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

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

René M. van den Wijngaard*, Oana I. Stanisor*, Sophie A. van Diest, Olaf Welting, Mira M. Wouters, Wouter J. de Jonge, Guy E. Boeckxstaens

*These authors contributed equally to this work.


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. 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. In animal models, stress not only leads to visceral hypersensitivity but also induces intestinal permeability changes. 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). Animal experiments indicated that stress-induced barrier and sensitivity changes may be caused by activation of intestinal mucosal mast cells. Investigations performed with supernatants of submerged intestinal biopsies from IBS patients and normal controls confirmed that these cells may indeed be relevant. 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 H1-receptor antagonist ketotifen reduced threshold of discomfort and IBS-symptoms and improved health related quality of life. 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 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. 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. These results were confirmed when CRF was added ex vivo in Ussing-chamber experiments with mast cell-sufficient and -deficient colonic rat tissue. Finally, Ussing-experiments performed by Wallon et al. indicated that human colonic mast cells are also susceptible to CRF-mediated mast cell degranulation. 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 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.\textsuperscript{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 macaron 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 °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 °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.\textsuperscript{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.\textsuperscript{19-21} Similar to other publications\textsuperscript{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. 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). 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.

**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.](image)

**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% NaHCO₃/0.9% 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/kg² (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.
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.
Chapter 3

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.

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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.\textsuperscript{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\textsuperscript{27, 28} and the maternal separation model in rats is often used to mimic such predisposing factor.\textsuperscript{10} In this model, adverse early life experience predisposes for complaints like visceral hypersensitivity and barrier dysfunction later in life\textsuperscript{20, 22, 23, 29} and these features are also important in IBS.\textsuperscript{2, 3, 7-9} In contrast to others who reported differences in baseline responsiveness to distension between nonhandled and maternally-separated rats\textsuperscript{22, 29} our adult separated Long Evans rats need an acute WA stress to bring out the hypersensitive phenotype.\textsuperscript{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.\textsuperscript{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\textsuperscript{15, 24-26, 29, 30} prevented elicitation of colonic hypersensitivity to distension.\textsuperscript{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).\textsuperscript{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.\textsuperscript{15, 18, 24-26} CRF interacts with two receptors, CRF\textsubscript{1} and CRF\textsubscript{2}, but with highest affinity to CRF\textsubscript{1}.\textsuperscript{14} In brain, CRF/CRF\textsubscript{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.\textsuperscript{18} Similarly, in rat mast cell studies the use of selective receptor antagonists for CRF\textsubscript{1}\textsuperscript{24} and CRF\textsubscript{2}\textsuperscript{16} implicated both receptors. On the other hand, the non-selective receptor antagonist \(\alpha\)-helical CRF(9-41) was shown to preferentially block CRF\textsubscript{2}-receptors\textsuperscript{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\textsuperscript{15, 24-26} and human\textsuperscript{18}. When administered peripherally, this particular antagonist has poor penetration into brain\textsuperscript{14} thus ruling out the modulation of central CRF-signalling pathways as much as possible. In our experiments intraperitoneal pre-WA \(\alpha\)-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 \textit{in situ} RMCP2 expression in colonic mucosal mast cells of MS rats which decreased to normal nonhandled-level upon WA.\textsuperscript{19} These results are corroborated by the present Western blot quantifications of stripped colonic mucosa. Compared to vehicle pre-treated MS-rats, \(\alpha\)-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.\textsuperscript{33} This may be relevant because loss of occludin induces barrier dysfunction\textsuperscript{34} and protease-induced occludin degradation was suggested to play a role in IBS.\textsuperscript{6} Our occludin quantifications are in line with the observed RMCP2 expression levels: a significant difference only occurs when rats are pre-treated with \(\alpha\)-helical CRF(9-41). Although we have not performed extensive barrier studies these data suggest that \(\alpha\)-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 \(\alpha\)-helical CRF(9-41) administrations. However, it was shown earlier that \(\alpha\)-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.\textsuperscript{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 CRF2-receptor antagonist and we did not apply specific CRF1-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 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 CRF1-receptor antagonist CP-154,526.38 This confirmed earlier observations that central CRF1-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, 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, 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. Pilot experiments performed in our own laboratory confirm that humoral immune responses may have a role in the maternal separation model and recent observations of increased anti-flagellin antibody titers suggest that humoral mechanisms may also be relevant to IBS. 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.

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REFERENCE LIST


7. Dunlop SP, Hebden J, Campbell Eet al. Abnormal intestinal permeability in subgroups of diarrhea-predominant irritable bowel syndromes. *Am J Gastroenterol* 2006;101:1288-94.


39. Dukes GE, Mayer EA, Kelleher DL, Hicks KJ, Boardley RL, Alpers DH. A randomised double blind, placebo controlled, crossover study to evaluate the efficacy and safety of the corticotrophin releasing factor 1 (CRF1) receptor antagonist GW876008 in IBS patients. *Neurogastroenterol Motil* 2009;21(Suppl.).

