Pathophysiology of stress-induced visceral hypersensitivity
Stanisor, O.I.

Citation for published version (APA):

General rights
It is not permitted to download or to forward/distribute the text or part of it without the consent of the author(s) and/or copyright holder(s), other than for strictly personal, individual use, unless the work is under an open content license (like Creative Commons).

Disclaimer/Complaints regulations
If you believe that digital publication of certain material infringes any of your rights or (privacy) interests, please let the Library know, stating your reasons. In case of a legitimate complaint, the Library will make the material inaccessible and/or remove it from the website. Please Ask the Library: http://uba.uva.nl/en/contact, or a letter to: Library of the University of Amsterdam, Secretariat, Singel 425, 1012 WP Amsterdam, The Netherlands. You will be contacted as soon as possible.
Essential role for TRPV1 in stress-induced (mast cell-dependent) colonic hypersensitivity in maternally separated rats


Neurogastroenterology and Motility Volume 21, Number 10, October 2009
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, acute stress 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. 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 pathophysiological 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. 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, and ex vivo supernatant injection into mesenteric arteries supplying rat jejunum lead to enhanced firing of the mesenteric nerves. 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 and hypersensitivity to colonic distension in rats. Acute stress was also shown to induce peripheral release of mast cell mediators in humans and is known to induce enhanced visceral perception in IBS patients. A potential mechanism via mast cell mediators induce visceral hypersensitivity could be the activation or modulation of transient receptor ion channel 1 (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. In addition, several mast cell mediators capable of inducing enhanced sensitivity to colonic distension [e.g. serotonin, tryptase and nerve growth factor (NGF)] are known to modulate TRPV1-mediated responses. Animal experiments suggest that TRPV1 is involved in mechanosensation and visceral hypersensitivity induced by neonatal irritation of the colon and hypersensitivity after intracolonic administration of zymosan. Recently, increased TRPV1 expression on nerve fibres in recto-sigmoid biopsies of IBS patients was shown to correlate with the degree of abdominal pain. 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
Chapter 2

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. 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 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. 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, 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. 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 and TRPV1+ nerve fibres in the gastrointestinal tract are mainly spinal in origin, DRG T13-L2 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 Agnès 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. 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, Pinicuk, 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 percent 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. 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. 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) 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 percent 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
TRPV1

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. We recently showed that all neurons labelled through subserosal administration of retrograde tracer were also labelled by i.p. injection. Moreover, the validity of i.p. tracer administration to identify visceral afferents was also shown by others. Thus, we preferred i.p. injections with the fluorescent tracer Fluoro-Gold (1.0 mg kg-1; Fluorochrome, Denver, 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 μM were cut and fluorescent cells were laser microdissected (PALM Microsystems, Bernried, Germany). Cells were catapulted into 100 μL 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 μL 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://genomics毫不logy.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. Statistical differences in immunohistochemical, western and RTQ-PCR evaluations were assessed by Mann–Whitney U-test.

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
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 and indications are that peripheral stress-induced degranulation of mast cells may be involved. Early life stressors are known to contribute to IBS in adults and the rat neonatal MS model is one of the different animal models used to investigate the role of early life trauma. 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. 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 and several studies suggested that increased intestinal mast cell numbers were relevant to the pathophysiology of IBS. 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

![Image](image-url)
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. 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. 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. 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. 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. It was shown that blocking of this pathway abrogated NGF-dependent capsaicin sensitivity and heat hyperalgesia. 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, additional experiments were then carried out with the potent and selective antagonist SB-705498. 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. 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, increased TRPV1-expression in individual neurons, (ii) via TRPV1 phosphorylation or (iii) via enhanced TRPV1 trafficking to the cell membrane.

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. 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. 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. Thus, we speculate that stress-induced and TRPV1-dependent hypersensitivity to colonic distension in the separation model may be due to enhanced TRPV1 phosphorylation and/or trafficking to the surface membrane of spinal sensory neurons. Obviously, the exact TRPV1 modulatory mechanisms have to be explored in further investigations.

In a recent publication, Winston et al. 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. 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 depends 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

RvdW and DvdC are supported by the Technology Foundation STW, Applied Division of NWO and the Technology Program of the Ministry of Economic Affairs (NWO-STW, grant AKG 5727). TKK is supported by a grant from The Netherlands Digestive Diseases Foundation (MLDS), grant number: MWO 05-42; WJdeJ is supported by a Marie Curie Intra-European Fellowship within the 6th European Community Framework Programme. GEB is supported by a grant (Odysseus program, Grant no.: G.0905.08) of the Flemish Scientific organization (FWO). Preliminary results of these investigations were presented at Digestive Disease Week 2005 (DDW, Chicago, IL, USA) 61.

REFERENCES


12 Sugiuira T, Bielefeldt K, Gebhart GF. TRPV1 function in mouse colon sensory neurons is enhanced by metabotropic 5-hydroxytryptamine receptor activation. J Neurosci 2004; 24: 9521–30.


Chapter 2


37 Anderson CR, Edwards SL. Intraperitoneal injections of Fluorogold reliably labels all


59 Bhave G, Hu HJ, Glauner KS et al. Protein kinase C phosphorylation sensitizes but does not activate the capsaicin receptor transient receptor potential vanilloid 1 (TRPV1). Proc Natl Acad Sci USA 2003; 100: 12480–5.
