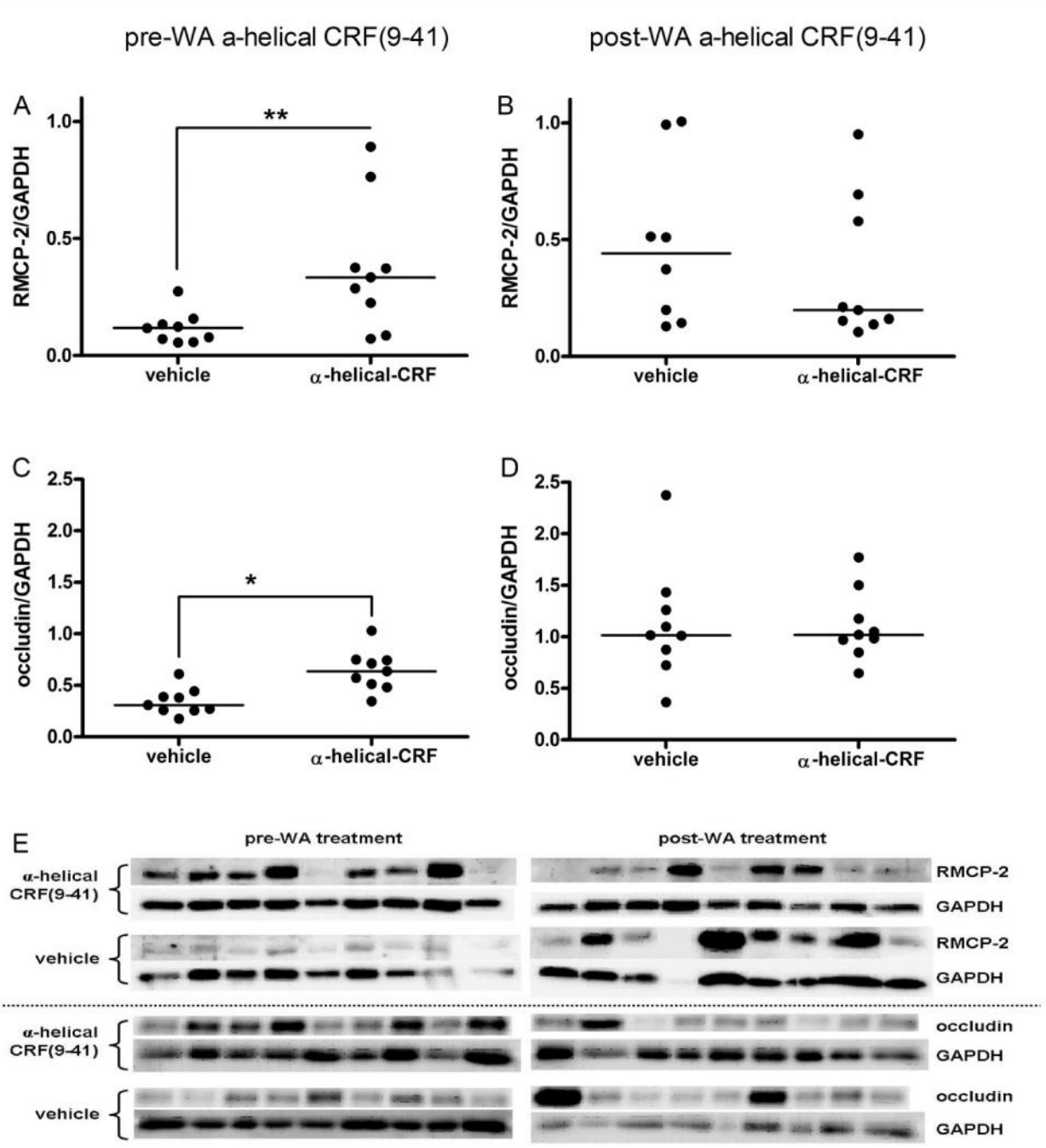


Figure 4. *Pre- but not post-WA α-helical-CRF(9-41) administration leads to differences in mucosal RMCP-2 and occludin protein levels.* Distal colonic mucosa collected from the pre- and post-WA α-helical-CRF(9-41) treatment groups was evaluated by Western-blotting. Protein expression levels (relative to GAPDH) were quantified by densitometry. Compared to vehicle alone, antagonist pre-treated maternally-separated rats display higher RMCP-2 *(panel A)* and occludin *(panel C)* protein levels. Comparison of post-WA treatment groups (vehicle vs antagonist) does not show differences in expression of RMCP-2 *(panel B)* and occludin *(panel D)*. Significant differences: **P*<0.05 and ***P*<0.01. Original Western blots in panel E.

DISCUSSION

Pre-stress administration of CRF-receptor antagonists was previously shown to prevent mast cell degranulation and subsequent barrier dysfunction and development of visceral hypersensitivity in animal models.[15, 24-26] Our study confirms that the receptor antagonist α-helical-CRF(9-41) potently prevents the development of visceral hypersensitivity when administered before acute stress in adult pre-disposed rats. In contrast, established post-WA hypersensitivity could not be reversed by this antagonist although hypersensitivity was reversed by mast cell stabilization. These results indicate that factors other then CRF may contribute to sustained post stress mast cell activation.

Early life stressors are known to contribute to IBS in adults[27, 28] and the maternal separation model in rats is often used to mimic such predisposing factor.[10] In this model, adverse early life experience pre-disposes for complaints like visceral hypersensitivity and barrier dysfunction later in life[20, 22, 23, 29] and these features are also important in IBS.[2, 3, 7-9] In contrast to others who reported differences in baseline responsiveness to distension between nonhandled and maternally-separated rats[22, 29] our adult separated Long Evans rats need an acute WA stress to bring out the hypersensitive phenotype.[20] This discrepancy may be explained by the use of different rat strains and by our use of radiotelemetry for EMG measurements. This technique allows measurement of baseline sensitivity while rats are freely moving in their cage, herewith minimizing unwanted stress that is induced by the measurement procedure itself. Because others often use restraint during measurements their baseline response may be incorrect because it will, unwontedly, reflect restraint stress induced hypersensitivity to distension. We regard the need for stress in our model a positive feature because also in IBS acute stress is a known trigger for visceral hypersensitivity.[2] In adult maternally-separated Long Evans rats we earlier showed an essential role for stress-induced mast cell degranulation in the acute phase. Pre-WA treatment with the broadly used mucosal mast cell stabilizer doxantrazole[15, 24-26, 29, 30] prevented elicitation of colonic hypersensitivity to distension.[19] We now extended this observation to long lasting post-WA visceral hypersensitivity: doxantrazole treatment at post-WA day 30 was able to reverse hypersensitivity established earlier. Most other studies focussed on stress-induced mast cell activation in the acute phase and never evaluated possible long term phenotypical consequences of acute stress. However, chronic mast cell activation was shown to play a role in maternally-separated Wistar rats that also display an IBS-like phenotype (barrier dysfunction and visceral hypersensitivity).[24, 29] But, in contrast to Long Evans rats, this rat strain does not require additional acute stress at adult age to induce this mast cell dependent phenotype in maternally-separated animals. Accordingly, it is unclear whether the observed long term effects are specific for Long Evans rats or also apply to other rat strains.

Our pre-WA CRF receptor-antagonist data confirm earlier studies which indicated that, upon acute stress, initial mucosal mast cell degranulation is triggered by peripheral CRF.[15, 18, 24-26] CRF interacts with two receptors, CRF_1- and CRF_2, but with highest affinity to CRF_1.[14] In brain, CRF/CFR_1 was shown to be the most relevant interaction for stress-related alterations in colonic function. In contrast, functional studies on human intestinal mast cells indicated that both receptors are expressed- and relevant for their CRF-induced activation.[18] Similarly, in rat mast cell studies the use of selective receptor antagonists for CRF_1[24] and CRF_2[16] implicated both receptors. On the other hand, the non-selective receptor antagonist α-helical CRF(9-41) was shown to preferentially block CRF_2-receptors[31, 32] and several groups successfully used this compound to inhibit stress-induced mast cell degranulation and subsequent changes in intestinal phenotype in both rat[15, 24-26] and human[18]. When administered peripherally, this particular antagonist has poor penetration into brain[14] thus ruling out the modulation of central CRF-signalling pathways as much as possible. In our experiments intraperitoneal pre-WA α-helical CRF(9-41) administration blocked mast cell dependent visceral hypersensitivity but, despite the observation that mast cell stabilization reversed post-WA effects, failed to counteract chronic post-WA hypersensitivity. These results suggest that chronic post-WA visceral hypersensitivity involves alternative mast cell dependent mechanisms that are less dependent on CRF-receptor activation.

We observed earlier enhanced *in situ* RMCP2 expression in colonic mucosal mast cells of MS rats which decreased to normal nonhandled-level upon WA.[19] These results are corroborated by the present Western blot quantifications of stripped colonic mucosa. Compared to vehicle pre-treated MS-rats, α-helical CRF(9-41) pre-treated animals displayed higher post-WA RMCP2 protein-expression. This most likely reflects RMCP-2 being retained in mast cells. In contrast, no difference was observed when post-WA treated rats were compared (vehicle vs antagonist), suggesting that RMCP-2 release was equal in these treatment groups. RMCP2 is a chymase analogue and it is known that chymase can degrade the tight junction protein occludin.[33] This may be relevant because loss of occludin induces barrier dysfunction[34] and protease-induced occludin degradation was suggested to play a role in IBS.[6] Our occludin quantifications are in line with the observed RMCP2 expression levels: a significant difference only occurs when rats are pre-treated with α-helical CRF(9-41). Although we have not performed extensive barrier studies these data suggest that α-helical CRF(9-41) may prevent stress-induced mast cell degranulation and subsequent barrier dysfunction but is unable to reverse it.

A possible limitation of this study could be the timing of the post-WA α-helical CRF(9-41) administrations. However, it was shown earlier that α-helical CRF(9-41) is not only capable of preventing CRF-mediated effects in rat paw skin, but was also able to reverse such effects within minutes after antagonist application.[35] The immediateness of this event suggests that our protocol, 3

times i.p. administration in a 24 hour timeframe (each dose equal to the successful pre-WA protocol) and the last dose given 30 minutes before post-treatment distensions, should suffice to antagonize CRF-receptor mediated mast cell degranulation. Another concern may be that α-helical CRF(9-41) is mainly a CRF_2-receptor antagonist and we did not apply specific CRF_1-antagonists. However, in this paper we focus on the role of mast cells in relation to stress-induced visceral hypersensitivity and several earlier studies showed that α-helical CRF(9-41) is capable of inhibiting CRF-induced mast cell degranulation.[15, 19, 24-26, 29, 30] Further, in a model of repeated WA-exposure (10 consecutive days, 1 hour/day) it was shown that a) mast cells are relevant for chronic stress induced barrier dysfunction[36] and b) that daily pre-WA α-helical CRF(9-41) treatment can prevent mast cell dependent antigen uptake.[37] The same model was also used to investigate the development of visceral hypersensitivity which was prevented by daily pre-WA administration (subcutaneously) of the BBB-crossing CRF_1-receptor antagonist CP-154,526.[38] This confirmed earlier observations that central CRF_1-receptors are essential in the acute peri-stress time frame. However, reversal of hypersensitivity by post stress CP-154,526 administration at day 11 alone was only partially successful. The combined above data suggest that ongoing post-stress hypersensitivity to distension may, at least to some degree, be due to non CRF-receptor dependent triggers.

Unlike rats that are exposed to one hour of water avoidance only, IBS-patients most likely experience repeated stress episodes that are considered as chronic stress. Although this suggests a continuous role for CRF in patients, our data support the outcome of two recent clinical trials in which CRF-receptor antagonists showed no patient benefit.[39, 40] Together with the observation that mast cell stabilization may be an effective treatment in IBS[13], these trial results support our proposed mechanism that, following initial stress-induced mast cell activation by CRF, other mast cell triggers become relevant as well. Since stress-induced barrier dysfunction is known to be associated with influx of luminal bacteria/antigens which can lead to antigen specific immunity,[36, 37] we expect humoral immune responses to be involved. In this respect, it was shown that in maternal separated Sprague-Dawley rats WA at adult age induced increased transepithelial transport of macromolecules which could be blocked by α-helical CRF(9-41) pre-treatment.[23] Using the same rat strain it was later shown that exposure to maternal separation induces bacterial adherence to- and penetration into colonic epithelium during and shortly after the separation period.[41] Culture of washed and homogenized segments of distal colon confirmed increased bacterial presence in colonic-wall of maternally-separated rats and penetration was accompanied by increased translocation to the spleen. These data show that, in the maternal separation model, early neonatal mucosal antigen-exposure can facilitate antigen-priming of the humoral immune response. At adult age subsequent CRF-induced barrier dysfunction can challenge

this response, which may explain how mast cells can then, after challenge, be relevant without further role of CRF.[5] Pilot experiments performed in our own laboratory confirm that humoral immune responses may have a role in the maternal separation model[42] and recent observations of increased anti-flagellin antibody titers suggest that humoral mechanisms may also be relevant to IBS.[43]

In summary, our investigations were performed in the rat model of maternal separation in which acute stress induces, similar to IBS, visceral hypersensitivity. Although the stressor used was one hour of WA only, the observed hypersensitivity to distension lasted for at least one month and was mast cell dependent. The CRF-receptor antagonist α-helical CRF(9-41) could prevent but not reverse this stress-induced hypersensitivity. If these results also apply to IBS, they suggest that antagonizing CRF receptors alone will not be sufficient for the reversal of stress-induced and mast cell dependent complaints in this disorder.

ACKNOWLEDGEMENTS AND DISCLOSURES

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Chapter 4

Stress-induced visceral hypersensitivity in maternally separated rats can be reversed by peripherally restricted histamine-1-receptor antagonists

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ABSTRACT

Background

The histamine-1 receptor (H1R) antagonist ketotifen increased the threshold of discomfort in **hypersensitive IBS patients. The use of peripherally restricted and more selective H1R** antagonists may further improve treatment possibilities. We examined the use of fexofenadine and ebastine to reverse post-stress visceral hypersensitivity in maternally separated rats.

Methods

The visceromotor response to colonic distension was assessed in adult maternally separated and nonhandled rats pre- and 24 hours post water avoidance. Subsequently rats were treated with vehicle alone or different dosages of fexofenadine (1.8 and 18 mg/kg) or ebastine (0.1 and 1.0 mg/kg) and re-evaluated. Colonic tissue was collected to assess relative RMCP-2 and occludin expression levels by Western blot and histamine-1 receptor by RT-qPCR. β-hexosaminidase release by RBL-2H3 cells was used to establish possible mast cell stabilizing properties of the antagonists.

Key results

Water avoidance only induced enhanced response to distension in maternally separated rats. This response was reversed by 1.8 and 18 mg/kg fexofenadine. Reversal was also obtained by 1.0 but not 0.1 mg/kg ebastine. RMCP-2 expression levels were comparable in these two ebastine treatment groups but occludin was significantly higher in 1.0 mg/kg treated rats. There were no differences in histamine-1 receptor expression between nonhandled and maternally separated rats. Fexofenadine but not ebastine showed mast cell stabilizing quality.

Conclusions

Our results indicate that the peripherally restricted 2nd generation H1-receptor antagonists fexofenadine and ebastine are capable of reversing post stress visceral hypersensitivity in rat. These data justify future IBS patient trials with these well tolerated compounds.

INTRODUCTION

The functional gastrointestinal disorder irritable bowel syndrome (IBS) is characterized by abdominal pain or discomfort associated with defecation or change in bowel habit.[1] Increased perception to gastrointestinal stimuli, so called visceral hypersensitivity, and barrier dysfunction are considered important pathophysiological mechanisms in IBS. Stress is an important trigger for IBS-symptoms and preclinical investigations suggest that barrier- and sensitivity changes may relate to stress-induced degranulation of intestinal mucosal mast cells.[2–4] A recent clinical trial with the mast cell stabilizer and histamine-1-receptor (H1R) antagonist ketotifen confirmed the possible relevance of this cell type.[5] Ketotifen not only decreased abdominal pain and other IBS symptoms but also improved health related quality of life and increased the threshold of discomfort in hypersensitive patients. However, the exact working mechanism of ketotifen remained elusive. Investigations comparing pre- and post-therapy mediator release by submerged rectal biopsies did not support a role for mast cell stabilization. Consequently, it was suggested that H1R antagonism was the main molecular mode of action in this trial.

Ex vivo investigations performed by Barbara *et al.* indicated that a mediator present in IBS biopsy-supernatants induced H1R-dependent mesenteric afferent nerve discharge and Ca^{2+}-mobilisation in cultured rat DRG neurons.[6] In addition, mucosal biopsies from IBS patients showed a significant increase in H1R mRNA levels over controls.[7] Similar to the ketotifen trial, these results suggested that H1R-targeting may be an attractive treatment option in IBS. However, ketotifen has low H1R selectivity and is known to cross the blood-brain barrier and cause central side effects.[8,9] Consequently, possibilities to increase therapeutic dose for enhanced effectiveness are limited and evaluation of other, peripherally restricted, H1-receptor antagonists may proof beneficial. In the nineteen eighties second generation non-sedating H1-antihistamines became available and by now this group of antihistamines comprises more than 45 different compounds, including fexofenadine and ebastine.[8] In clinical trials these compounds appeared to be safe, effective and well tolerated and they are now routinely being used in the treatment of allergic rhinitis and urticaria.[10,11] To establish whether these antagonists hold promise for therapeutical interventions in IBS we evaluated them in the IBS-like rat model of maternal separation. Similar to patients, acute stress induces enhanced sensitivity to colorectal distension in previously separated Long Evans rats.[12] This change in sensitivity was shown to be long lasting, one hour of water avoidance induced enhanced sensitivity for up to one month, and could be reversed by the mast cell stabilizer doxantrazole.[13] In the present study we investigated whether fexofenadine and ebastine were also capable of reversing post stress, mast cell

dependent, visceral hypersensitivity in the rat maternal separation model. Our results suggest that peripheral H1Rs may be a safe new target for therapeutical intervention in IBS.

MATERIAL AND METHODS

Ethic statement

All procedures were conducted in accordance with the institutional guidelines and approved by the Animal Ethical Committee of the AMC/University of Amsterdam (reference protocol number 100998).

Animals and maternal separation (MS) protocol

Long-Evans rats (Harlan, Horst, The Netherlands) were bred and housed at the animal facility of the AMC (Amsterdam Medical Centrum, Amsterdam, The Netherlands). Rats were maintained on a normal 12:12-h dark/light cycle and temperature (20-22 ^{0}C) and provided with food and water *ad libitum*. Separation was accomplished by placing the dams into another cage in a separate room for 180 minutes per day from postnatal day 2 to 14. During separation, cages were placed on a heating pad (30-34 °C) to help pups regulate normal body temperature. Pups were weaned on day 22 and subsequently raised in pairs of two. NH pups were nursed normally.

Colonic distension protocol and water avoidance (WA).

In IBS patients investigations of visceral sensitivity are performed by colorectal distensions: hypersensitive patients perceive pain during luminal distensions at lower volumes or pressures than normal controls.[14] In our investigations in rat, colonic distensions were performed with a latex balloon (Ultracover 8F, International Medical Products, Zutphen, The Netherlands) at the minimum age of 4 months and carried out as described before.[12,13,15] A catheter was placed during short isoflurane anesthesia 20 minutes before distensions with graded volumes of water (1.0, 1.5 and 2.0 mL). Length and diameter of the balloon during maximum volume distension were 18 mm and 15 mm respectively. After each 20 second distension period, water was quickly removed and 80 seconds rest was exercised. At adult age rats were subjected to WA stress during which they were positioned on a pedestal surrounded by water for one hour. Earlier investigations indicated that, in contrast to NH rats, WA induces enhanced sensitivity to colonic distension in MS rats.[12]

Measurement of the visceromotor response to colonic distension and data analysis

Distension of the colon induces contractions of the abdominal musculature, the so called visceromotor response. Quantification of these contractions by electromyography (EMG) is often used to assess visceral pain responses in rodents. We used radiotelemetric transmitters (Physiotel Implant TA10AE-F20; DSI, St Paul, MN, USA) to record these EMG signals in freely moving rats.[12] In short, the transmitter was positioned in the abdominal cavity and two connected electrodes were placed in abdominal muscles. During distension protocols, animals were placed in a standard macralon cage (exact size of the receiver) that was positioned on top of a receiver (Data Sciences International). The receiver was linked to a Biopac MP100 data acquisition system (Biopac Systems Inc., Santa Barbara, CA, USA) and a personal computer via a raw data analog converter (Data Sciences International). Data were acquired with AcqKnowledge software (Biopac Systems Inc., Santa Barbara, CA, USA) and analyzed as described before. Briefly, each 20-s distension period and its preceding 20-s of baseline recording were extracted from the original raw EMG data file. After correction for movement and breathing, data were rectified and integrated. Absolute data sets were then obtained by subtracting the 20-s baseline recording from the 20-s distension result. Similar to earlier publications the final results are given as normalized data sets, which were calculated from the absolute data by setting the 2mL value of the first (pre-stress) distension at 100%.[12,13,15] Area under the curve (AUC) of relative responses was calculated for individual rats and used to show possible changes in visceromotor response within treatment groups. Relative response data were also used to evaluate possible changes on a per volume basis.

Design of in vivo pharmacological intervention protocols

Animal experiments were performed while the investigator was blinded to administration of drug or vehicle alone (disclosed after evaluation of all tracings). Directly after measuring baseline sensitivity to distension (10:00 AM, day 0), rats were subjected to WA stress and measured again 24 hours post-WA. Subsequently, rats were treated with intraperitoneal fexofenadine hydrochloride (Tocris Bioscience, Bristol, UK), ebastine (Sigma-Aldrich, Zwijndrecht, The Netherlands) or vehicle alone (10% alcohol). Compounds were administered two times at day 1 (11:00 AM and 05.00 PM) and one time at day 2 (30 minutes before the last distension protocol at 09:30 AM). Cumulative dosages (total of 3 intraperitoneal injections in 24 hour timeframe) were 1.8 mg/kg or 18 mg/kg for fexofenadine and 0.1 mg/kg or 1 mg/kg for ebastine.

RT-qPCR

To avoid possible distension related effects on H1R expression levels, vehicle treated NH and MS rats were sacrificed 7 days after the last distension protocol. Total RNA was isolated from colonic tissue of NH and MS rats using TRIzol (Invitrogen, Breda, The Netherlands) according to manufacturer's protocol. Following DNAse treatment, cDNA was obtained by using RevertAid First Strand cDNA Synthesis Kit (Fermentas, Waltham, MA, USA) Quantitative PCR was performed with SYBR Green in the LightCycler480 system (Roche) using a default 60° program. Primer pairs used for H1R were; sense, CTTCTACCTCCCCACTTTGCT , antisense: TTCCCTTTCCCCCTCTTG and for the housekeeping gene Ppib[15]: sense, GCAAGCACGTGGTTTTCGGC, antisense: TGTGAGGGAATCGACAGGACCC.

Western blotting.

In contrast to tissues used for RT-qPCR H1R evaluation, ebastine treated rats were sacrificed directly after the last distension protocol. This tissue was than used to semi quantitatively assess direct effects of ebastine treatment on RMCP-2 and occludin expression levels. Distal colon was dissected, homogenized in lysis buffer (Cell Signaling, Danvers, MA, USA) and assessed by SDS-polyacrylamide gel electrophoresis and Western blotting. Blots were cut at appropriate kD and evaluated for expression of the rat chymase analogue RMCP-2 (polyclonal anti-RMCP-2, Moredun Scientific, Penicuik, Scotland), the tight junction protein occludin (rabbit-anti-occludin, Zymed, San Francisco (CA), USA) and GAPDH (mouse-anti-GAPDH, Millipore, Amsterdam, The Netherlands). Peroxidase-labelled secondary antibody was visualized with Lumi-light plus (Roche Diagnostics, Almere, The Netherlands) and densitometric analyses were carried out with the image processing program ImageJ (http://rsb.info.nih.gov/ij/).

In vitro mast cell degranulation experiments and beta-hexosaminidase asay

RBL-2H3 cells were used to evaluate the possible mast cell stabilizing effect of fexofenadine and the active metabolite of ebastine; carebastine[11] (Santa Cruz, Heidelberg, Germany). After 30 minutes pre-treatment with these compounds (10 µM, 100 µM or vehicle alone)[16] cells were stimulated with compound C48/80 (Sigma-Aldrich, 100 µg/ml, 500 µg/ml, 1 mg/ml or vehicle alone) for 1 hour. β-hexosaminidase release was quantified by using 4-methylumbelliferyl glucaosaminide as a substrate. Fluorescence was measured at an emmision wavelength of 450 nm and an excitation wavelength of 360 nm. Release of β-hexosaminidase was calculated as a percentage of total cellular content.

Statistical analysis

Statistical calculations were performed using SPSS for windows (version 11.5.2). VMR data were analysed with the Wilcoxon signed ranks test which was applied for the area under the curve (AUC) of the relative response (normalized data) to colonic distension. Possible statistical differences in Western blot and RT-qPCR evaluations were assessed by Mann-Whitney test.

RESULTS

***In vivo* fexofenadine treatment**

We established whether a) WA induced post stress hypersensitivity to distension in NH and MS rats and b) whether fexofenadine was capable of reversing sensitivity changes. As published before[12], WA was unable to induce post stress visceral hypersensitivity in NH rats (figures 1A and 1B, white vs black bars) and intraperitoneal post stress administration of high dose fexofenadine (18 mg/kg) did not induce sensitivity changes in these animals (figure 1B, black vs grey bar). In MS rats WA led to increased post-WA AUC in all 3 treatment groups (figure 1C, D and E; $*P<0.05$, $**P<0.01$). Enhanced post-stress sensitivity levels were not affected by vehicle treatment alone (figure 1C) but treatment with 18 and 1.8 mg fexofenadine/kg effectively reversed visceral hypersensitivity (figure 1D and 1E respectively).

Per volume comparisons (right side line-diagrams and accompanying statistics-boxes in figure 1A-E) corroborated AUC-data for all MS groups except rats treated with 1.8 mg fexofenadine/kg. In the latter group fexofenadine-induced reversal of hypersensitivity was not significant for 1.0 and 2.0 ml distension volumes. Antagonist treatment did not lead to changes in compliance as assessed by pressure-volume curves (data not shown).

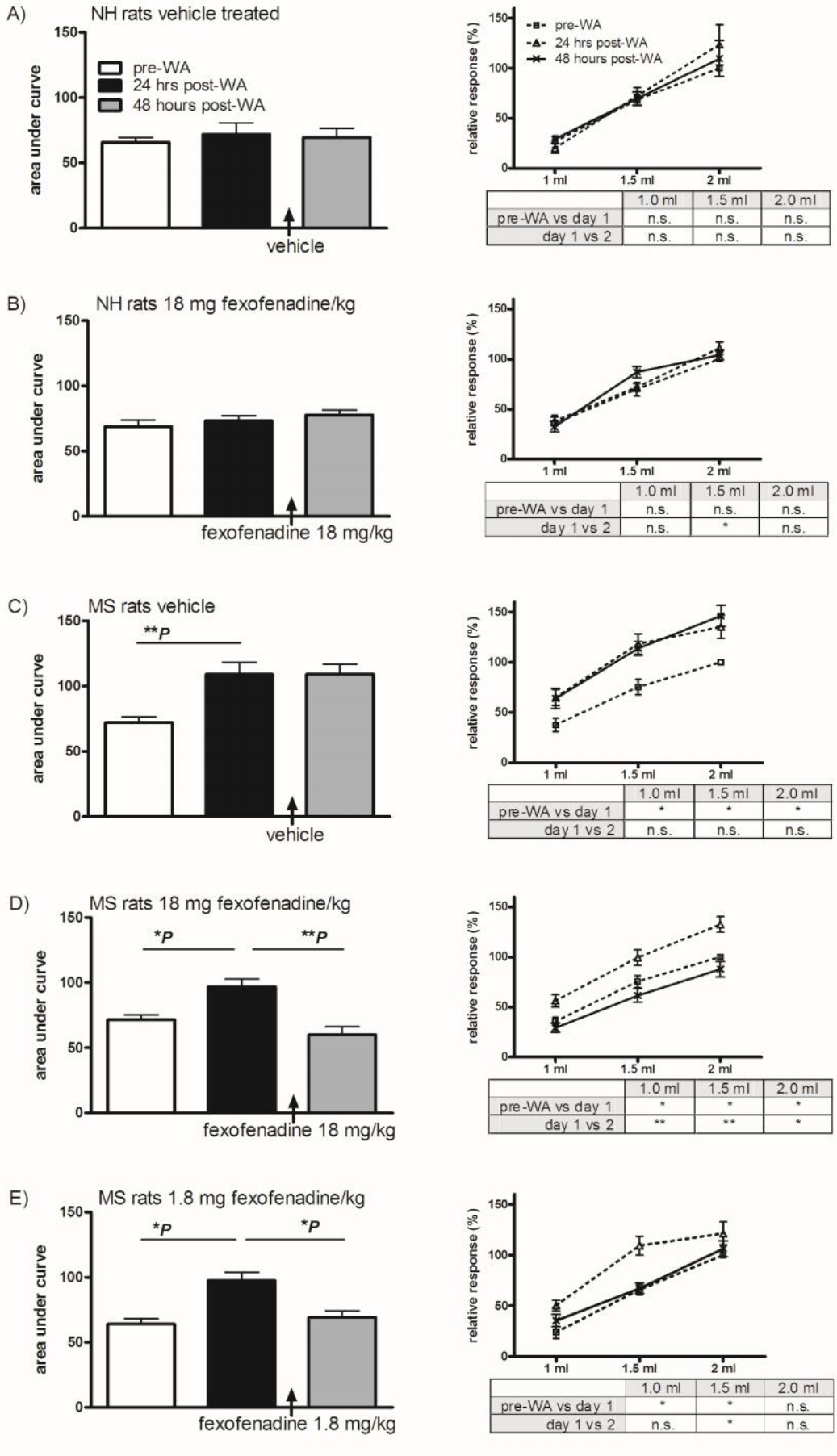

A)
NH rats vehicle treated
area under curve
pre-WA
24 hrs post-WA
48 hours post-WA
vehicle
relative response (%)
pre-WA
24 hrs post-WA
48 hours post-WA
1 ml
1.5 ml
2 ml
1.0 ml 1.5 ml 2.0 ml
pre-WA vs day 1 n.s. n.s. n.s.
day 1 vs 2 n.s. n.s. n.s.
B)
NH rats 18 mg fexofenadine/kg
fexofenadine 18 mg/kg
1.0 ml 1.5 ml 2.0 ml
pre-WA vs day 1 n.s. n.s. n.s.
day 1 vs 2 n.s. * n.s.
C)
MS rats vehicle
**P
vehicle
1.0 ml 1.5 ml 2.0 ml
pre-WA vs day 1 * * *
day 1 vs 2 n.s. n.s. n.s.
D)
MS rats 18 mg fexofenadine/kg
*P
**P
fexofenadine 18 mg/kg
1.0 ml 1.5 ml 2.0 ml
pre-WA vs day 1 * * *
day 1 vs 2 ** ** *
E)
MS rats 1.8 mg fexofenadine/kg
*P
*P
fexofenadine 1.8 mg/kg
1.0 ml 1.5 ml 2.0 ml
pre-WA vs day 1 * * n.s.
day 1 vs 2 n.s. * n.s.

Figure 1. In vivo post stress fexofenadine treatment.
The visceromotor response to distension was measured pre-WA and 24 and 48 hours post-WA in NH and MS rats. Fexofenadine or vehicle was administered 3 times between 24- and 48 hours measurements (cumulative dosages 18 and 1.8 mg/kg). Responses to distension are depicted as AUC (left side histograms) and per volume (right side line-diagrams, corresponding statistics in lower right side tables). NH rats did not become hypersensitive to distension and fexofenadine treatment did not change sensitivity levels (figures A and B). In MS rats WA induced enhanced sensitivity to distension in all 3 treatment groups (figures C, D and E). Treatment with 18 and 1.8 mg fexofenadine/kg (figure D and E respectively) but not vehicle alone (C) was able to reverse stress induced visceral hypersensitivity. All data are presented as mean ± SEM, all groups n=8 or 9 rats, **P*<0.05 and ***P*<0.01.

H1R gene expression was then determined in colonic tissue of vehicle treated NH and MS rats. Tissue was collected 7 days after the final distension series to avoid protocol induced effects on receptor expression levels. Sufficient yield of RNA was obtained from all but 2 vehicle treated nonhandled rats. As shown in figure 2 there were no significant differences between NH and MS rats.

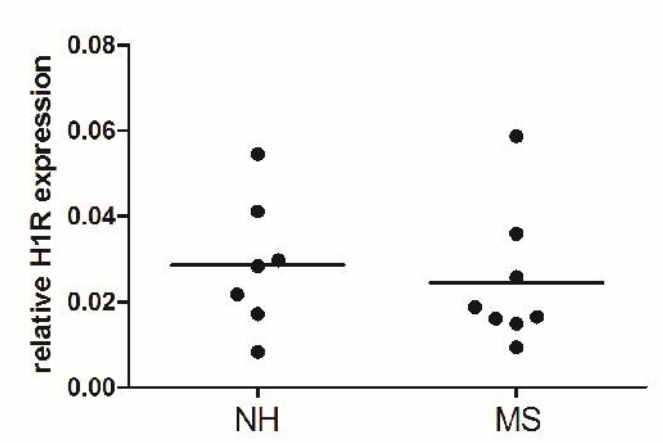
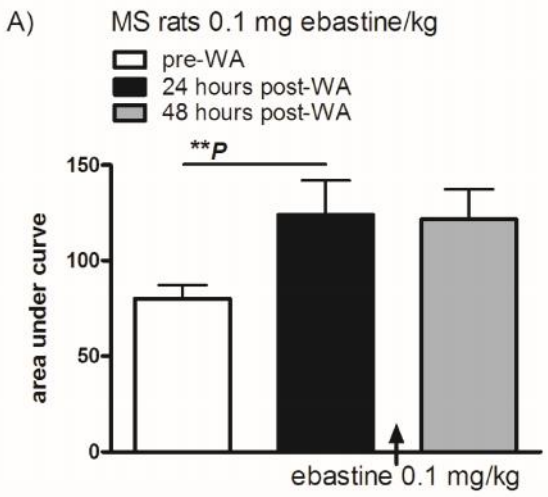
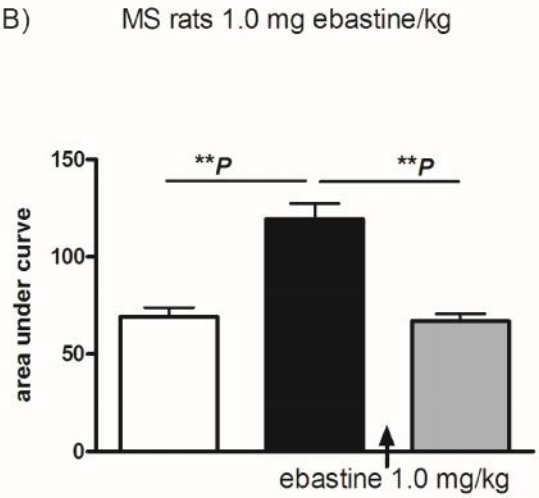


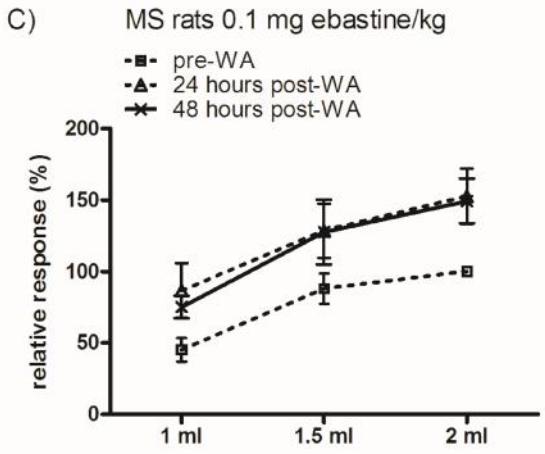
Figure 2. Relative colonic expression values for the *histamine H1 receptor* gene. H1R mRNA expression was evaluated relative to the housekeeping gene *Ppib* in colonic samples of NH and MS rats. Tissue was collected 7 days post vehicle treatment and distensions. There were no significant differences.

In vivo ebastine treatment

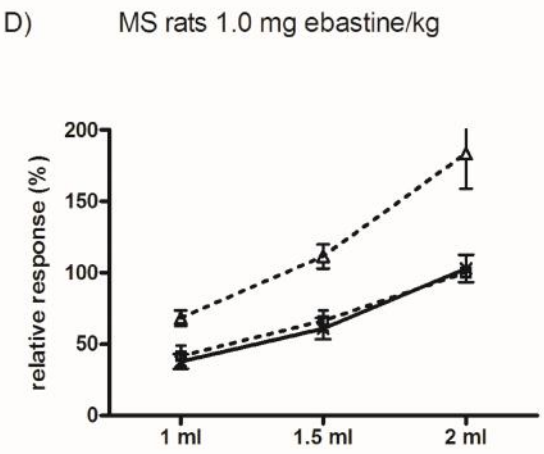
Post WA hypersensitivity to distension did not occur in NH rats and remained unaltered upon fexofenadine treatment. Further, due to their broad clinical use, we know that fexofenadine as well as ebastine are well tolerated in the human setting. Therefore, we choose not to sacrifice additional NH rats to reconfirm results obtained with fexofenadine; ebastine was only evaluated in MS rats. AUC comparisons indicated that WA-induced hypersensitivity to distension could be reversed by an accumulative dose of 1.0 mg ebastine/kg (figure 3B, black vs grey bars, ***P*<0.01) but not 0.1 mg/kg (figure 3A). Statistical evaluations on a per volume basis (line-diagrams and statistics boxes in figures 3 C and D) showed similar results: we observed a significant post-WA increase for all 3 distension volumes in both treatment groups but these responses could only be reversed in 1.0 mg/kg treated rats.



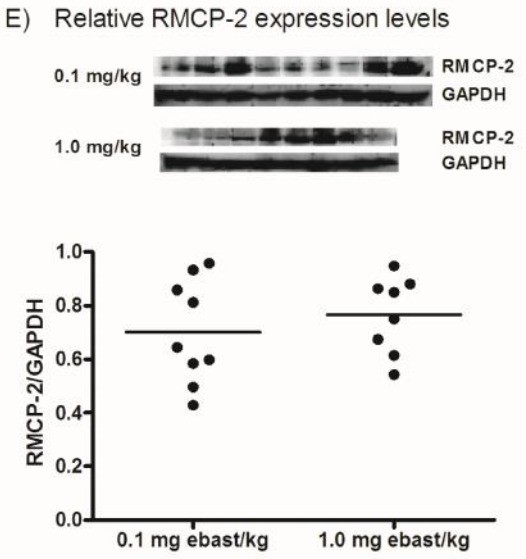




	1.0 ml	1.5 ml	2.0 ml
pre-WA vs day 1	*	**	*
day 1 vs 2	n.s.	n.s.	n.s.



	1.0 ml	1.5 ml	2.0 ml
pre-WA vs day 1	*	**	**
day 1 vs 2	**	**	*



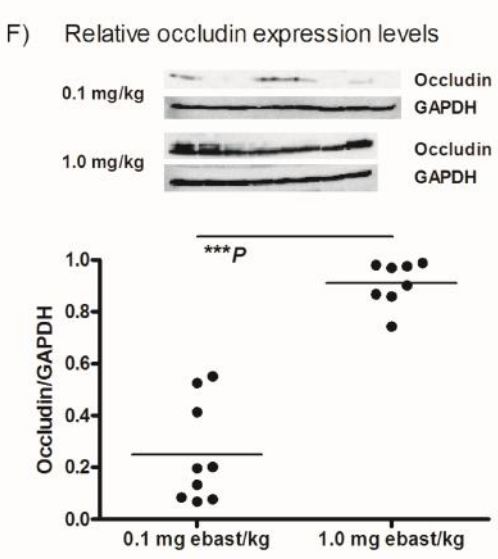


Figure 3. In vivo post stress ebastine treatment.
Sensitivity to distension was measured pre-WA and 24 and 48 hours post-WA in MS rats. Ebastine (cumulative dose 0.1 or 1.0 mg/kg) was administered 3 times between 24- and 48 hours measurements (please refer to figure 1C for vehicle treatment group). WA induced increased AUC in both groups (figure A and B, white vs black bars) and a cumulative dose of 1.0 (B) but not 0.1 mg ebastine/kg (A) was able to reverse post-WA hypersensitivity. Line-diagrams of per volume responses show similar results: significantly increased, WA-induced, response to distension for all volumes that were reversed in 1.0 mg/kg but not 0.1 mg/kg treated rats. Semi-quantitative evaluation of (distal) colonic RMCP-2 levels by Western blot showed no difference in expression (E). Compared to 0.1 mg/kg treated rats, occludin expression was significantly higher in 1.0 mg/kg treated rats (F). All data are depicted as mean ± SEM (n=9 and 8 rats per group). Statistical differences: ***P*< 0.01 and ****P*<0.001.

Rats were sacrificed directly after distensions at the 48 hours time point and selected tissue samples (i.e. tissue not distended by balloon) from distal colon were gathered and evaluated by semi quantitative Western blot. Densitometric analysis of RMCP-2 expression levels (figure 3E) showed no differences between two treatment groups. However, compared to 0.1 mg/kg treated rats, occludin levels were significantly higher in rats treated with 1.0 mg ebastine/kg (figure 3F, ****P*<0.001).

H1R antagonist mediated modulation of C48/80 induced RBL-2H3 degranulation.

We monitored C48/80-induced release of β-hexosaminidase by RBL-2H3 cells to demonstrate possible mast cell stabilizing qualities of fexofenadine and ebastine. C48/80 induced a dose dependent release of the enzyme from RBL-2H3 cells (figures 4A and B). 30 minutes fexofenadine pre-treatment reduced baseline release as well as release induced by 500 and 1000 µM C48/80 (figure 4A, **P*<0.05). In contrast, the ebastine metabolite carebastine was unable to prevent degranulation in any of the C48/80 concentrations tested (figure 4B).

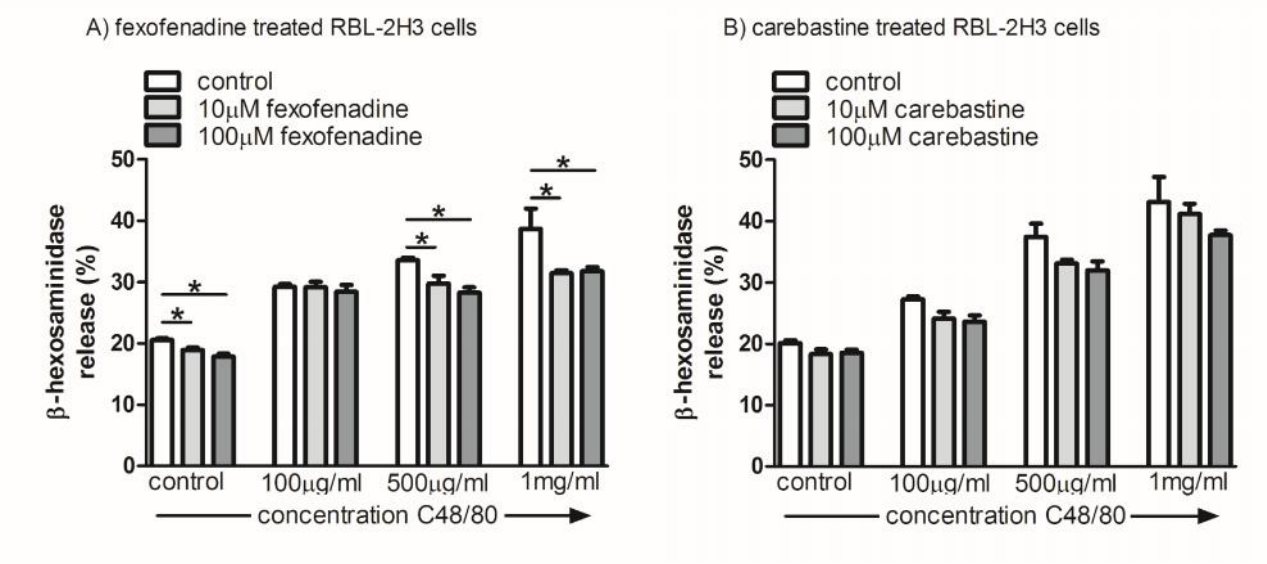


Figure 4. Modulation of Compound 48/80-induced RBL-2H3 degranulation.
Compound 48/80 treatment of RBL- 2H3 cells induced a concentration dependent release of β-hexosaminidase. 30 minutes pre-incubation with 10 and 100 µM fexofenadine reduced β-hexosaminidase release in control cells and cells treated with 500 and 1000 µg Compound 48/80/ml (figure A). Carebastine pretreatment did not show an effect on Compound 48/80 stimulated RBL-2H3 cells (figure B). Results are a representative example of 3 independent experiments and expressed as mean ± SEM (4 wells per condition, **P*<0.05).

DISCUSSION

In a recent clinical trial the use of ketotifen was shown to improve health related quality of life, increase threshold of discomfort and decrease IBS symptoms in hypersensitive IBS patients. Ex vivo evaluations of pre- and post therapy rectal biopsies suggested that positive trial outcome was not due to the mast cell stabilizing effect of ketotifen but may be related to the H1R-antagonistic properties of this compound. Because ketotifen treatment may be associated with central side effects we evaluated, in the rat MS-model, the potential use of peripherally restricted H1R-antagonists. Our data show that the selective antagonists fexofenadine and ebastine are both capable of reversing post-WA visceral hypersensitivity.

Early life stressors are known to predispose for IBS at adult age[17,18] and MS in rat is a well accepted animal model to mimic such predisposing factors.[2] Although in some rat strains neonatal MS as such is enough to induce an IBS-like phenotype, Long Evans rats have to be subjected to an additional acute stress (e.g. WA) at adult age to bring out the hypersensitive phenotype.[12] This is similar to what is observed in IBS patients were acute stress is a known trigger for visceral hypersensitivity. In MS Long Evans rats we were able to show that mast cell degranulation plays a pivotal role in the development of stress-induced visceral hypersensitivity and loss of barrier integrity. Pre stress treatment with the mast cell stabilizer doxantrazole prevented and post-stress treatment reversed WA-induced hypersensitivity to distension and occludin degradation.[13,15] Although histamine may be one of the mediators released upon mast cell activation direct evidence for a role in MS associated visceral hypersensitivity was not available so far. Histamine is however one of the mast cell mediators implicated in the activation of afferent-expressed TRPV1[19] and post stress treatment with the selective TRPV-1 antagonist SB-705498 reversed visceral hypersensitivity in the MS model.[15] Thus, we considered MS a suitable model to evaluate the possible use of H1R antagonists in the treatment of stress-induced IBS-like phenotypical changes. Importantly, because a possible future treatment protocol would aim to reverse complaints in IBS patients we evaluated these compounds in a post stress treatment protocol. Fexofenadine as well as ebastine were capable of reversing post-WA visceral hypersensitivity.

The outcome of these experiments contradicts earlier investigations involving the in vivo use of the calcium ionophore BrX-537A (Bromolasolacid). Coelho *et al.* showed that intraperitoneal administration of BrX-537A led to mast cell degranulation and enhanced sensitivity to colorectal distension.[20] The observed visceral hypersensitivity was prevented by 5-HT_{1a} receptor antagonist

but not by histamine receptor-1, -2 and -3 antagonists. In these experiments only one dosage of H1R antagonist (1 mg chlorophenizamine/kg) was used and we can not rule out the possibility that it was to low to be effective. Alternatively, histamine release may occur in both experimental conditions but release only has consequences relevant to visceral sensitivity when rats are predisposed to react to this mediator (e.g. by increased H1R expression). In relation to this, mucosal biopsies of IBS patients were indeed shown to have increased H1R mRNA over controls.[7] Therefore, we investigated the possibility of enhanced post-WA H1R expression in MS rats but expression was not increased over NH rats. Although the same approach to H1R evaluation was successfully used by Sander *et al.*[7], we can not exclude the possibility that existing differences between groups were diluted out because we evaluated whole tissue specimens instead of isolated sensory neurons. Another explanation for the observed discrepancy with the earlier BrX-537A investigations may be found in an often neglected aspect of in vivo visceral sensitivity investigations. The calcium ionophore study evaluated prevention of mast cell induced hypersensitivity whereas our H1R antagonist data describe reversal of mast cell dependent hypersensitivity. The difference is not 'just semantics'. Recent data on the use of α-helical-CRF (9-41) showed that pre-WA targeting of CRF receptors prevented, but post-WA targeting could not reverse stress induced visceral hypersensitivity.[13] Similarly, histamine may play a role in prolonged mast cell dependent hypersensitivity but not during an acute phase such as investigated in the BrX-537A experiments.

Because ketotifen that was used in the IBS clinical trial has H1R antagonistic as well as mast cell stabilizing qualities we also evaluated fexofenadine and carebastine (the active metabolite of ebastine) for possible mast cell stabilizing effects. Data obtained with RBL-2H3 cells indicated that fexofenadine had some weak mast cell stabilizing quality and carebastine, although results did not reach significance, showed the same trend. However, the level of stabilization was far from complete and can never explain the successful in vivo reversal of post stress visceral hypersensitivity by these compounds. Further, our results on RMCP-2 tissue expression levels suggest that in vivo mast cell degranulation is not altered by their use. In an earlier study we showed that in vivo post stress mast cell degranulation is associated with a decrease in tissue RMCP-2 levels.[13] In the current investigations high (1.0 mg/kg) but not low dose (0.1 mg/kg) ebastine effectively reversed visceral hypersensitivity whereas semi-quantitative evaluation of corresponding colonic tissues did not show differences in RMCP-2 expression levels. The latter data suggest equal level of mast cell degranulation in treatment groups and confirmed the lack of in vitro stabilization by ebastine. Therefore, at least for ebastine, we suggest that H1R antagonism rather than mast cell stabilization was the in vivo mechanism of action.

In addition to RMCP-2, homogenized colonic tissue samples of ebastine-treated rats were evaluated for occludin expression. In a previous study we observed post-stress degradation of this tight junction protein in stripped colonic mucosa of MS Long Evans rats.[13] Here, ebastine-induced reversal of visceral hypersensitivity was associated with high- and failure to reverse with low- level occludin expression. How this change is relevant for the observed visceral hypersensitivity is not clear yet. However, barrier dysfunction is thought to be an important pathophysiological mechanism in IBS and in patient biopsies occludin degradation was shown to correlate with abdominal pain intensity scores.[21,22] The latter may be explained by enhanced mucosal influx of luminal antigens and/or bacteria leading to subsequent immune cell and afferent activation.[4] In relation to this, *in vivo* occludin depletion by selective siRNA-induced knock down in mouse intestine was indeed shown to enhance macromolecular flux across intestinal epithelial cells.[23] A direct link between barrier dysfunction and hypersensitivity to distension was shown in rats where intra-colonic infusion of a tight junction blocker prevented stress induced rectal hypersensitivity.[24] In the present investigations we only obtained a limited dataset on occludin expression. Future investigations should aim to establish whether ebastine treatment, next to possible effects on afferent expressed H1R[6], can also lead to antagonist mediated restoration of barrier function.

At present peripherally restricted H1R-selective antihistamines are the most broadly used medications in the treatment of allergic diseases. In consequence, compounds like ebastine and fexofenadine have been extensively investigated regarding clinical pharmacology and safety. The present study indicates that these compounds are capable of reversing post stress visceral hypersensitivity. Since this trait may be relevant to IBS we suggest that peripheral H1Rs can be a safe new target for IBS therapy.

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Chapter 5

Susceptibility to stress induced visceral hypersensitivity in maternally separated rats is transferred across generations

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ABSTRACT

Background:

In IBS, familial clustering and transfer across generations may largely depend on environmental factors but this is difficult to establish in the human setting. Therefore, we aimed to set up a relevant animal model. We investigated whether susceptibility to stress induced visceral hypersensitivity in maternally separated (MS) Long Evans rats can be transferred across generations without further separation protocols and, if so, whether this depends on maternal care.

Methods:

At adult age, we evaluated pre- vs post water avoidance (WA) changes in visceromotor response to distension in nonhandled second filial generation offspring (NH-F2) of previously separated MS-F1 dams. Further, the role of maternal care was evaluated by cross fostering F2 offspring of NH-F1 and MS-F1 dams and subsequent sensitivity measurements at adult age. Involvement of mast cells in post stress hypersensitivity of NH-F2 rats was evaluated by mast cell stabilization.

Key Results:

In adult NH-F2 offspring of MS-F1 dams, post-WA hypersensitivity to colorectal distension was observed in 80% of rats compared to 19% in offspring of NH-F1 dams. Cross-fostered pups adapted to the phenotype of the foster mother: pups of NH-F1 dams nursed by MS-F1 dams showed post-WA hypersensitivity to distension at adult age and vice versa (100% and 20% respectively). In NH-F2 rats, post-WA hypersensitivity was reversed by mast cell stabilizer doxantrazole.

Conclusions and inferences:

MS-induced susceptibility to stress-triggered visceral hypersensitivity is transferred across generations and this transfer depends on maternal care. Thus, MS is a suitable model to evaluate environmental triggers relevant to IBS clustering in families.

INTRODUCTION

The irritable bowel syndrome (IBS) is a functional bowel disorder characterized by abdominal pain or discomfort associated with defecation or a change in bowel habit.[1] Poor understanding of mechanisms relevant to this disorder hamper research efforts to develop effective treatment strategies. Directions in research seem influenced by the question whether the most important etiopathogenic influences are genetic or environmental in nature. In relation to this it was suggested that identification of disease–susceptibility loci for IBS may lead to better understanding of disease aetiology and facilitate drug-development.[2] The search for single nucleotide polymorphisms associated with an increased risk for IBS resulted in several genetic associations. However, most of these studies were performed on relatively small cohorts and never replicated.[3] Although a genetic aetiology for IBS was also put forward because IBS tends to cluster in families,[4-8] it should be noted that familial aggregation is not necessarily explained by shared disease susceptibility genes. Clustering may also relate to common environmental factors. Indeed, most twin-studies suggested that, next to a genetic component, environmental factors have equal or perhaps even greater influence on development of IBS.[9-13] Risk-factors such as infection,[14] diet,[15] childhood affluence,[16] illness behaviour of parents,[17] physical and sexual abuse[18] and adverse parent-child interactions[19-21] may all be considered possible environmental triggers relevant to familial clustering.

In a previous twin-study it was shown that having a mother with IBS or having a father with IBS are independent predictors of irritable bowel status.[11] Although in this study it was shown that heredity also contributed, the environment was shown to have equal or even greater influence on the development of IBS. These data may suggest that clustering in families or even transfer across generations can occur independently of changes in DNA sequence. Whether this is true and, if so, is due to social learning or other factors such as aberrant parent child interactions, transmission of milk born factors or even vertical transmission of an 'IBS prone microbiome' from parent to offspring remains to be established. Since human studies in these directions are difficult to perform, we aimed to set up a relevant animal model and decided to take increased sensitivity to rectal distension (so called visceral hypersensitivity) as readout.

In IBS, visceral hypersensitivity is considered a possible pathophysiological mechanism. Visceral hypersensitivity is observed in the majority of patients and can be triggered by stress.[22;23] The latter was also shown in the maternal separation model in rat: when maternally separated (MS) Long Evans rats were subjected to acute stress at adult age they displayed post-stress visceral hypersensitivity.[24;25]

In the present study we tested the hypothesis that susceptibility to stress-induced visceral

hypersensitivity in MS Long Evans rats can be transferred across generations and, if so, whether this depends on maternal care. Moreover, because mast cell degranulation is essential to the post-stress phenotype in MS rats[26] and possibly IBS patients,[27] we also assessed the relevance of this cell type in second generation animals.

MATERIAL AND METHODS

Animal ethics statement. All procedures were conducted in accordance with the institutional guidelines and approved by the Animal Ethical Committee of the AMC/University of Amsterdam (reference protocol number 100998).

Animals. Long-Evans rats (Harlan, Horst, the Netherlands) were housed at the animal facility of the AMC (Amsterdam, The Netherlands) under conditions of controlled light (06:00-18.00), temperature (20-22 ^{0}C) and humidity (45%) and kept in standard macralon cages with a layer of wood shavings. Water and food (SDS; Technilab BMI, Someren, The Netherlands) were available *ad libitum*.

Colonic distension protocol and acute water avoidance (WA) stress. Colonic distensions were performed with a latex balloon (Ultracover 8F, International Medical Products, Zutphen, The Netherlands) and carried out as described before.[25] Insertion of the catheter was performed under brief isoflurane anaesthesia. Distensions started after a 20 minute recovery period. They were performed at the minimum age of 3 months (weight > 300 grams) and achieved by inflation of graded volumes of water (male rats: 1.0, 1.5 and 2.0 mL, female rats: 0.8, 1.2 and 1.6 mL). Length and diameter of the balloon during a 2 mL maximum volume distension were 18 and 15 mm respectively. For each volume a 20-s distension period followed by an 80-s resting period was exercised. For acute stress at adult age (WA stress) rats were positioned on a pedestal surrounded by water for a time-period of one hour. Sensitivity to distension was evaluated pre- and 24 hours post-WA.

Measurement of the visceromotor response to colonic distension and data analysis. Distension of the colon induces contractions of abdominal musculature: the visceromotor response. Quantification by means of electromyography (EMG) reflects visceral sensitivity. We previously validated a radio-telemetry technique in freely moving rats to record these signals[25] and further details on techniques and data analysis system have been published extensively.[25;28-30] Data analysis was performed by extracting, from the raw EMG data file, each 20-s distension period and its preceding 20-s of baseline recording. After correction for movement and breathing, data were rectified and integrated. Absolute

data sets were then obtained by subtracting the 20-s baseline recording from the 20-s distension result. Similar to our earlier publications, final results are given as normalized data sets, which were calculated from the absolute data by setting the 2mL value of the first (pre-stress) distension at 100%. Subsequently, area under the curve (AUC) of relative responses was calculated for individual rats and used to 1) establish whether, based upon a predefined cut off value, individual rats were hypersensitive to distension, and 2) to calculate average AUC within treatment groups. Relative response data were also used to evaluate possible changes on a per volume basis. Data are always depicted as mean +/- SEM.

Historical data and definition of AUC cut off value. Although not all MS rats will become hypersensitive to distension upon WA, we always included all evaluated animals when presenting averaged results in previous publications.[25;28-30] In the current study we followed the same procedure and, in addition, defined the absolute number of hypersensitive rats in different groups and generations tested. To this end we defined an AUC cut off value based on historical data. In the results section we compiled data on sensitivity of first filial generation (F1) nonhandled (NH) and MS rats (n=72 for each group). These data are gathered from earlier experiments[25;28-30] and concern adult rat groups that were either unresponsive to vehicle treatment or received no pharmacological treatment at all. Because of possible impact of the estrous-cycle on visceral sensitivity,[31] data were obtained in male rats only, female pups were always eliminated from the nest on post natal day 2 (please refer to figure 1A for schematic representation of groups). The arbitrary AUC cut off value was defined by calculating the upper 10% pre-WA AUC (distension-vs-response) of the 72 NH rats. Subsequently, all individual rats (NH and MS) with an AUC above cut off were considered hypersensitive to distension.

Maternal separation. Female pups were eliminated on post natal day 2. During MS, dams were separated from the nest from post natal day 2 to 14 for 3 hours/day. Separation was achieved by placing the dams into another cage in a separate room. During separation, cages were placed on a heating pad (30-34 °C) to help pups regulate normal body temperature. Weaning was performed on post natal day 22 and rats were then raised in pairs of two. NH pups were nursed normally.

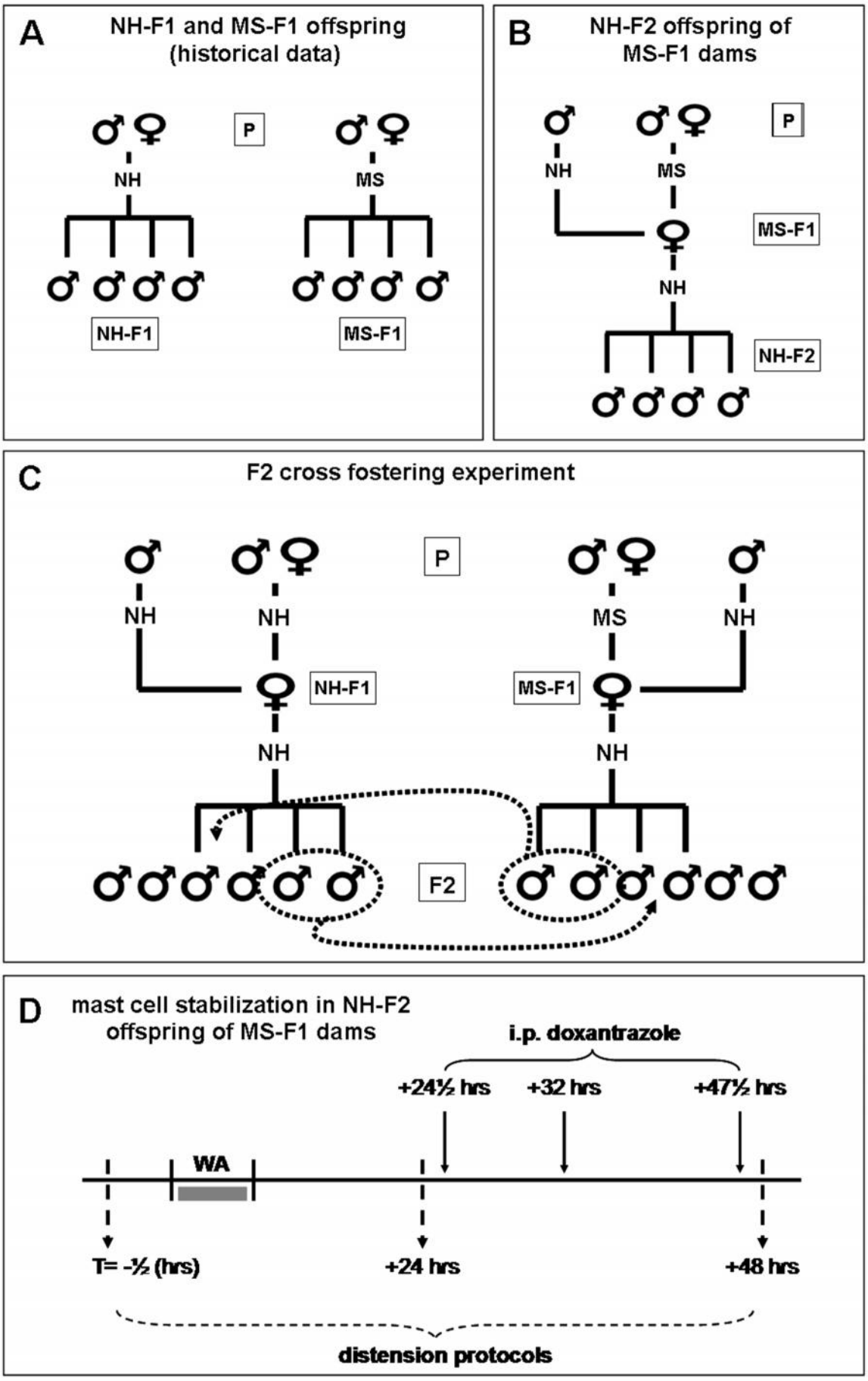

A
NH-F1 and MS-F1 offspring
(historical data)
P
NH
MS
NH-F1
MS-F1
B
NH-F2 offspring of
MS-F1 dams
P
NH
MS
MS-F1
NH
NH-F2
C
F2 cross fostering experiment
P
NH
NH
MS
NH
NH-F1
MS-F1
NH
NH
F2
D
mast cell stabilization in NH-F2
offspring of MS-F1 dams
i.p. doxantrazole
+24½ hrs
+32 hrs
+47½ hrs
WA
T= -½ (hrs)
+24 hrs
+48 hrs
distension protocols

Figure1. Schematic representation of experimental protocols. All parental generation male and female animals (P) were normal nonhandled (NH) rats. Visceromotor response to distension was determined before and 24 hours after WA in adult rats. 1A) Historical data: nests subjected to the maternal separation (MS) protocol rendered MS first filial generation (MS-F1) offspring. Nests that were left undisturbed rendered NH-F1 control rats. 1B) MS-F1 females mated with NH males. NH-F2 offspring was not subjected to the separation protocol. 1C) F2 offspring of MS-F1 dams was cross-fostered to the nest of NH-F1 dams and vice versa. None of the F2 offspring was subjected to MS protocol. 1D) Visceromotor response to colonic distension was determined before and 24 hours after the NH-F2 offspring of MS-F1 dams was subjected to water avoidance (WA). After treatment with the mast cell stabilizer doxantrazole or vehicle alone the last distension protocol was carried out 48 hours post-WA.

Nonhandled second filial generation (NH-F2) offspring of MS-F1 dams. Because female pups are usually culled on post natal day 2, extra separation nests were used in which MS-F1 females were allowed to grow up together with male littermates. Adult MS-F1 females were then allowed to mate with NH males and the subsequent male F2 offspring was not subjected to the maternal separation protocol (NH-F2; see figure 1B for diagram). Pre- and post-WA distension protocols were performed in adult NH-F2 as well as in the MS-F1 dams (at least one month after they gave birth to NH-F2 offspring). All dams were measured in the same stage of the estrous cycle (diestrus stage) as detected by evaluation of vaginal smears.

Cross fostering experiments. F2-offspring of MS-F1 and NH-F1 dams was cross fostered within 24 hours after pups were being born. In short, after removal of female pups from the nests, 2 male pups were marked and then switched from one nest to the other, remaining pups were left with their own dams (litters were not culled to equal numbers of rats). Switching of not more than 2 pups was chosen because cross fostering of whole litters is known to influence maternal behaviour[32] and this can be prevented by limiting the number of cross fostered rats.[33] Weaning was performed on post natal day 22 and pre- vs post-WA visceromotor response to distension was evaluated in adult F2 offspring. Importantly, none of the F2 offspring was subjected to the maternal separation protocol (see figure 1C for schematic representation).

Mast cell stabilization experiments. Our previous investigations showed that stress-induced hypersensitivity to distension in male MS-F1 Long Evans rats depends on mast cell degranulation. When administered pre-WA, the mast cell stabilizer doxantrazole prevented stress-induced visceral hypersensitivity[29] whereas post-WA administration reversed increased sensitivity.[30] Here we determined whether WA-induced hypersensitivity in adult male NH-F2 offspring of MS-F1 dams can also be reversed by mast cell stabilization. In short, after establishing pre-WA sensitivity status, the visceromotor response was measured again at T= +24 hours and T= +48 hours post-WA. In between these two post-WA time-points (at T= +24½, 32 and 47½) rats (n=9) received i.p. doxantrazole (10 mg kg^{-1}, gift of Agnès Francois, Institut Gustave Roussy, Villejuif, France) dissolved in 0.5%

$NaHCO_3$/0.9% saline pH 7.5, or vehicle alone (n=9). The experimenter (OW) was blinded to the different treatment groups that were only disclosed after interpretation of the individual tracings. See figure 1D for schematic representation of the experiment.

Statistical analysis. Statistical calculations were performed using SPSS for windows (version 16.0.1, Chicago, Il, USA). Visceromotor response data were analysed with the Wilcoxon signed ranks test that was applied to the normalized data sets. All data are presented as mean ± standard error of mean and $P<0.05$ was considered statistically significant.

RESULTS

MS-F1 dams At least one month after giving birth, seven out of nine MS-F1 dams were subjected to WA and distension protocols (other two dams were mistakenly sacrificed before measurements took place). The volume/relative-response data for this group are depicted in figure 2A. Post WA AUC was significantly increased over pre-WA AUC (108.2 ± 7.1 *vs* 57.4 ± 2.3, $^{\#}P<0.05$). Similar results were obtained by per volume comparisons; we observed a significant increase in response for all 3 volumes tested ($^{*}P<0.05$).

NH-F2 offspring of MS-F1 dams Nine MS-F1 female rats mated with NH males and gave birth to n=30 NH-F2 male offspring that were not subjected to the separation protocol. Figure 2B shows the volume/relative-response relationship for the NH-F2 group. AUC of the 24 hours post-WA time point was significantly increased over pre-WA AUC; 100.9 ± 4.2 *vs* 68.8 ± 1.5 $^{\#\#\#}P<0.001$. Per volume comparisons corroborated AUC-data and showed increased post-WA response at 1.0, 1,5 and 2.0 ml ($^{***}P<0.001$).

Cut off value and % hypersensitive rats in different groups The volume/relative response data of 72 NH-F1 and 72 MS-F1 male rats measured in previous studies are depicted in Figure 2C and D respectively. Pre-WA vs post-WA comparisons of AUC as well as per volume differences show significantly increased post-WA responses in MS-F1 rats (pre-WA vs post-WA AUC; 63.8 ± 1.4 vs 96.3 ± 2.8, $^{\#\#\#}P<0.001$ and pre-WA vs post-WA per volume comparisons of 1.0, 1.5 and 2.0 ml all $^{***}P<0.001$). Figure 2E shows the individual pre-WA AUC data for the 72 NH rats. The 90 percentile AUC of their pre-WA response was 77.8. This number was then used as an arbitrary cut-of-value to define hyper- or normo-sensitivity status in individual rats (AUC>77.8 defined as hypersensitive). Based on this, figure 2F depicts the percentage of hypersensitive animals in the different groups. By definition, 10% of the 72 NH rats showed pre-WA hypersensitivity, this number increased to 19.4% post-WA. In contrast, in the 72 MS-F1 rats 8.3% was hypersensitive pre-WA and this increased to

76.4% post-WA. Similar results were obtained for the 30 NH-F2 rats; 13.3% was hypersensitive pre-WA and 80% post-WA.

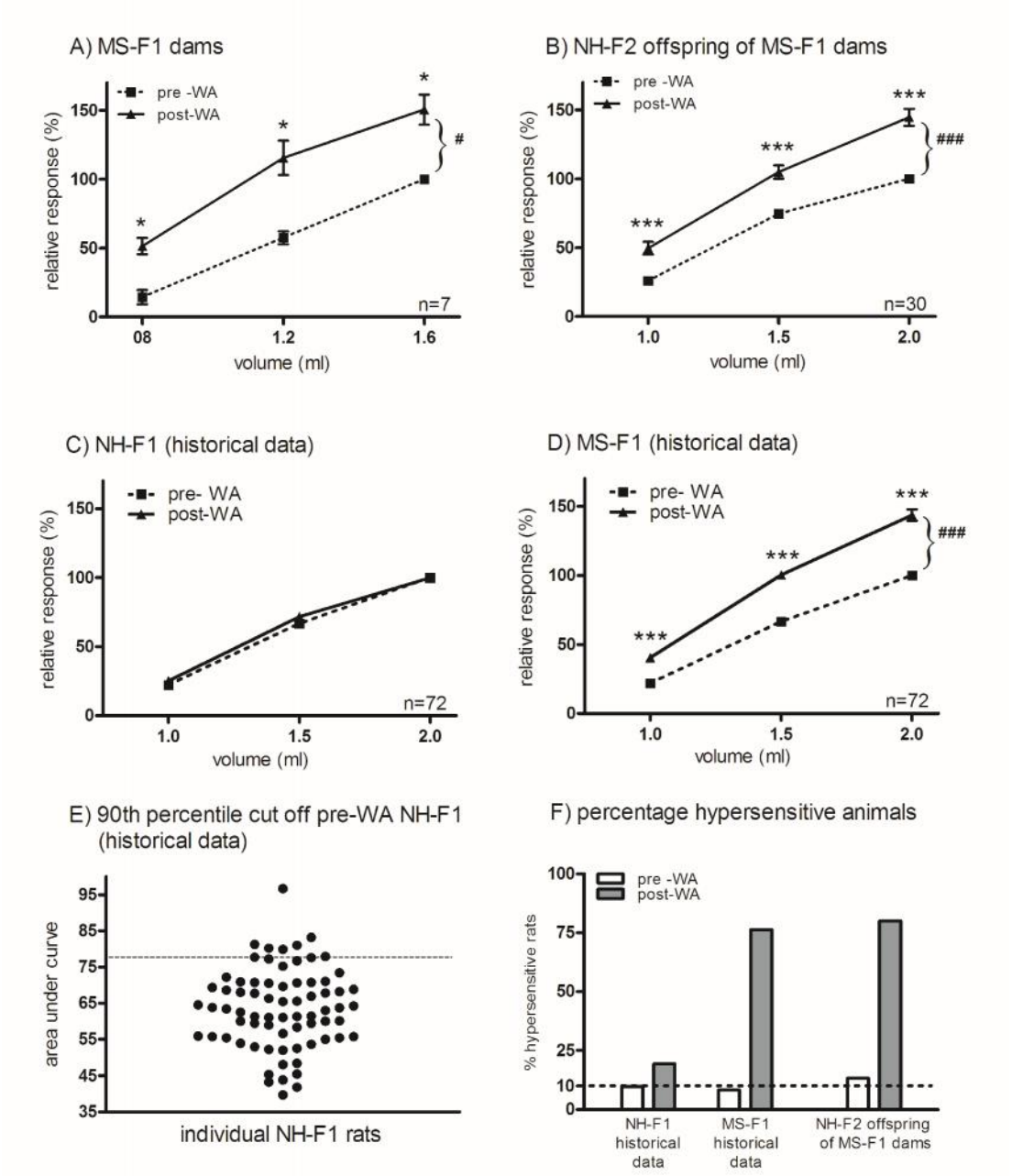


Figure 2. Effect of WA-stress on visceromotor response to colonic distension, all data in 2A-2D are given as mean +/- SEM. Figure 2A) depicts enhanced post-WA response to distension in n=7 MS-F1 dams (evaluated after giving birth to NH-F2 offspring shown in 2B); pre-WA vs post-WA AUC, $^{\#}P<0.05$ and increased post-WA response in all 3 per volume comparisons ($^{*}P<0.05$). Figure 2B) shows enhanced post-WA response to distension in 30 NH-F2 male offspring of MS-F1 dams; pre-WA vs post-WA AUC, $^{\#\#\#}P<0.001$ and similar results in pre-WA vs post-WA per volume comparisons ($^{***}P<0.001$ for all 3 volumes). Historical data of n=72 NH-F1 male rats (Figure 2C) and 72 MS-F1 male rats (Figure 2D) show enhanced post-WA AUC in MS-F1 rats (pre-WA *vs* post-WA AUC; $^{\#\#\#}P<0.001$ and pre-WA *vs* post-WA per volume comparisons all $^{***}P<0.001$). Figure 2E) shows the individual pre-WA AUC data for the 72 NH-F1 rats. The 90 percentile pre-WA AUC value was calculated (77.8) and used as an arbitrary cut off value for further evaluations. In Figure 2F) the AUC cut off value was used to define the % hypersensitive rats in different investigated groups. In the NH-F1 group WA stress only induced a moderate increase in the number of hypersensitive rats. In contrast, strongly increased numbers were observed in post-WA MS-F1 and NH-F2 groups.

Cross fostering experiment In these cross fostering experiments none of the F2 offspring was subjected to the maternal separation protocol. Average volume/relative-response data of the 4 different groups are given in figure 3. When nursed by their natural NH-F1 mothers, WA was unable to induce an enhanced response to distension in NH-F2 offspring (n=7, Figure 3A). In contrast, when pups of NH-F1 dams were nurtured by MS-F1 foster mothers there was a significant increase in post-WA AUC (pre-WA *vs* 24 hours post-WA AUC; 59.7±5.6 *vs* 115.8±7.9, $^{\#}P<0.05$, n=5) as well as increased responses based on per volume comparisons ($^{*}P<0.05$ for all 3 distension volumes, Figure 3B). Figures 3C confirms that, when raised by their natural mother, pups of MS-F1 dams become hypersensitive to distension upon WA; pre-WA vs post-WA AUC; 77.6±6.1 vs 109.7±5.9, $^{\#}P<0.05$ (n=6) and $^{*}P<0.05$ for 1.5 and 2.0 ml distension volumes. When, on the other hand pups of MS-F1 dams are switched to NH-F1 foster mothers, the enhanced post-WA response to distension does no longer occur (Figure 3D, n=5).

The individual sensitivity status of cross fostered rats is depicted in Table 1. Again, rats with volume/relative response AUC>77.9 were considered to be hypersensitive to distension (marked with an asterisk). When nurtured by NH-F1 dams, all but two rats (one delivered by a MS-F1 dam and one by a NH-F1 dam) were normo-sensitive upon WA. In contrast, all animals reared by MS-F1 dams showed post-WA visceral hypersensitivity.

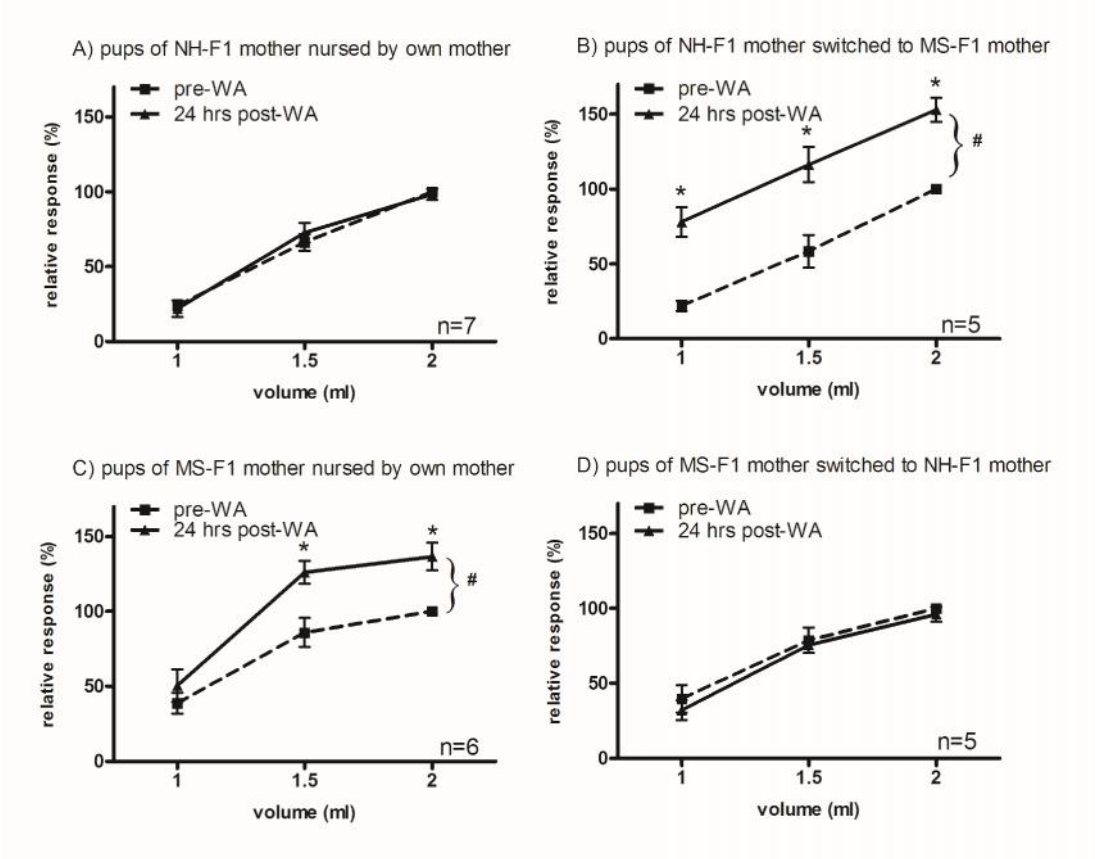


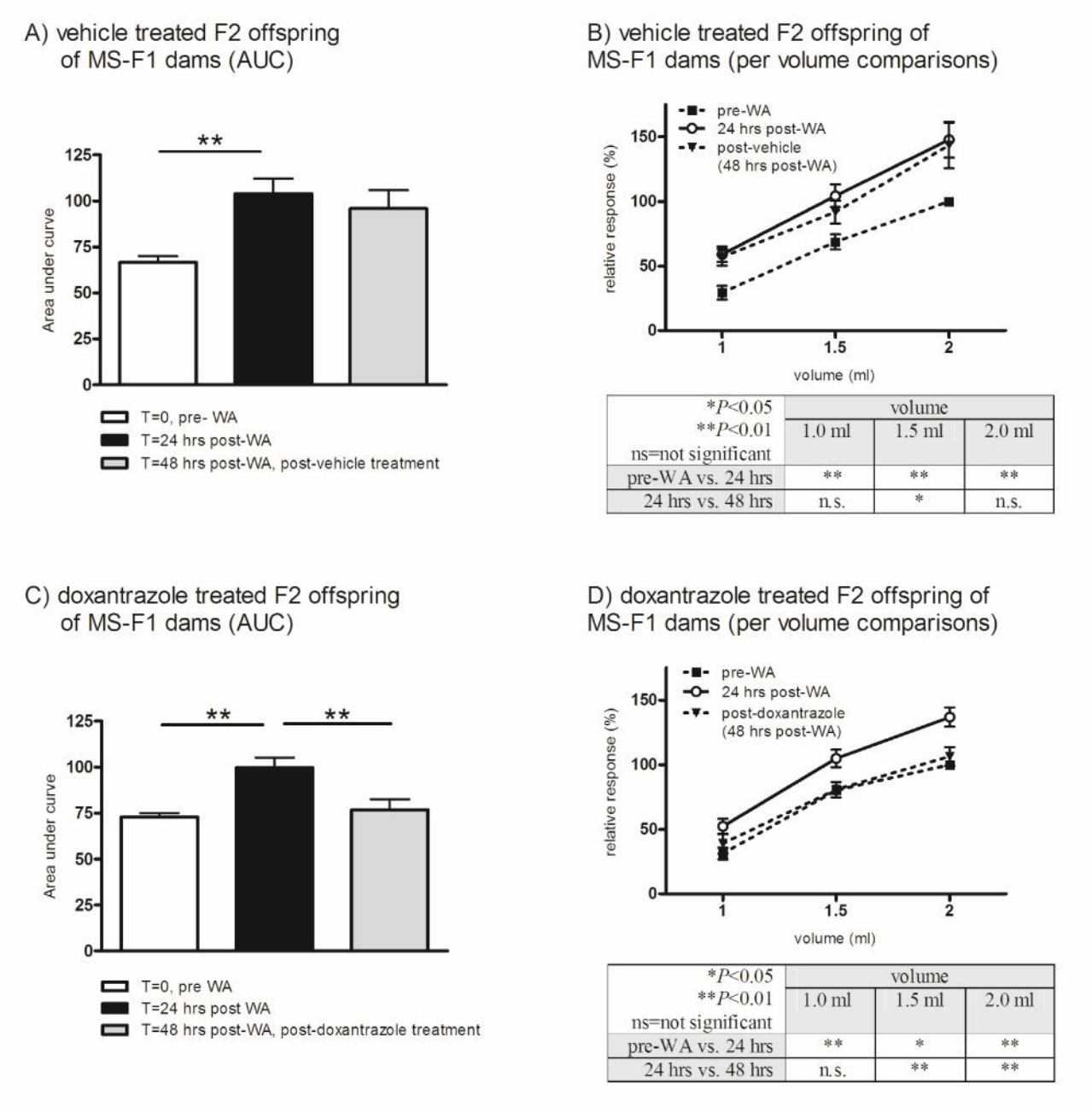
Figure 3. Volume/relative response data of cross fostering experiments, data are mean ± SEM. A) Absence of WA-induced visceral hypersensitivity when NH-F2 offspring (n=7) was nursed by own NH-F1 dams. B) NH-F2 offspring (n=5) switched to MS-F1 dams. WA induced an enhanced response to distension; pre-WA *vs* 24 hours post-WA AUC; $^{\#}P<0.05$ and $^{*}P<0.05$ for pre-WA *vs* post-WA comparisons of all 3 distension volumes. C) MS-F2 offspring (n=6) nursed by own MS-F1 dams. WA induced an enhanced response to distension; pre-WA vs post-WA AUC; $^{\#}P<0.05$ and $^{*}P<0.05$ for 1.5 and 2.0 ml distension volumes. D) Absence of post-WA hypersensitivity to distension when MS-F2 offspring (n=5) was switched to NH-F1 dams.

	nurtured by NH-F1 dams		nurtured by MS-F1 dams	
	pre-WA	Post-WA	pre-WA	Post-WA
offspring of MS-F1 dams	85.5 *	78.0 *	68.9	119.3 *
	75.0	63.3	81.0 *	111.3 *
	66.0	75.4	64.9	96.2 *
	84.5 *	62.4	73.2	107.2 *
	60.3	69.5	106.2 *	131.6 *
			71.4	92.8 *
offspring of NH-F1 dams	70.3	63.8	70.7	106.5 *
	65.9	67.1	44.6	90.7 *
	76.4	81.6 *	58.0	125.2 *
	59.6	76.0	51.2	121.0 *
	50.1	58.5	73.8	135.7 *
	60.6	47.3		
	65.5	69.9		

Table 1. Individual results of cross fostering experiments. Cells in grey represent pre- and post-WA data (AUC) of rats reared by foster mothers, white cells are animals reared by their natural mother. AUC > 77.8 (predefined cut of value) are marked with an asterisk and represent hypersensitive rats.

Mast cell stabilization in male NH-F2 offspring of MS-F1 dams Previous investigations showed that post stress visceral hypersensitivity in MS-F1 Long Evans rats depends on mast cell degranulation.[30] Since NH-F2 offspring of MS-F1 dams also showed post-stress visceral hypersensitivity we evaluated the role of mast cells in this response by post-WA application of doxantrazole or vehicle alone. Figure 4A depicts relative response/volume AUC data of vehicle treated rats; WA induced enhanced post stress sensitivity to distension and this was not reversed by vehicle alone (pre-WA *vs* post-WA *vs* post vehicle; 66.7±3.3 *vs* 103.9±8.2 ($^{**}P<0.01$) *vs* 95.9±9.8). Per volume comparisons (depicted in the line graph and accompanying statistics box in Figure 4B) show similar results: increased post-WA response for all 3 volumes ($^{**}P<0.01$) that was only reversed at 48 hrs for the 1.5 ml distension volume ($^{*}P=0.03$). Figure 4C shows AUC data corresponding to doxantrazole treatment (pre-WA vs post-WA vs post doxantrazole 72.9±2.1 vs 99.7±5.5 ($^{**}P<0.01$) vs 76.7±5.8 ($^{**}P<0.01$)). Per volume evaluations show similar results: for all 3 volumes comparison of pre-WA vs 24 hrs post-WA reveals significantly enhanced sensitivity (1.0, 1,5 and 2.0 ml;

**P<0.01, *P<0.05 and **P<0.01 respectively) that was reversed by doxantrazole treatment at the 1.5 and 2.0 ml volumes (both **P<0.01).



*P<0.05 **P<0.01 ns=not significant	volume		
	1.0 ml	1.5 ml	2.0 ml
pre-WA vs. 24 hrs	**	**	**
24 hrs vs. 48 hrs	n.s.	*	n.s.

*P<0.05 **P<0.01 ns=not significant	volume		
	1.0 ml	1.5 ml	2.0 ml
pre-WA vs. 24 hrs	**	*	**
24 hrs vs. 48 hrs	n.s.	**	**

Figure 4. Mast cell stabilization experiments in NH-F2 offspring of MS-F1 dams. In histograms A) and C) data are depicted as AUC. Both treatment groups show enhanced 24 hours post-WA sensitivity to distension (**P<0.01) but only doxantrazole treated rats (Figure 4C) are reversed at the 48 hour time point (24 hours post-WA vs 48 hours post-WA, **P<0.01). Figure 4B) and 4D) show the same results as volume/relative response data with corresponding statistics boxes for per volume comparisons. All data are mean ± SEM.

DISCUSSION

IBS clustering in families and transfer across generations may largely depend on environmental factors. The nature of these factors is unclear and difficult to pinpoint in the human setting: often conclusions beyond 'may be associated with' are not possible. Although animal investigations can never fully reflect the human situation, rodent studies do have the advantage that subsequent generations can be investigated in a relatively short timeframe. Maternal separation in rat is a model with IBS-like features. In this study we show for the first time that susceptibility to stress induced visceral hypersensitivity in maternally separated rats can be transferred to the next generation without further separation protocols. Our cross-fostering experiments indicated that the observed transfer depends on maternal care. Moreover, and similar to earlier findings in F1 rats, we were able to show that mast cell degranulation is essential to the observed post stress F2 phenotype. Together these findings indicate that maternal separation can be used to investigate cross-generational effects of environmental IBS-triggers.

IBS clusters in families (i.e. aggregation in parents, siblings and offspring of patients)[4-8] and twin studies suggested that there is an important environmental component to the emergence of this disorder.[9-13] In case environmental factor(s) are essential to aggregation in offspring of patients, each generation must have been exposed to this factor independent of the previous generation, or, the environmentally-induced phenotype is transferred vertically from one generation to the next. Our investigations in rats suggest that the latter may be the case: enhanced susceptibility to stress-induced visceral hypersensitivity (a hall mark of IBS) was induced by maternal separation and, subsequently, transferred to the next generation without further separation protocols. Importantly, although we did not exclude possible separation-induced genetic mutations, it is known that population-wide Mendelian-inheritance of mutations is a slow process that needs not just one, but many generations. Thus, the observed transfer is more likely the result of soft-inheritance which, in contrast to Mendelian 'hard' inheritance, is well suited to quickly adapt to environmental changes.[34] The latter is corroborated by our cross-fostering results that showed rapid adaptation to the foster-mother phenotype.

Epigenetic modifications are often put forward as an ideal mechanism to facilitate soft inheritance; they refer to functionally relevant modifications to the genome that do not involve a change in the nucleotide sequence. In a recent opinion article by Dinan *et al.* it was suggested that the absence of a clear genetic phenotype for IBS is supportive of the view that the disorder is epigenetic in nature and that epigenetics may help explain familial clustering and transgenerational impact of IBS.[35] Methylation of cytosine residues of DNA is among the most investigated epigenetic effects. Since MS rats were shown to have increased HPA-axis responsiveness[36] it is tempting to compare our 'transfer-

results' with those obtained in another transfer model were methylation effects seem to play an important role. In this model Meaney and colleagues studied naturally occurring variations in rat maternal care. Not only were extremes in maternal care transmitted across generations[33] but also enhanced HPA-axis responsiveness. The latter was linked to hyper-methylation of the hippocampal glucocorticoid-receptor promoter.[37] However, when Daniels *et al.* compared the methylation status of the same promoter region between MS and NH-rats, they failed to detect differences.[38] Our own data on GCR protein levels confirm equal hippocampal expression in MS and NH rats (supporting information S1). These data suggest that, in contrast to the behavior-selection model, behavior may not be the determining factor in the maternal separation model. Indeed, Macrì *et al.* convincingly showed that, in contrast to the behavior-selection model, active maternal care is not decreased in MS vs NH rats.[39;40] However, other non-behavioral factors associated with maternal care should not be ruled out as an explanation for results observed in our cross fostering experiments. In relation to this it is known that even brief maternal separations induce increased corticosterone release in dams and indications are, albeit in a different setting, that enhanced corticosterone plasma concentrations can be transmitted to offspring via lactation.[41] Whether this holds true for the MS model remains to be investigated.

Another possible route of phenotype transmission is the microbiome. Evidently the high incidence of post-infectious IBS triggered an increased interest in the possible role of the gut flora in IBS.[42] Microbiota profiling indicated dysbioses of fecal and mucosal microbiota in patients and pre-biotics, pro-biotics and anti-biotics treatment strategies suggested that the observed dysbiosis may be relevant. Although it is not clear when the observed dysbiosis occurs, we do know that bacterial colonization and shaping of the intestinal microbiome begin at birth and are greatly influenced by environmental factors. Among these factors, vertical transmission of the mother's microbiota is considered highly relevant.[43] O'Mahoney *et al.* showed altered fecal microbiota in maternally separated (i.e. MS-F1) rats when compared to NH rats.[44] Thus, in our 'F2-model', transfer of altered microbiome from MS-F1 to NH-F2 is theoretically possible. Future investigations in this direction should first confirm the O'Mahoney report and subsequently establish whether the microbiome of the NH-F2 offspring resembles that of the MS-F1 dams. Finally, transfer of disease associated microbiome from MS-F1 to normal NH adult rat could provide definitive proof for the hypothesized relevance of microbiome transfer. In relation to this, recent evidence obtained by Crouzet *et al.* indicated that germfree rats, when incubated with fecal microbiota of hypersensitive IBS patients, adapt to the IBS-microbiome and concurrent hypersensitivity to distension.[45]

Negative results on the epigenetic modulation of hippocampal GCR in MS[38] do not rule out a role for epigenetic changes in other target tissue or cells. In relation to this, several lines of evidence suggest that mast cells play an important role in IBS. Barbara *et al.* not only showed enhanced numbers of degranulated mucosal mast cells, but mast cell proximity to nerves also correlated with severity and frequency of abdominal pain/discomfort.[46] When supernatants of submerged intestinal human biopsies were used, IBS but not normal control supernatants induced histamine-dependent firing of rat mesenteric afferents[47] and serine-protease dependent colonic hyperalgesia in mice.[48] In a recently performed double-blind placebo controlled trial with the mast cell stabilizer ketotifen, this compound reduced threshold of discomfort and IBS symptoms and improved health related quality of life.[27] In MS-F1 Long Evans rats we previously showed that pre-WA administration of the mast cell stabilizer doxantrazole prevented[29] and post-WA administration reversed[30] stress-induced hypersensitivity to colonic distension. Furthermore, our most recent data indicate that peripherally restricted histamine-1 receptor antagonists can effectively reverse post-WA hypersensitivity in MS-F1.[49] In the present investigation doxantrazole was able to reverse post-WA hypersensitivity in NH-F2-offspring of MS-F1 dams. Although we did not evaluate possible epigenetic modulation of F2 mast cells, future investigations should certainly consider this possibility. Results obtained by Pallinger *et al.* suggested that experimentally induced phenotypical changes in mast cells can be transmitted across generations without further experimental interference.[50] In these experiments female rats were treated with intra-muscular β-endorphin on day 19 of pregnancy. At 7 weeks of age, peritoneal mast cells obtained from the F1-offspring contained significantly more histamine than offspring of control dams and similar results were obtained in their non-treated F2 progeny. Irrespective of the underlying mechanisms, our data provide further evidence that mast stabilizers may be an interesting therapeutic approach for IBS no matter which generation is being targeted.

In conclusion, we showed that separation-induced susceptibility to stress-triggered-visceral-hypersensitivity can be transferred across generations (i.e. from MS-F1 dams to their NH-F2 male offspring). Similar to separated male F1-rats, the stress-induced phenotype of these NH-F2 rats seems to depend on activation of mast cells. Finally, cross fostering of F2-pups indicated that maternal care was essential to the observed transfer. Our data suggest that this model can be used to further delineate environmental triggers and mechanisms relevant to IBS transfer across generations.

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Chapter 6

Summary and conclusions

SUMMARY AND CONCLUSIONS

Next to being one of the most common, irritable bowel syndrome (IBS) is also one of the least understood gastrointestinal disorders. It is a functional bowel disorders identified only by symptoms. Patients experience abdominal pain or discomfort, associated with defecation or a change in bowel habit.[1] Importantly, visceral hypersensitivity (i.e. increased perception of gastrointestinal stimuli) is observed in the majority of patients and considered a pathophysiological mechanism. Although reversal of visceral hypersensitivity may proof beneficial for these patients, effective treatment options are lacking because mechanisms relevant to hypersensitivity are ill defined. Stress, however, was shown to induce enhanced sensitivity to distension in IBS patients[2, 3] and this knowledge can be used as a starting point for novel directions in research. We used the maternal separation model in rat, combined with acute stress at adult age,[4] to obtain a better understanding of mechanisms relevant to visceral hypersensitivity.

In **chapter 2** we confirmed earlier reports[5, 6] on the relevance of mast cell degranulation for post stress visceral hypersensitivity; pre-stress administration of the mast cell stabilizer doxantrazole prevented its occurrence. A similar result was obtained by peri-stress nerve growth factor (NGF) neutralization with antiserum. These results substantiated earlier observations obtained by Barreau et al.[5, 7], who already suggested that mast cell derived NGF is relevant to the observed phenotype. Importantly, NGF is a modulator of the transient receptor ion channel 1 (TRPV1)[8] and, on the basis of enhanced immunohistochemical staining in tissue samples, Akbar *et al.* suggested that TRPV1 may be relevant in IBS pain perception.[9] Therefore, we next assessed TRPV1 expression levels and its *in vivo* relevance in our rat model. Evaluating retrograde labelled and microdissected dorsal root ganglia sensory neurons we could not show enhanced TRPV1 transcription in MS rats. Neither did we observe increased numbers of TRPV1 positive neurons by immunohistochemical staining. Nevertheless, the use of capsazepine as well as the selective antagonist SB-705498 indicated that mast cell induced hypersensitivity to distension does depend on TRPV1. Together these data suggested that mast cells, possibly via the release of NGF, modulated TRPV1 responses without affecting TRPV1 expression levels. We herewith identified mast cells and TRPV1 as possible targets for future therapeutical intervention strategies in IBS.

Based on investigations in preclinical stress models others suggested that peripheral corticotrophin releasing hormone (CRH) is an important trigger for stress-induced mast cell degranulation in colon.[10] Earlier animal experiments already indicated the relevance of brain expressed CRH for the post stress

phenotype. Consequently, two large clinical trials were performed with CRH-receptor antagonists but, surprisingly, they both failed to show the expected results.[11, 12] In response to these failures, Professor Michael Camilleri (Mayo Clinic, Rochester, USA) suggested that 'the degree of stress experienced by patients attending a clinic is not as severe as that of a rat avoiding water!'.[13] In other words, animal models of stress-induced IBS-like complaints may not be relevant for the human situation. In **chapter 3** we set out to establish why these earlier experiments failed to predict the negative outcome of CRH-receptor antagonist studies in IBS patients. Interestingly, although a range of different stress protocols was used in these previous studies, they showed one important commonality; successfully tested CRH-receptor antagonists were always given in a pre-stress setting. Using α-helical CRF (9-41) we were able to confirm these findings. However, when we used the same antagonist in a post-stress treatment protocol we failed to reverse established visceral hypersensitivity. In contrast, the mast cell stabilizer doxantrazole was capable of reversing the hypersensitive phenotype. Two important conclusions can be drawn from this study a) results obtained in older CRH receptor antagonist experiments were correct but probably not relevant because patients require reversal instead of prevention. b) If CRH is not required for post stress mast cell activation, other factors are. We hypothesized, but did not investigate, that these could be luminal antigens (bacterial or food derived). Future identification of receptors relevant to recognition of such antigens may provide novel therapeutic targets for IBS.

In line with preclinical animal investigations, several lines of evidence obtained with human tissue also suggested that mast cells are relevant in IBS.[14-16] Therefore, our group at the AMC conducted a double blind placebo controlled trial with the mast cell stabilizer ketotifen.[17] Although clinical results obtained in this study were positive, *ex vivo* investigation also suggested that ketotifen did not act as a mast cell stabilizer. Since ketotifen is also a histamine-1-receptor (H1R) antagonist we hypothesized that this was the more likely mechanism of action. However, ketotifen does not have high specificity for the H1R and, because it crosses the blood brain barrier, may cause central side effects. Therefore, in **chapter 4**, we decided to evaluate second generation H1R antagonists in our animal model. These antagonists show enhanced H1R specificity and are peripherally restricted.[18] In chapter 3 we already showed that 1 hour of water avoidance stress was able to induce long term (up to one month) mast cell dependent visceral hypersensitivity in maternal separated rats. Since reversal of hypersensitivity is the ultimate goal in patients, we evaluated fexofenadine and ebastine in a post stress intervention protocol. Both compounds successfully reversed the IBS-like phenotype. Further, although we only obtained a limited set of data (on expression of occludin), our results also suggested that barrier function is restored by antagonist treatment. Based on these results we suggested that H1R antagonists, initially developed for the treatment of allergic rhinitis, should also be evaluated in IBS. This trial was recently

conducted at the Catholic University of Leuven by the group of Prof GE Boeckxstaens. The use of ebastine resulted in significant improvement in global symptom relief, abdominal pain and quality of life compared to placebo.[19]

Whether the most important contributing factors in IBS are genetic or environmental in nature is an important question that influences general directions in IBS research. So far, the search for single nucleotide polymorphisms associated with increased risk for IBS was not very successful[20, 21] and twin studies also suggested that environmental triggers are probably more important than genetic factors.[22, 23] Nevertheless, IBS does cluster in families.[24, 25] This may suggest that each generation is exposed to a trigger factor independent of the previous generation, or, the environment induced phenotype can be transferred vertically from one generation to the next. We investigated the latter hypothesis in our rat model of maternal separation (discussed in **chapter 5**). In contrast to humans, a rodent model has the advantage that subsequent generations can be investigated under controlled conditions and in a relatively short timeframe. We could show that susceptibility to stress induced visceral hypersensitivity in maternally separated (F1) rats can be transferred to the next (F2) generation without further separation protocols. Our cross-fostering experiments indicated that the observed transfer depends on maternal care: pups adapted to the phenotype of the foster mother. This suggests that *in utero* mechanism do not play an important role. In our experiments we did not investigate other, possibly causal, mechanisms like e.g. transfer of milk born factors or an 'IBS micro biome' from mother to child. We did however explore the role of mast cells in post-stress visceral hypersensitivity of the F2 offspring. Similar to earlier findings in F1 rats, the mast cell stabilizer doxantrazole was capable of reversing the IBS-like phenotype in F2 animals. Together these findings indicate that maternal separation can be used to investigate cross-generational effects of environmental IBS-triggers.

In conclusion, pre-clinical investigations presented in the current thesis indicated that stress-induced visceral hypersensitivity depends on mast cell activation, initially triggered by peripheral CRH. We also showed that prolonged, post stress activation of these cells no longer depends on this stress hormone and suggested that this may explain the failure of clinical trials that were carried out with CRH-receptor antagonists. Consequently, we strongly feel that future identification of post stress triggers for mast cell activation will lead to novel therapeutic strategies. An alternative approach to the identification of novel treatments is to define mast cell mediator/receptor interactions relevant to the observed hypersensitive phenotype. Here, we presented evidence that the H1R is an imported target and this was recently confirmed in a clinical trial. Histamine as well as NGF may exert their effect via modulation of TRPV1. Although our results with a specific TRPV1 antagonist suggest that this ion channel could also be a target, its role in the regulation of body temperature may block future use of

this type of drug in IBS. Finally, an important strategy that is often neglected in IBS research is to aim for prevention instead of treatment. Our model of transfer across generations can be used to delineate and intervene with environmental triggers and mechanisms that may be relevant to IBS clustering in families.

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SAMENVATTING EN CONCLUSIES

Het prikkelbare darm syndroom (PDS) is niet alleen een van de meest voorkomende, maar ook een van minst begrepen gastro-intestinale aandoeningen. Het is een zogenaamde functionele darm aandoening die aan de hand van alleen klinische criteria gediagnostiseerd wordt.[1] Patiënten ervaren abdominale pijn of ongemak in associatie met defecatie of een veranderde stoelgang. Viscerale hypersensitiviteit (d.w.z. verhoogde perceptie van gastro-intestinale stimuli) is waarneembaar bij de meerderheid van deze patiënten en wordt gezien als een pathofysiologisch mechanisme. Omkeren van de waargenomen overgevoeligheid is wellicht gunstig voor deze patiënten. Desondanks blijven effectieve behandel opties vooralsnog uit, waarschijnlijk doordat de mechanismen relevant voor hypersensitiviteit nauwelijks bekend zijn. Duidelijk is echter wel dat stress bij PDS patiënten een verhoogde gevoeligheid voor distensie teweeg brengt[2, 3] en dit gegeven kan worden gebruikt als startpunt voor nieuwe onderzoeksrichtingen. Voor ons onderzoek hebben wij daarom gebruik gemaakt van het maternaal separatie model in rat, gecombineerd met stress op adulte leeftijd[4], om zodoende een beter begrip te krijgen van mechanismen relevant voor stress geïnduceerde viscerale hypersensitiviteit. In **hoofdstuk 2** hebben wij eerst eerder onderzoek bevestigd waaruit bleek dat mest cel degranulatie relevant is voor het post-stress fenotype;[5, 6] pre-stress toedienen van de mest cel stabilisator doxantrazole voorkwam het ontstaan van viscerale hypersensitiviteit. Een soortgelijk resultaat werd bereikt door het peri-stress toedienen van anti-'nerve growth factor' (NGF) antilichamen. Deze resultaten bevestigden onderzoek van Barreau *et al.* die eerder al suggereerde dat van mest cellen afkomstig NGF relevant is voor het ontstane fenotype.[5, 7] Van NGF is bekend dat het een modulator is van het 'transient receptor ion channel 1' (TRPV1).[8] Op basis van verhoogde immunohistochemische aankleuring, suggereerden Akbar *et al.* dat dit ion kanaal relevant zou kunnen zijn voor verhoogde pijn perceptie bij PDS.[9] Daarom hebben wij vervolgens het expressie niveau en de *in vivo* rol van TRPV1 in ons rat model vastgesteld. Evaluatie van retrograat gelabelde en door laser microdisectie verkregen materiaal toonde geen verhoogde TRPV1 transcriptie aan bij sensibele neuronen. Ook konden wij immunohistochemisch geen verhoogde aantallen TRPV1-positieve neuronen aantonen. Desalniettemin, bleek uit *in vivo* gebruik van capsazepine en de selective antagonist SB-705498 dat mest cel geïnduceerde overgevoeligheid voor distensie wel degelijk TRPV1 afhankelijk was. Tezamen suggereren deze resultaten dat mest cellen, mogelijk via het vrijstellen van NGF, de TRPV1-respons moduleren zonder de TRPV1 expressie te veranderen. Hiermee hebben wij mest cellen en TRPV1 geïdentificeerd als mogelijke targets voor toekomstige behandeling van PDS.

Anderen suggereerden, gebaseerd op preklinische stress modellen, dat perifeer 'corticotrophin releasing hormone' (CRH) een belangrijke trigger is voor stress geïnduceerde mest cel degranulatie in het colon.[10] Eerdere proefdier experimenten hadden al aangetoond dat ook CRH-expressie in het brein relevant is voor het post stress fenotype. Op basis van deze gegevens werden twee grote klinische trials uitgevoerd die, helaas, geen van beide tot het gewenste resultaat leiden.[11, 12] In antwoord op deze mislukkingen stelde professor Michael Camilleri (Mayo Clinic, Rochester, USA) dat "de mate van stress die een patiënt ondergaat bij kliniek bezoek niet zo ernstig is al de stress die een rat ondergaat tijdens een 'water avoidance' stress!".[13] Met andere woorden, proefdiermodellen voor stres-geïnduceerde IBS-achtige klachten zouden wel eens niet relevant kunnen zijn voor de humane situatie. In **hoofdstuk 3** hebben wij daarom onderzocht waarom de eerder door anderen uitgevoerde experimenten niet konden voorspellen dat klinische trials met CRH receptor antagonisten zouden falen. Ondanks dat in deze onderzoeken een verscheidenheid aan protocollen gebruikt was, bleek er ook een belangrijke overeenkomst te zijn; succesvol geteste CRH receptor antagonisten werden altijd voorafgaande aan de stressor toegediend. Met behulp van de CRH receptor antagonist α-helical CRF (9-41) konden wij deze proeven inderdaad herhalen. Echter, wanneer dezelfde antagonist gebruikt werd in een post-stress behandelingsprotocol dan kon de al ontstane overgevoeligheid niet worden omgekeerd. Dit terwijl een mest cel stabilisator hier wel toe in staat was. Uit deze gegevens kunnen twee belangrijke conclusies worden getrokken a) de resultaten verkregen in eerdere studies waren wel correct maar waarschijnlijk niet relevant omdat bij patiënten niet het voorkomen maar het omkeren van de respons van belang is. b) Als CRH niet nodig is voor het voort laten duren van de post stress mest cel activatie dan is iets anders dat wel. Wij veronderstellen, maar hebben niet aangetoond, dat luminale antigenen (bacterieel of afkomstig van voedsel) hiervoor verantwoordelijk kunnen zijn. Toekomstige identificatie van receptoren relevant voor deze antigeen herkenning kan mogelijk nieuwe therapeutische targets opleveren.

Gelijk aan het preklinische proefdieronderzoek toonden verschillende onderzoekslijnen met humaan weefsel aan dat mest cellen relevant zijn in PDS.[14-16] Daarom voerde de AMC onderzoeksgroep eerder een dubbel blind placebo gecontroleerde studie uit met de mest cel stabilisator ketotifen.[17] Hoewel het klinische resultaat positief was toonden *ex vivo* metingen aan dat het effect van ketotifen niet beruste op de stabilisatie van mest cellen. Omdat deze stof ook een histamine-1-receptor (H1R) antagonist is hypothetiseerden wij dat dit het mogelijke mechanisme van actie is geweest in de klinische trial. Ketotifen heeft geen hoge specificiteit voor de H1R en passeert ook de bloed-brein-barrière. Omdat dit laatste tot centrale bijwerkingen kan leiden, besloten wij om in ons model twee tweede-generatie H1R antagonisten te testen (beschreven in **hoofdstuk 4**). Deze antagonisten hebben een hoge H1R specificiteit en passeren de bloed-brein-barrière doorgaans niet.[18] In hoofdstuk drie hadden we al

aangetoond dat een uur ‘water avoidance’ stress aanleiding geeft tot langdurige (tot een maand) mest cel afhankelijke viscerale hypersensitiviteit in voorheen maternaal gesepareerde ratten. Omdat bij patiënten het omkeren van deze hypersensitiviteit het ultieme doel is, hebben wij fexofenadine en ebastine geëvalueerd in een post stress interventie protocol. Beide stoffen bleken in staat het PDS fenotype om te keren. Hoewel gebaseerd op een beperkte hoeveelheid data (expressie van occludin) suggereerden onze resultaten ook dat behandeling met deze antagonisten de barrière functie van de darm hersteld. Naar aanleiding van deze resultaten stelden wij dat H1R antagonisten, ontwikkeld voor gebruik bij allergieën, ook bij PDS getest zouden kunnen worden. Deze trial is recent uitgevoerd door de groep van Professor Boeckxstaens op de Katholieke Universiteit Leuven (België). In vergelijking met placebo resulteerde gebruik van ebastine in een significante verbetering van globale symptomen, abdominale pijn klachten en kwaliteit van leven.[19]

Of genetische dan wel omgevingsfactoren de meest belangrijke bijdrage leveren aan het ontstaan van PDS is een belangrijke vraag die in hoge mate de richting van het internationale PDS onderzoeksveld bepaalt. Tot dusverre is de zoektocht naar met PDS geassocieerde ‘single nucleotide polymorphisms’ niet bijzonder succesvol geweest.[20, 21] Ook tweelingstudies suggereren dat niet genetische maar omgevingsfactoren belangrijker zijn.[22, 23] Desalniettemin clustert PDS in families.[24, 25] Dit kan er op duiden dat iedere generatie is blootgesteld aan een trigger onafhankelijk van de vorige generatie, of, het omgeving geïnduceerde fenotype kan verticaal van de ene op de andere generatie worden overgedragen. In **hoofdstuk 5** hebben wij deze laatste hypothese in ons rat model onderzocht. In tegenstelling tot de mens, bieden ratten het voordeel dat opeenvolgende generaties in relatief korte tijd en onder redelijk stabiele omstandigheden kunnen worden onderzocht. Wij konden aantonen dat gevoeligheid voor stress geïnduceerde viscerale hypersensitiviteit in maternaal gesepareerde ratten (F1) ratten naar de volgende generatie (F2) kan worden overgedragen zonder het toepassen van verdere separatie protocols. Onze zogenaamde ‘cross fostering’ experimenten toonden verder aan dat deze overdracht over generaties afhangt van de maternale zorg; pups adapteerden aan het fenotype van de foster moeder. Dit geeft aan dat *in utero* mechanismen geen belangrijke rol spelen. Wij hebben geen andere, mogelijk causale, mechanismen zoals overdracht van melk-gebonden factoren of een ‘PDS microbioom’ van moeder naar kind onderzocht. Wel hebben wij met behulp van doxantrazole aangetoond dat, gelijk aan de eerdere resultaten bij F1-ratten, het post stress fenotype ook bij deze F2 generatie mest cel afhankelijk is. Samen laten deze resultaten zien dat het maternaal separatie model gebruikt kan worden om PDS overdracht over generaties te bestuderen.

Samenvattend kunnen we concluderen dat het in dit proefschrift beschreven preklinische onderzoek aantoont dat stress geïnduceerde viscerale hypersensitiviteit afhangt van mest cel degranulatie en dat deze initieel getriggerd word door CRH. Wij toonden ook aan dat de post stress activatie van deze

cellen niet langer van dit stress hormoon afhangt en suggereerden dat dit wellicht de verklaring is voor het falen van recent uitgevoerde klinische trials met CRH receptor antagonisten. Hieruit concludeerden wij ook, dat de toekomstige identificatie van post stress mest cel triggers mogelijk nieuwe therapeutische targets zal opleveren. Een alternatieve manier om tot nieuwe targets te komen is de identificatie van mest cel mediator/receptor interacties relevant voor het optreden van het hypersensitieve fenotype. Wij toonden aan dat de H1R een mogelijke target is en dit is recent bevestigd in een klinische trial. Zowel histamine als NGF bewerkstelligen hun effect wellicht door modulatie van TRPV1. Hoewel onze experimenten (met een selectieve antagonist) inderdaad aantonen dat dit mechanisme van belang zou kunnen zijn, is het door de rol die TRPV1 speelt bij de regulatie van de lichaamstemperatuur onwaarschijnlijk dat TRPV1 antagonisten in de toekomst gebruikt gaan worden voor de behandeling van PDF. In plaat van behandeling zou preventie een andere, vaak genegeerde, strategie kunnen zijn. Ons model van overdracht over generaties zou gebruikt kunnen worden om hierin belangrijke omgevingsfactoren te identificeren en waar mogelijk moduleren.

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