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Publication date
2019
Document Version
Final published version
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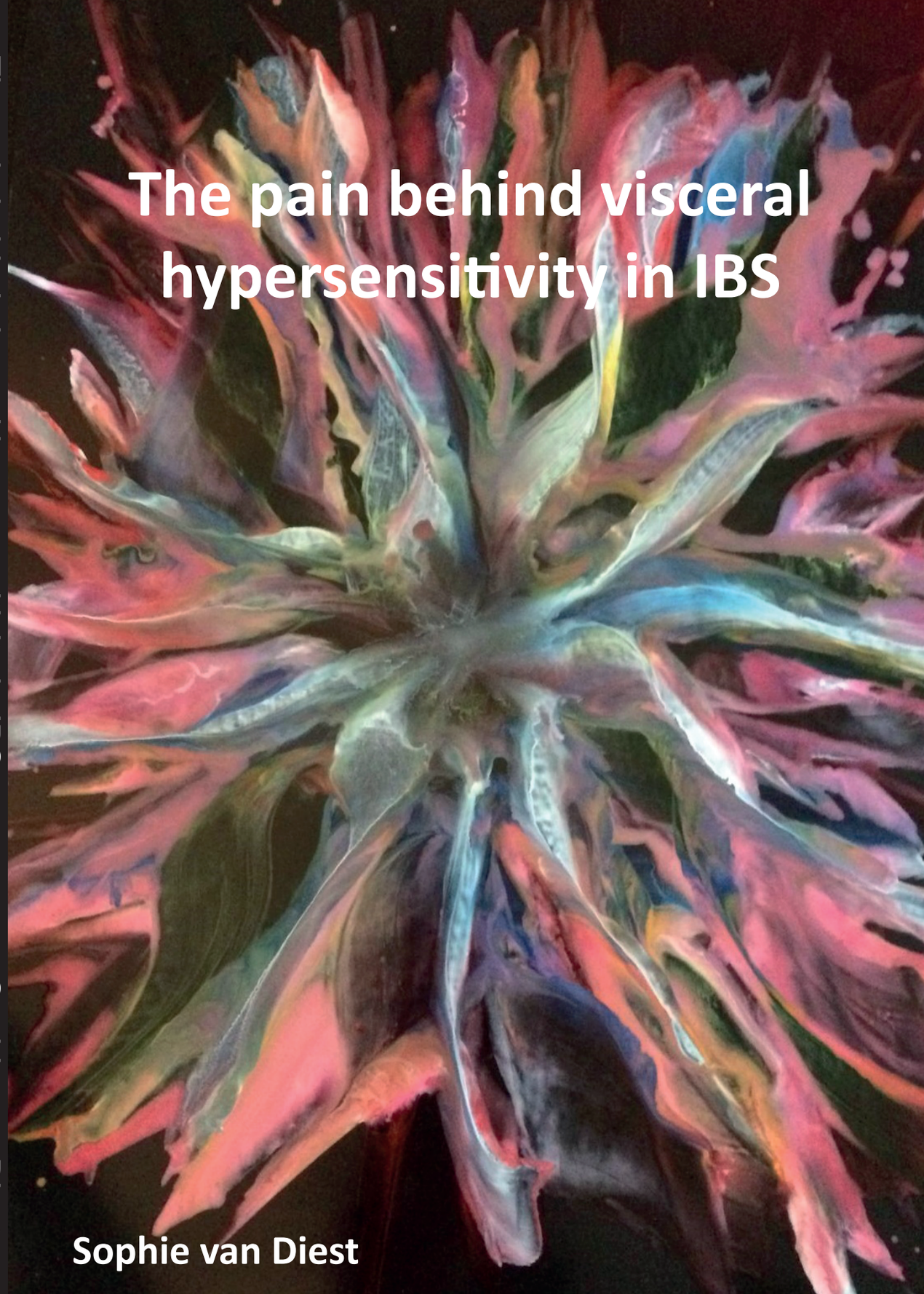

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Cover: Encaustic art by Gonnie Rond
Design and layout: Legatron Electronic Publishing, Rotterdam
Printing: Ipskamp Printing, Enschede

June 2019

ISBN: 978-94-028-1549-8

Publication of this thesis was financially supported by:
Universiteit van Amsterdam

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ACADEMISCH PROEFSCHRIFT

ter verkrijging van de graad van doctor

aan de Universiteit van Amsterdam

op gezag van de Rector Magnificus

prof. dr. ir. K.I.J. Maex

ten overstaan van een door het College voor Promoties ingestelde

commissie, in het openbaar te verdedigen in de Agnietenkapel

op dinsdag 25 juni 2019, te 12:00 uur

door

Sophie Alexandra van Diest

geboren te Saint James

Promotiecommissie:

Promotor:	prof.dr. W.J. de Jonge	AMC – UvA
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Chapter 1

General introduction and outline of this thesis

Irritable Bowel Syndrome (IBS) is one of the most prevalent gastrointestinal disorders in the Western world, affecting approximately 9−23% of the population with substantial health care costs.[1] Although IBS is not life-threatening, symptoms such as abdominal pain and/or discomfort, bloating and abnormal bowel function (either diarrhoea or constipation) lead to considerable morbidity by causing a reduced quality of daily life. Although the prevalence is relatively high, IBS remains an important medical challenge in terms of diagnosis and treatment.[2] Treatment success is often disappointing as effective therapies are limited by poor side-effect profiles (such as fatigue, constipation, nausea and liver damage).[3] The development of new therapeutical interventions is difficult due to incomplete knowledge of the underlying pathophysiology. Currently, most of the IBS related experimental investigations focus on the basis of enhanced pain perception, using controlled mechanical distension of the colorectal part of the intestine in rodents and in humans. This so-called visceral hypersensitivity is observed in 33 to 65% of IBS patients and is regarded as an important pathophysiological mechanism. Although the causes underlying this altered sensory motor function are only partially understood, stress is a known trigger for visceral hypersensitivity in IBS.[4-7] In this post-stress visceral hypersensitivity, the activation of mast cells is implicated as the relevant relay event. In **Chapter 2** we will summarize knowledge on the interaction between mast cells and nerves relevant to visceral hypersensitivity.

Several clinical studies suggest that a history of early life stress (such as impaired parent-child interaction, childhood neglect, physical abuse and sexual abuse) is a risk factor for the development of adult gastrointestinal pathologies, including IBS.[3,8,9] Interestingly, a history of childhood abuse has even been correlated with abnormal bidirectional communication between brain and gut, providing a potential explanation for the linkage between early life stress and abdominal pain symptoms of gastrointestinal disorders.[2,3,10] Although they can never fully reflect the complex human parent-child interactions, animal models with early life stress are considered important tools to better understand the aetiology of gastrointestinal disorders like IBS and to explore possible therapeutical targets to tackle abdominal pain symptoms in these disorders. This thesis describes investigations performed in the rat maternal separation model for IBS, which reflects an impaired parent-child interaction as neonatal pups are separated from their mothers for a time period of 3 hours daily for 12 consecutive days (postnatal day 2−14). Because in IBS patients acute stress is one of the major factors involved in the onset and exacerbation of IBS symptoms, we combined the maternal separation protocol with acute stress at adult age.

Patients with IBS often report a positive family history of IBS.[11-13] However, the question remains whether this familial clustering can be explained by shared environmental factors or by shared genetic background. Twin studies suggest that, although they both contribute, intra-familial environmental factors have a greater influence on the aetiology of IBS than genetic causes.[14,15] When environmental factors are essential for familial clustering of IBS, either each generation must have been exposed to this same factor(s) independent of the previous generation, or the environmentally-induced phenotype is transferred from one generation to the next. In **Chapter 3** we investigated the latter option and we established 1) whether this so called transfer across

generations is possible, in maternal separated rats, 2) whether possible phenotype transfer occurs during the post-natal phase and depends on the foster mother and 3) whether triggering of the transferred phenotype depends on the degranulation of mast cells similar to that observed in F1 offspring.

As mentioned earlier, stress is one of the major factors involved in the onset and exacerbation of IBS symptoms.[16] It has been well described that the hypothalamic-pituitary-adrenal (HPA) axis is activated by stress through the secretion of corticotropin-releasing hormone (CRH) by neurons in the hypothalamus. CRH is generally considered a central stress hormone, however it has been demonstrated that, rather than central CRH, peripheral-released CRH contributes to visceral hypersensitivity and intestinal barrier dysfunction through the activation of mast cells.[17-21] Although this suggests that CRF-receptors are an attractive therapeutical target in IBS, it is important to note that, almost without exception, these animal studies were aimed at preventing stress-induced phenotypical changes whereas the reversal of these changes may be more relevant to patients. At present it remains unclear whether post-stress CRH receptor-antagonist treatment is able to reverse mast cell dependent visceral hypersensitivity. Therefore, in **Chapter 4** we used the CRH receptor antagonist α-helical CRF (9–41) and compared effectivity of pre- and post-stress treatment strategies. Our results question the use of CRH receptor antagonists in a clinical setting.

Earlier Klooker *et al*. evaluated the role of mast cells in IBS patients. In this experiment, treatment with the mast cell stabiliser and histamine-1 receptor (H1R) antagonist ketotifen was shown to increase the threshold of discomfort, reduce IBS symptoms and to improve health-related quality of life.[22] 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. Importantly, ketotifen has low H1R selectivity and is known to cross the blood-brain barrier and cause central side effects.[9,23] Therefore, possibilities to increase therapeutic dose for enhanced effectiveness are limited and evaluation of other, peripherally restricted, H1R antagonists may proof beneficial. In **Chapter 5** we investigated the effects of two different H1R receptor antagonists on visceral hypersensitivity and barrier function in maternal separated rats. Like in the clinical study using Ketotifen, our data suggest that H1R receptors are a promising target in treating IBS symptoms.

When the role of mast cells and the H1R were firmly established, my group designed studies to address whether triggers other than CRH may be involved in mast cell activation. An aberrant gut fungal microbiome (i.e. mycobiome) was shown in maternal separated rats as well as in hypersensitive IBS patients. Faecal transfer experiments in fungicide treated rats indicated that mycobiome dysbiosis was functionally relevant for visceral hypersensitivity. Additional *in vivo* and *in vitro* evidence then indicated that fungal cell wall components (i.e. β-glucans) activate mast cells via the Dectin-1/SYK pathway.[24] Combined with earlier findings on the essential role of the nociceptive transient receptor ion channel 1 (TRPV1) and TRPV1-sensitization via the H1R,[25,26] the following

sequence of events was suggested; fungal β-glucans induce the release of mast cell derived histamine, which then, via afferent expressed H1Rs, potentiates TRPV1 responses. In consequence, increased neuronal responses may be targeted by modulating the gut mycobiome as well as by intervening with mast cell and TRPV1 activation. In **Chapter 6** we evaluated Miltefosine, which is an FDA-approved compound for the treatment of Leishmanias. Miltefosine is a prototype lipid raft modulator. Lipid rafts are primarily composed of tightly packed sphingolipids, cholesterol and saturated phospholipids. In addition to regulating the activity of raft associated signal proteins, rafts are also known to regulate gating properties of ion channels, suggesting that miltefosine may be able to affect *in vivo* TRPV1 activation. In addition to that, miltefosine was shown to have fungicidal activity[27,28] and can be a direct inhibitor of mast cell activation.[29-32] Thus, we addressed miltefosine's potential to act on multiple levels of fungal induced visceral hypersensitivity, and concluded that it may proof to be an attractive compound for repurposing to IBS.

As mentioned before, effective therapies in IBS are often limited by poor side-effect profiles. Therefore a dietary intervention is an attractive treatment option for IBS as it renders a low risk of side effects and its costs are relatively low. And importantly, as lipid rafts are lipid based, their composition, structure and function are susceptible to manipulation by dietary components. It has been shown previously that omega 3 (n-3) polyunsaturated fatty acids (PUFAs) reduce the lipid raft sphingolipid content and thereby alter the raft fatty acid composition.[33-35] Interestingly, n-3 fatty acid-rich tuna oil shows promising results in dietary intervention studies that aim to alter inflammatory responses. Besides acting as a lipid raft modulator, n-3 PUFAs are thought to exert their anti-inflammatory effects through activation of peroxisome proliferator-activated receptor γ (PPARγ), activation of which negatively regulates inflammatory cytokine production by interfering with the activation of inflammatory transcription factors. In mast cells, treatment with both a PPARγ[36,37] agonist and n-3 PUFAs[38,39] was shown to reduce the production and release of histamine. The effects of both PPARγ activation by its agonist Rosiglitazone and dietary supplementation of n-3 fatty acids through with tuna oil enriched diet on mast cell activation and visceral hypersensitivity are evaluated in **Chapter 7.**

Taken together, in this thesis underlying triggers for mast cell activation in stress-induced visceral hypersensitivity were investigated and possible targets for therapeutical intervention strategies aimed at reducing abdominal pain were tested. Although not all strategies described here proofed beneficial, we do show interesting and promising strategies that could be used for treatment of abdominal pain in IBS patients.

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Chapter 2

Review: Relevance of mast cell-nerve interactions in intestinal nociception

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Biochimica et Biophysica Acta, 2012 Jan; 1822(1):74-84

Abstract

Cross-talk between the immune- and nervous-system is considered an important biological process in health and disease. Because mast cells are often strategically placed between nerves and surrounding (immune)-cells they may function as important intermediate cells. This review summarizes the current knowledge on bidirectional interaction between mast cells and nerves and its possible relevance in (inflammation-induced) increased nociception. Our main focus is on mast cell mediators involved in sensitization of TRP channels, thereby contributing to nociception, as well as neuron-released neuropeptides and their effects on mast cell activation. Furthermore we discuss mechanisms involved in physical mast cell-nerve interactions.

Introduction

It has been long known that the nervous system and the immune system are able to influence each others responses through bidirectional communication.[32,56,58,67-69,124,134,141,145,178,179] The immune system can influence nervous system activity through the release of inflammatory mediators and cytokines. In turn, the nervous system, including the brain and peripheral neurons, can stimulate or inhibit activities of the innate and adaptive immune response, by acting on inflammatory cells. Mast cells are often found in close proximity to nerves,[13,127,172,173] placing them in a key position to act as the intermediate cells between the nervous- and immune system. Several mast cell mediators act on receptors present on sensory afferent nerve terminals. Receptors present on these afferents, also called nociceptors, detect and respond to potentially damaging physical and chemical stimuli (including inflammatory mediators), usually resulting in pain perception. When stimulated, these neurons send signals to the central nervous system and concomitantly release neuropeptides, such as substance P and calcitonin-gene related peptide (CGRP). These neuropeptides can further stimulate mast cell activation creating a bidirectional positive feedback-loop that may eventually result in neurogenic inflammation.[9,110]

In this review we will discuss the bidirectional interaction between mast cells and sensory afferent nerves. Since mast cells from different tissues are shown to be morphologically, biochemically and functionally distinct from one another,[19] we will restrict this review and mostly focus on mast cells present in the gastrointestinal tract. In this setting the mechanism of both mast cell-mediated activation of sensory afferent neurons resulting in pain perception, as well as some mechanisms by which neurons can modulate mast cell function will be discussed. We will conclude with a short summary of mechanisms discussed in this review and some possible implications in gastrointestinal diseases like inflammatory bowel syndrome (IBD) and irritable bowel syndrome (IBS).

Physical mast cell-nerve interactions

Although close contact between mast cells and neurons has been well established,[13,127,172,173] little is known about the mechanisms involved in their interaction. In *in vitro* co-culture experiments nerve cells extended neurits toward rat basophilic leukemia cells (RBL-2H3), with the majority of RBL cells attached to neurites within 17 hours.[23] Using electron microscopy, actual membrane-membrane contact between mast cells and nerves was shown *in vitro*[23] and *in vivo*.[10] Importantly, degranulation of mast cells in close proximity to nerves was shown to correlate to abdominal pain in IBS patients. In the following section, we discuss methods for cell to cell communication between mast cells and neurons, as well as adhesion molecules that are relevant for interaction between both cell types.

Adhesion molecules in direct cell-cell contact

Recently, the cell adhesion molecule 1 (CADM1) was shown to be intensively expressed at the contact site of both neurites and mast cells. This molecule is suggested to mediate the adhesion between

both cell types.[17,99] Over-expression of CADM1 was shown to increase susceptibility of IC-2 cells (a murine cell line with mast cell progenitor characteristics) to substance P-mediated activation.[24] The authors suggest that homophilic binding between CADM1 expressed on mast cells and neurons, results in either up-regulation of NK-1 receptors on mast cells or an increase in release of substance P by neurons, promoting the enhancement of neuron-induced mast cell activation.[74,98,100] Since substance P is among the factors released during stress, this could have major implications in stress-induced exacerbation of disease, such as is seen in IBD and IBS.

Besides CADM1, N-cadherin is expressed by both mast cells and neurons and is implicated to play a role in the establishment of membrane-membrane contact in various celltypes. In mast cells N-cadherin is diffusely distributed in the cytoplasm. Association between a mast cell and neuron causes N-cadherin to traffic to sites of interaction at the plasma membrane.[100,181,215] Cleavage of N-cadherin in heterologous cells is mediated by MT5-MMP,[130] which is a matrix metalloprotease expressed in both neuronal and inflammatory cells.[12,88,115,146] In both neurons and mast cells, deficiency in MT5-MMP has been shown to result in abnormal processing of N-cadherin, with higher levels of full length N-cadherin at the cell membrane.[73] In monocultures of bone marrow derived mast cells (BMMC) this abnormal processing did not affect baseline mast cell degranulation but in mast cell-neuron co-cultures it resulted in an increased percentage of degranulating cells. In contrast to wild-type co-cultures, degranulation in mutant co-cultures could not be further increased by subsequent treatment with nerve growth factor (NGF) or nociceptor TRPV1 (transient receptor potential) agonist capsaicin. Since thermal responsiveness after inflammation was diminished in MT5-MMP deficient mice, the authors suggest that this is due to the inability of mast cells to release mediators required for fiber sensitization. Further investigations suggested that absence of MT5-MMP results in altered physical contact between mast cells and sensory fibers.[73] Thus, although more research on this subject is needed, direct contact between nerves and mast cells, mediated by adhesion molecules, seems to play an important role under physiological and pathological conditions.

Transgranulation and exosome transfer

In 1983, Greenberg and Burnstock described 'a novel cell-to-cell interaction between mast cells and other cell types', which they named transgranulation.[83] In co-cultures with fibroblasts the authors observed formation of specialized mast cell pseudopods that flattened against the ventral surface of adjacent fibroblasts. These pseudopods filled with granules and were eventually cast off and left on the surface of the adjacent cell. In addition, these investigators observed granule exocytosis by mast cells and subsequent endocytosis by adjacent fibroblasts. Although not necessarily referring to the same phenomenon (involving pseudopod formation), Wilhelm *et al.* also used the term transgranulation to describe mast cell-nerve interactions in the medial habenula in the brain of doves.[210] The habenula region of the brain is, among many other functions, involved in the processing of pain and stress responses.[7] Uptake of mast cell products by neurons in the brain correlated with mast cells undergoing piecemeal degranulation (based on granular morphology and characterized by the presence of variable losses of dense contents from mast cell granules). Products shown

to be captured by neurons were granule remnants and intragranular particles. The exact role of this neuronal uptake is not clear but the authors suggested that it may represent a mechanism to rapidly terminate the stimulus of mast cell secretary products. However, it has to be taken into account that up to date, only a few publications concerning this method of communication exist. Further research needs to be performed to evaluate the role of such mechanism in mast cell-nerve interactions in the gut.

Another manner of mast cell-nerve communication may occur via the release of exosomes. Relative to mast cell granules, exosomes are small vesicles that are secreted from multivesicular bodies upon fusion with the plasma membrane. Because exosomes express specific cell surface proteins, including cell adhesion molecules and integrins, they are able to bind selectively to specific recipient cells.[72,196,202] Interestingly, investigations performed by Skokos *et al.* showed that, within mast cells, exosomes can be localized in mast cell granules.[165] This suggests that the description of transgranulation by Wilhelm *et al.* (neuronal uptake of intragranular mast cell particles) may also involve neuronal uptake of exosomes. Mast cell-derived exosomes are implicated in immunomodulation, as they have been shown to influence dendritic cell maturation[165] and B- and T-cell activation.[166] Furthermore, mast cell-derived exosomes transport functional RNA to recipient cells, suggesting a new pathway for cell communication.[196] Exosomal signaling has been described in the central nervous system, where it is suggested that exosomal secretion of proteins and RNA may be a fundamental mechanism of communication.[167] To our knowledge, no study was performed to evaluate exosome-mediated communication between mast cells and neurons in gut or any other peripheral tissue. It would be interesting, however, to evaluate if exosomes of both mast cells and peripheral neurons can bind to and be integrated in one another, and if so, what are the consequences for cell activation and function.

Activation of sensory afferent nerves by mast cells

For the gastrointestinal tract, there are 3 main pathways through which sensory afferent nerves can signal to the central nervous system; the vagal nerves with cell bodies in the nodose and jugular ganglia, the splanchnic nerves with cell bodies in the thoracolumbar dorsal root ganglia (DRG) and the pelvic nerves with cell bodies in the lumbosacral DRG.[22] In literature, there seems to be a discrepancy in which of these pathways is predominantly involved in pain perception,[28,29] which may be explained by differences in time courses studied. For example, in a mouse model for TNBS (trinitrobenzene sulfonate) colitis, acute post-inflammatory visceral hypersensitivity, present only for 7 days after TNBS treatment, was shown to predominantly involve splanchnic nerve activation, whereas in delayed post-inflammatory hypersensitivity both splanchnic and pelvic afferent pathways were involved.[93] Besides differences in time course, discrepancies may also be explained by differences in subsets of sensory afferent nerves studied.[93]

There are three types of afferent nerve fibers; Aβ fibers, Aδ fibers and unmyelinated C fibers. Expressing major receptors involved in pain perception, these C fibers are probably the most important subtype in mast cell-mediated sensory afferent activation in the gastrointestinal tract. Based on histological markers, C-fibers can be further sub-divided into two main groups. In the first group neurons contain binding sites for isolectin B4 (IB4) and are dependent on glial cell line-derived neurotrophic factor (GDNF). The second group consists of peptidergic neurons that contain the neuropeptides calcitonin-gene related peptide (CGRP) and substance P and are regulated by NGF acting on the TrkA receptor. For the enhanced pain responsiveness seen after inflammation it has been long appreciated that activation of these NGF-dependent C-fibers exerts a major contribution.[95] Therefore it is not surprising that these C-fibers abundantly express nociceptors involved in pain perception, with the most extensive studied member being the TRPV1 receptor. Several mast cell mediators are known to modulate nociceptor function,[197] a selection of which will be discussed in this review.

Although from early research it was thought that mast cell mediators could directly activate nociceptors, more recent studies suggest that mast cell-derived compounds sensitize nociceptors by acting on co-expressed receptors. Probably the best studied intermediate in this mast cell-mediated sensitization is proteinase activated receptor 2 (PAR2). Members of the PAR family play an important role in responses to injury, inflammation and repair.[61] As PARs are G-protein coupled receptors, activation of a PAR itself is not sufficient to induce action potentials in primary afferent neurons. However, several PARs have been shown to functionally interact with transient receptor potential (TRP) ion channels, thereby causing depolarization.[5,52,81,163,204] PAR2 is the only member of the PAR family that can be activated by mast cell tryptase[50,97,104,129,137,151,206] and is predominantly expressed by dorsal root ganglia that co-express TRPV1, TRPV4, TRPA1 (members of the TRP family) and substance P and CGRP.[5,81,175,216] It is well established that tryptase-mediated cleavage of sensory afferent expressed PAR2 contributes to neurogenic inflammation and hyperalgesia in rodents, partly by stimulating the release of substance P and CGRP from afferent terminals, causing plasma extravasation, granulocyte infiltration and neurogenic inflammation in skin and intestine,[42,135,175,205] and partly by sensitizing co-expressed TRP channels.[5,52,81,163]

TRPV1

The transient receptor potential vanilloid 1 (TRPV1) is a member of the TRP family of cation-selective channels. TRP receptors may have various functions, from regulation of blood circulation to pain signal transduction and hypersensitivity reactions in bladder, airway and intestine.[96] The relevance of TRPV1 in pain perception has been validated in studies concerning deletion of the TRPV1 gene,[18,24,36,54,101,153] RNA interference[44] and TRPV1 antagonism.[59,198,212] In different rat models in which visceral sensitivity is increased by either TNBS[59] or colonic neonatal irritation,[212] sensitivity to colorectal distension was reduced by TRPV1 antagonism. These results were confirmed in a study using the maternal separation model for IBS performed in our department.[198] In this model stress induces a mast cell-dependent increase in sensitivity to colorectal distension in adult maternally separated rats, which could be prevented and reversed by treatment with TRPV1 antagonists. In

some animal models, TRPV1 expression is increased and this increase is thought to contribute to the observed increase in sensitivity.[22] In the maternal separation model, however, there were no differences in TRPV1 expression levels between maternally separated rats and non-handled control rats.[198] In human, increases in pain perception are thought to correlate with increases in TRPV1 expressing fibers, as is shown by Chan *et al.* who demonstrated that an increase in TRPV1-expressing fibers in the intestine is associated with increased rectal sensitivity.[43] Furthermore, in patients with non-erosive reflux disease[16] and IBS,[3] TRPV1-expressing fibers may increase up to 3.5 fold in number. In the pathogenesis of both diseases a role for mast cell activation is implicated.

TRPV1 is abundantly present in the gastrointestinal tract, with expression on nerves in the mucosa, muscle layer and blood vessels within the intestinal wall.[75,208] Activation of TRPV1 channels on trkA$^+$ (NGF-dependent) primary sensory neurons stimulates the release of substance P and CGRP in peripheral tissues, contributing to neurogenic inflammation. Concomitantly, activation of TRPV1 leads to cation influx and action potential firing, resulting in pain sensation.[75,183] TRPV1 can be directly activated by low extracellular pH, noxious temperature, bioactive lipids and the active component of chili peppers, capsaicin. In addition, several mast cell mediators have been shown to modulate TRPV1 functions as will be discussed below. Interestingly, TRPV1 expression is also shown on human skin mast cells[169] and mouse BMMCs,[20] where activation of TRPV1 channels caused release of IL-4, but not IL-6, TNF-α and serotonin.[20] Since serotonin and histamine are released differentially,[188] the inability of capsaicin to induce serotonin release does not rule out direct TRPV1-mediated mast cell degranulation. Thus, next to inhibiting neuronal TRPV1, TRPV1 antagonists may also interfere with mast cell-expressed TRPV1 and herewith contribute to successful inhibition of pain responses in TRPV1-dependent *in vivo* models. This further emphasizes that TRPV1 is an attractive target for pain suppression in mast cell-dependent pathologies.

Upon mast cell activation, several mediators both pre-formed and *de novo* synthesized, can be released. One of the major pre-stored compounds that is released during mast cell degranulation is histamine. Interestingly, neurons that are sensitive to histamine have been shown to mostly overlap neurons that are sensitive to capsaicin,[105] indicating a possible role for histamine in the activation of TRPV1. Recently it has been shown *in vitro* that histamine is able to sensitize TRPV1 functions in mouse DRG.[105] In this study histamine was shown to enhance intracellular Ca^{2+} increases and membrane depolarization in response to acid in both NGF-dependent and GDNF-dependent sensory neurons. Agonist/antagonist experiments suggested involvement of histamine receptor 1 (HR1) in this sensitization process through PLC/PKC pathways. Up to date, four histamine receptors are known; HR1-HR4. Three of these receptors, HR1, HR3 and HR4, are expressed by dorsal root ganglia[34,136,177] and activation of these receptors is implicated in pain perception.[49,107,126,142,159] Although pharmacological intervention of HR3[126] and HR4[49] activation caused suppression of pain induced by chronic constriction injury and carrageenan respectively, HR1 seems to be mainly implicated in nociceptor sensitization. Also in the TRPV1-dependent maternal separation model for IBS, antagonism of HR1 was able to reverse stress-induced hypersensitivity,[170] suggesting that also in this model histamine acts on HR1 to sensitize TRPV1 channels and contribute to increased pain

perception. Another preformed mast cell mediator that is implicated to sensitize TRPV1 channels present on visceral sensory afferent neurons is serotonin (5-HT).[76,133,150] Receptors for 5HT ($5HT_1$-$5HT_{4)}$ are co-expressed with TRPV1[191,203] and activation of either the $5\text{-}HT_2$ receptor alone, or both $5\text{-}HT_2$ and $5\text{-}HT_4$ receptors was shown to enhance TRPV1 responses in rat spinal cord[108] and isolated sensory neurons.[140,180] Recently, it was shown *in vivo* that visceral hypersensitivity to colorectal distension induced by TRPV1 activation is modulated through 5-HT pathways.[150] However, this study did not determine which 5-HT receptor was responsible for regulating TRPV1 function.

Intestinal mast cells are considered to be the major source for tumor necrosis factor-α (TNF-α) in the human body.[77] TNF-α can be either *de novo* synthesized or preformed and stored constitutively within the mast cell granules and is co-released with histamine and tryptase upon IgE-mediated activation.[31,79,192] Several studies have shown a correlation between the level of TNF-α expression and the development of allodynia or hyperalgesia.[47,60,122,168] *In vitro,* stimulation of isolated neurons with TNF-α results in elevated responses to chemical TRPV1-activators.[84,92,109,168] Since rather long stimulation with TNF-α was needed to induce elevated responses, these effects are likely due to transcriptional up-regulation of TRPV1.[92] In line with this, two research groups show increased TRPV1 gene expression after TNF-α stimulation in isolated DRG neurons[89] and in a human synoviocyte cell line.[109] TNF-α acts through activation of two receptors (TNFR1 and TNFR2) and both can be detected on sensory neurons.[148,162] However, for TRPV1 sensitization in isolated DRG neurons, TNFR1 seems to be the most important.[89] Also in a mouse model for pulmonary hypersensitivity, TNFR1 is implicated in TNF-α-mediated increased responsiveness of sensory nerve endings to TRPV1 agonists.[200]

Two other mast cell mediators capable of modulating TRPV1 functions are NGF and tryptase. NGF is *de novo* synthesized upon mast cell activation and is implicated in the up-regulation of TRPV1 expression[6,105,213] and sensitization of TRPV1 channels,[46,160,161] probably through activation of PI3K pathways.[25,174,218] It is proposed by Stein *et al.* that binding of NGF to trkA facilitates TRPV1 trafficking to the plasma membrane through physical interaction between trkA, PI3K and TRPV1, thereby increasing TRPV1 function.[174] Sensitization of TRPV1 through activation of PAR2 by mast cell tryptase requires activation of PKCε and PKA. PAR2 agonists activate PKCε and PKA, causing their translocation from the cytosol to the plasma membrane, where they may phosphorylate and thereby sensitize ion channels that participate in nociception.[4] Activation of PAR2-sensitized TRPV1 activity in both mechanical allodynia and thermal hyperalgesia. However, in this study, where increased level of Fos-expression in the spinal cord was used as a marker for secondary spinal neuron activation, pain responses and Fos expression were not completely prevented in TRPV1-knockout mice, suggesting the involvement of other mechanisms down-stream of PAR2 activation.[52] Interestingly, in a following study, these authors demonstrate that interaction between PAR2 and TRPA1, in addition to TRPV1, might be an important mechanism underlying PAR2-mediated inflammatory pain.[138]

TRPV4

Another member of the vanilloid subfamily, TRPV4, can also be implicated in mast cell-induced pain perception. Different studies have defined a role for TRPV4 in the transduction of somatic pain[70,71,183]

and, recently, TRPV4 has been shown to play a major role in colonic visceral nociception.[40,163] In this study, intracolonic administration of a synthetic TRPV4 agonist caused a dose-dependent increase in visceral sensitivity to colorectal distension. TRPV4 is expressed by the majority of primary spinal afferent neurons innervating the colon, with a high expression on both pelvic and splanchnic DRG.[30] Consistent with this expression pattern, deletion of the TRPV4 gene increased both pelvic and splanchnic thresholds for mechanosensation, while vagal afferent functions did not change.[217] In the periphery, TRPV4 is expressed on NGF-dependent neurons that contain substance P and CGRP and activation causes neuropeptide release and Ca^{2+} mobilization and subsequent action potential firing.[81] Both histamine and 5-HT have been shown to sensitize responses of TRPV4 *in vivo* and *in vitro*.[41] Pre-treatment of isolated sensory neurons with either histamine or 5-HT enhanced Ca^{2+} signals in response to a TRPV4 agonist. Expression levels of TRPV4 at the plasma membrane were increased after treatment with histamine or 5-HT, concomitantly with a decreased expression in the nuclear and perinuclear compartment. Furthermore, histamine and 5-HT not only enhanced the amplitude of the response to the agonist, but also increased the number of responding neurons. The authors show that histamine-induced sensitization of TRPV4 is partially mediated by HR1, while 5-HT-induced sensitivity is mediated by the 5-HT_3 receptor. Intracolonic administration of histamine or 5-HT caused visceral allodynia and hyperalgesia to colorectal distension in mice. This hypersensitivity did not develop when mice were intrathecally injected with TRPV4 siRNA. Interestingly allodynia in response to histamine did not depend on TRPV4 expression.

TRPA1

The ankyrin subfamily of the TRP family of receptors contains only one member; TRPA1. TRPA1 is the mammalian orthologue of the *Drosophila* gene painless and studies using TRPA1 knockout mice, antisense knock-down of TRPA1 and pharmacological inhibition of TRPA1 demonstrate a significant role for TRPA1 in the processing of nociceptive information in inflammatory- and nerve injury models.[14,112,125,138,147] TRPA1 mediates cold, mechanical and chemical nociception and can be activated by temperatures under 17°C,[177] extracts of mustard, cinnamon, oregano and garlic,[11,15,48,102,177,214] as well as a wide range of environmental irritants.[14,120,125] TRPA1 is localized on GDNF-dependent sensory nerves of all three pathways innervating the intestine; vagal, splanchnic and pelvic afferents[22] and is extensively co-expressed with TRPV1.[11,62,102,138,176] Both TRPV1 and TRPA1 can be activated by intracellular[63,199] and extracellular Ca^{2+}.[1,39] Activation by extracellular Ca^{2+} results in a baseline supply of Ca^{2+} into the cell, which may maintain Ca^{2+}-dependent processes, such as transcription regulation and phosphorylation. Activation by intracellular calcium may account for mechanisms of TRPA1 gating by inflammatory mediators.[11,220] Interestingly, activation of TRPA1 by both intracellular and extracellular Ca^{2+} is shown to be controlled by the TRPV1 channel,[143] demonstrating functional interaction between both channels. Being a rather new member of the TRP family of receptors, not much is yet examined about the mechanisms of mast cell-mediated activation or sensitization of TRPA1. However, some studies provide evidence for PAR2-mediated sensitization of TRPA1 channels. TRPA1 is highly co-localized with PAR2 in rat DRG neurons and functional interaction depending on activation of PLC was shown in both TRPA1-transfected HEK293 cells as well as in DRG neurons.[53] Interaction between PAR2 and TRPA1 is also shown in

the gastrointestinal tract, where mast cell-induced activation of PAR2 was shown to contribute to mechanical hypersensitivity of esophageal vagal nerves, through sensitization of TRPA1.[216] In this study, however, possible involvements of TRPV1 and TRPV4 were not excluded.

MRG receptors

Mas-related G-protein-coupled (Mrg) receptors, first described in 2001, are predominantly, but not exclusively, expressed by $IB4^+$ (GDNF dependent) sensory neurons.[87] Because of their selective expression on sensory nerves, Mrg receptors are also called sensory neuron specific receptors (SNSR)[114] and are implicated to play a role in nociception. In human, seven Mrg receptors are expressed; MrgX1-MrgX7, while in rodents, the family of Mrg receptors can be further subdivided in MrgD and three subfamilies; MrgA, MrgB and MrgC based on homology analysis.[113] Several ligands such as; BAM peptides, neuropeptide FF (NPFF), dynorphin 14 and γ2-melanocyte-stimulating hormone.[64,87,90,106,114,116,131,132,139,152,185,220] Peptides known to activate the MrgC receptor have been shown to elicite pain-like behaviour when administrated to rats.[82] Furthermore deletion of both MrgD[38] as well as MrgE[51] in mice was shown to affect mechanosensation and the development of allodynia respectively. Interestingly approximately half of the Mrg-expressing neurons (both $IB4^+$ as well as $TrkA^+$) co-expressed TRPV1, suggesting a possible interaction between both receptors.[114] Indeed MrgC agonist BAM22 (bovine adrenal medulla peptide 22) was shown to facilitate CGRP release induced by TRPV1 agonist capsaicin. However, both deletion of the TRPV1 gene as well as antagonizing its activation with caspazepine had no effect on BAM22-induced CGRP release.[85] Therefore, to draw any firm conclusions on the possible interaction between Mrg receptors and TRP channels, more reseach is needed.

Interestingly, IgE-dependent activation of both mouse BMMC and RBL cells was shown to induce release of mediators capable of activating MrgC.[113] The authors suggest that, RF-amides, such as NPFF, released by mast cells may be responsible for activation of sensory neuron-expressed MrgC. Importantly, Mrg receptor expression was also found on human peripheral blood-derived connective tissue-type mast cells (MrgX1 and MrgX2 expression) as well as on rat peritoneal mast cells (MrgB expression). In both cell types, activation of the Mrg receptor (by several Mrg ligands) resulted in a dose-dependent increase in mast cell degranulation.[185] Therefore, *in vivo* effects on pain perception induced by ligand-mediated activation of Mrg receptors may, in addition to direct afferent activation, also involve mast cell activation and subsequent stimulation of nociceptive neurons by mast cell mediators,

Activation of mast cells by sensory afferent nerves

Among the earliest evidence for the involvement of neuronal mechanisms in mast cell activation is a study performed by MacQueen *et al.*[121] In this study antigen injections were paired with an audiovisual cue in rats. Re-exposure to only the audiovisual cue caused a RMCP2 (rat mast cell protease 2) release that was similar to the release of RMCP2 in rats that were re-exposed to both the

audiovisual cue and the antigen, as is seen in Pavlovian conditioning. Since then an overwhelming amount of evidence was published to indicate that neuronal activation can modulate mast cell function and thereby affect and regulate the immune system and its response under inflammatory conditions.[211] Here we discuss several of the neuropeptides released from afferent nerve endings which were shown to cause mast cell mediator release and functional alterations in mast cell receptor profile.

Modulation of mast cell function by neuropeptides

As mentioned earlier in this review, neurons can release mediators, such as neuropeptides, upon activation. Several of these mediators (such as substance P, CGRP, vasoactive intestinal protein (VIP), dopamine, arachidonic acid) are able to influence mast cell activation, a selection of which is discussed in this review.

Substance P is probably the most extensively studied neuropeptide in relation to mast cell activation. However, there seems to be a discrepancy whether intestinal mast cells express neurokinin (NK) receptors for substance P. Kulka *et al*. show protein expression of NK-1, NK2 and NK-3 in LAD2 cells and NK-1 in $CD34^+$-derived mast cells, which was up-regulated after antigen stimulation.[111] In contrast, Bischoff *et al*. were unable to detect any NK-1 expression in isolated human intestinal mast cells,[21] suggesting that mast cell susceptibility to substance P may depend on the local environment. Despite this, Shanahan *et al.* showed that high doses of substance P induce histamine release in mucosal mast cells.[158] Because of the high dosage needed these data may indicate that SP-induced histamine release by mucosal mast cells is irrelevant, but they simultaneously show that functional NK-receptors may be present. The latter is important because nanomolar concentrations of substance P were shown to induce TNF-α release without release of histamine or other mediators.[8] Importantly, in addition to mediator release, substance P has been shown to influence mast cell function by up-regulation of TLR receptors.[184] Substance P was shown to increase expression levels of TLR2, TLR4 and TLR8 in the LAD2 human mast cell line. It primed these cells for subsequent TLR2-agonist stimulation[184] and was shown to down-regulate the expression of the high affinity IgE receptor.[123] These changes in receptor profile render the mast cell more susceptible for direct activation by bacterial antigens in stead of indirect activation through anti-bacterial IgE, which may have major implications in various diseases.

Substance P and CGRP are not only considered as major initiators of neurogenic inflammation[91,194,201] but CGRP is also implicated to play a role in (mast cell-dependent) epithelial cell migration during wound healing.[33] Despite this, there is not much data available on the effects that CGRP may exert on mucosal mast cells. In a recent study however, CGRP stimulation induced release of relatively small amounts of mMCP-1 without degranulation, which was not dependent on extracellular Ca^{2+}, indicating piecemeal degranulation.[65,66,190] In this study subunits of the CGRP1 receptor were expressed on the majority of BMMCs,[149] which are thought to resemble mucosal mast cell morphology and mediator content.[119,128] The observed role of piecemeal degranulation may be highly relevant. Not only has it been shown to be involved in the release of other mast cell

mediators, but this mechanism of mast cell activation is also implicated in activation of mast cells in close proximity to nerves.[149]

Besides CGRP, also vasoactive intestinal protein (VIP) has been shown to induce piecemeal degranulation in mast cells.[195] VIP is a prominent neuropeptide, produced by nerves in the central and peripheral nervous system, with functions ranging from neurotransmission to immunomodulation.[154,155] Mast cells have also been shown to express a truncated form of VIP but the function of this peptide is unclear.[78,111,209] Expression of VIP receptor type 2 (VPAC2) but not VPAC1 has been shown on human mast cells (LAD and primary cultured mast cells).[111] IgE crosslinking increased VPAC2 expression and rendered cells more susceptible to VIP-induced degranulation of β-hexosaminidase. VIP also induced significant production of chemokines and cytokines like MCP-1, RANTES, Il-8 and TNF-α and Il-3. Interestingly, mast cell-derived tryptase is able to cleave VIP, while chymase was shown to degrade both VIP and substance P,[37] indicating a balancing reaction of mast cells to afferent nerve-induced activation.

Cholinergic modulation of mast cells

A close anatomic relation between vagal nerve endings and intestinal mucosal mast cells has been shown[172] and depletion of the vagal nerve resulted in approximately 25% fewer mast cells in the jejunal mucosa than in sham operated controls.[80] Based on the trophic effects vagal nerves can exert on stomach mucosa,[80,86] this observation lead to the suggestion that the vagal nerve exerts trophic effects on mast cells. The vagus nerve, via release of its principal neurotransmitter acetylcholine, is also involved in the control of immune responses. Activation of the so called 'cholinergic anti-inflammatory pathway' by electrical stimulation of the vagus nerve or nicotinic acetyl choline (nACh) agonists ameliorates inflammation and improves survival in various experimental models for sepsis and inflammation.[26,27,144,187,207] These effects are mediated by nACh receptors expressed on inflammatory cells. Mouse BMMCs express mRNA for α4, α7 and β2 nACh receptor subunits and nACh receptor agonists were shown to inhibit IgE-induced degranulation of mucosal mast cells.[103] It has to be taken in account however, that significance was only achieved at relatively high concentrations (≥3,2mM) of nicotine, compared to the concentration of nicotine found in plasma of smokers (100nM).[94] Furthermore, the authors state that differences in sensitivity for nicotine may exist between different mast cell subtypes. In line with their observation, also in basophiles millimolar concentrations of nicotine were needed to inhibit IgE-mediated histamine release.[193] *In vivo*, vagal stimulation caused an increase in histamine-immunoreactivity in intestinal mucosal mast cells.[171] Whether this involves increased histamine synthesis or a decrease in release remains to be established. Taking into account the inhibitory effect of nicotine treatment on histamine release *in vitro,* the latter seems most probable. Further evidence that vagal stimulation influences mast cell degranulation was obtained in a model for postoperative ileus which is a (mast cell-dependent[57,187]) post-operative complication that results from manipulation of the bowel during abdominal surgery. The cholinergic anti-inflammatory pathway can be physiologically activated by administration of lipid-rich nutrition, through activation of CCK-receptors.[118] When POI was induced in rats, administration of lipid-rich nutrition reduced release of RMCP-II, indicating that a high fat diet can

prevent manipulation-induced mast cell degranulation.[117] Recently, also in a rodent shock model high-lipid nutrition was shown to modulate mast cell activation,[55] providing strong evidence for inhibitory effects of vagal nerve activation on mast cells.

Stress-induced mast cell activation

Corticotropin releasing factor (CRH) has a major function in regulating the stress response by activation of the hypothalamic-pituitary-adrenal (HPA) axis and the sympathetic nervous system.[45] In addition, CRH was shown to induce degranulation of peripheral mast cells upon acute stress in rodent models. The exact source of mast cell-activating CRH is unknown, however, CRH has been shown to co-localize with substance P in capsaicin-sensitive neurons.[164] Release of CRH from activated neuronal terminals could provide a pro-inflammatory pathway for mast cell degranulation or mediator release during stress. Importantly, human umbilical cord-derived mast cells have also been shown to functionally express subunits of both types of CRH receptors; CRHR1 and CRHR2. In the human mast cell line, HMC-1, expression of only CRHR1 subunits was found[35] but it has to be noted that HMC-1 cells are considered immature mast cells and may not represent mast cells *in situ*. CRH-induced activation of umbilical cord-derived mast cells as well as HMC-1 cells caused selective secretion of vascular endothelial growth factor (VEGF), without secretion of tryptase, histamine, IL-6, IL-8 or TNF-α.[35] This differential release of VEGF may represent piecemeal degranulation[190] but at present no clear evidence exists that VEGF is among the mediators that can be released by this type of degranulation: granule density and other features of piecemeal degranulation were not determined after CRH-induced VEGF release. Thus, further investigations are needed, especially because Santos *et al*. showed that piecemeal degranulation may indeed be important in stress-induced mast cell activation.[157] Although most of the published stress/gut mast cell investigations concern models for IBS, signs for piecemeal degranulation have also been found in mucosal mast cells in the gastrointestinal tract of patients with IBD.[66] In IBD, stress-induced exacerbations of symptoms and relapse of disease are prominent and these observations indicate that neuron-mast cell interactions may play an important role.

Bidirectional mast cell-nerve interactions: summary and implications in intestinal disease

In this review we focused on the mechanisms for bidirectional interaction between mast cells and sensory afferent nerves, summarized in Figure 1. As is shown in this figure, several mast cell mediators can sensitize nociceptors through activation of receptors that are co-expressed by sensory afferent neurons and thereby reduce the threshold for activation of these nociceptors. Activation of these afferents stimulates release of neuropeptides, several of which can change the mast cell receptor profile, modulate mast cell function or stimulate mast cell mediator release, herewith creating a positive feedback loop for ongoing mast cell activation and nociceptor sensitization. This mechanism is considered relevant for pathologies where mast cells and changes in sensitivity are involved.

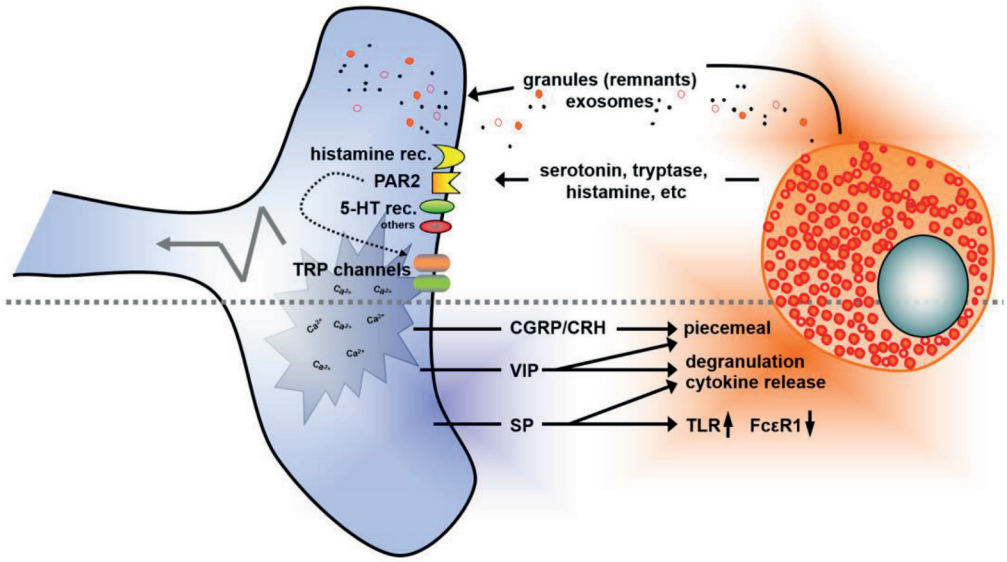

Figure 1. *Mast cell mediators such as serotonin, tryptase and histamine interact with their specific (sensory afferent expressed-) receptors which then, through phospholipase Ccoupled receptor pathways, induce TRP-channel sensitization. The lowered threshold for activation can result in TRP-channel activation, membrane depolarization and Ca^{2+} influx which in turn drives the release of pre-stored neuropeptides such as CGRP, CRH, VIP and substance P. Propagated action potentials can lead to increased pain perception and neuropeptide release to further induce mast cell activation/degranulation as well as other phenotypical changes in these cells (i.e. lowered FcεR1- and increased TLR-expression). Next to direct mast cell mediator-induced afferent activation there is convincing evidence of neuronal uptake of mast cell granules (complete as well as remnants) and perhaps mast cell-derived exosomes. It is not clear at present what the relevance of this phenomenon is.*

Stress can cause neuropeptide release (e.g. substance P and CRH) from peripheral nerve terminals. As discussed in this review, these neuropeptides can induce release of mast cell mediators either by piecemeal- or classic degranulation. Furthermore, substance P is shown to induce changes in mast cell mediator profile, making the mast cell more susceptible for direct activation by bacterial antigens. The latter could have major implications not only in IBS pathology, in which intestinal barrier dysfunction is implicated, facilitating the passage of bacteria through the lumen, but also in pathologies with stress-induced exacerbations of symptoms, like IBD. In both IBS and IBD, expression of TRPV1 is implicated to play a role in increased pain perception. In IBS patients mast cell activation in close proximity to nerves[13] and the amount of TRPV1 expressing fibers[3] are shown to correlate with abdominal pain. To our knowledge, in literature there is no evidence for up-regulation of both TRPV4 and TRPA1 expression in IBS patients. However, increased mRNA expression of both HR1 and HR2 was shown in biopsy material of IBS-patients,[156] suggesting that these receptors might be involved in the sensitization of TRPV1 channels in these patients. In IBD patients, activation of TRPV1 channels is implicated in increased pain sensation. In patients with quiescent IBD but with abdominal pain, TRPV1-expressing fibers were increased up to 5 fold when compared to

quiescent IBD without abdominal pain.[2] The latter data suggest that nociceptors like TRPV1 can be a pharmaceutical target in IBS as well as IBD patient-subsets.

In conclusion, and although we are only on the verge of understanding the exact pathways involved in mast-cell-nerve communication, it seems clear that these bidirectional interactions form a major contribution to gut disorders like IBS and IBD. Thus, furthering our knowledge on these interactions may lead to new treatment opportunities.

Acknowledgements

This work is supported by grants from the Netherlands Top Institute Pharma (TIPharma), grant number T1-215-1 and by funding for the IPODD consortium under the Seventh Research Framework Programme of the European Union.

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Chapter 3

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

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Neurogastroenterology & Motility, 2013 Dec; 25(12): e780-e790

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°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 anesthesia. Distensions started after a 20 minute recovery period. They were performed at the minimum age of 3 months 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. After each 20-s distension period water was quickly removed and an 80-s resting period was exercised. For acute stress at adult age (water avoidance stress; WA) 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 2 mL 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 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.

Protocols

Maternal separation

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.

Nonhandled second filial generation (NH-F2) offspring of MS-F1 dams

Because female pups are usually culled on post natal day 2, separate 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 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 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).

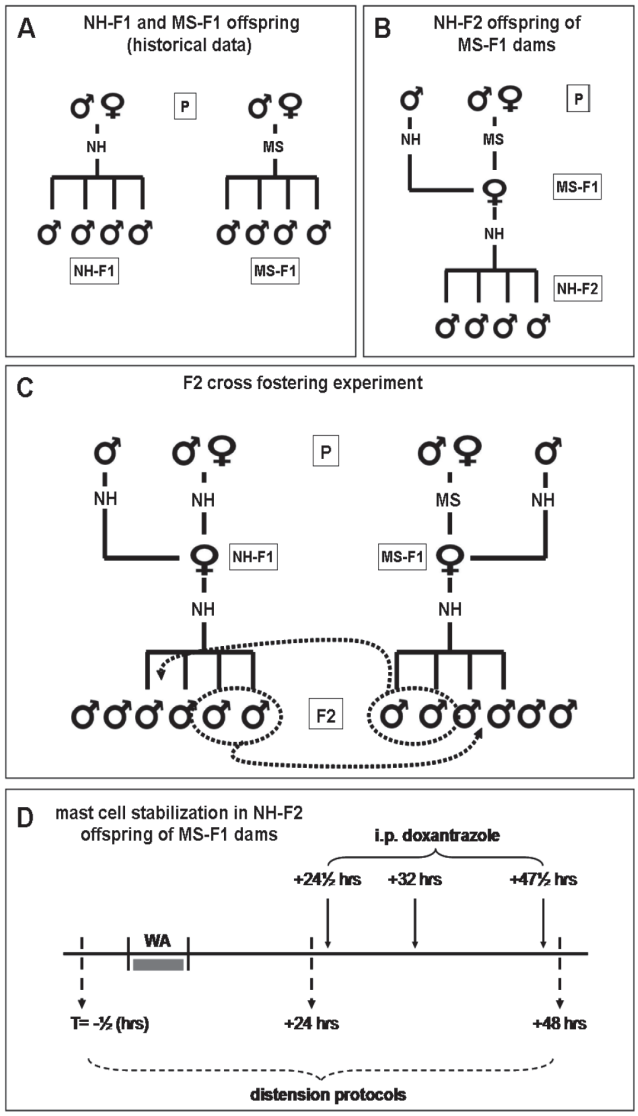


Figure 1. *Schematic representation of experimental protocols. All parental generation male and female animals (P) were normal nonhandled (NH) rats. Visceromotor response to distension was determined before and 24 hours after WA in adult rats. (1A) Historical data: nests subjected to the maternal separation (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.*

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 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 to n=30 NH-F2 offspring, seven out of nine MS-F1 dams were subjected to WA and distension protocols. 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.

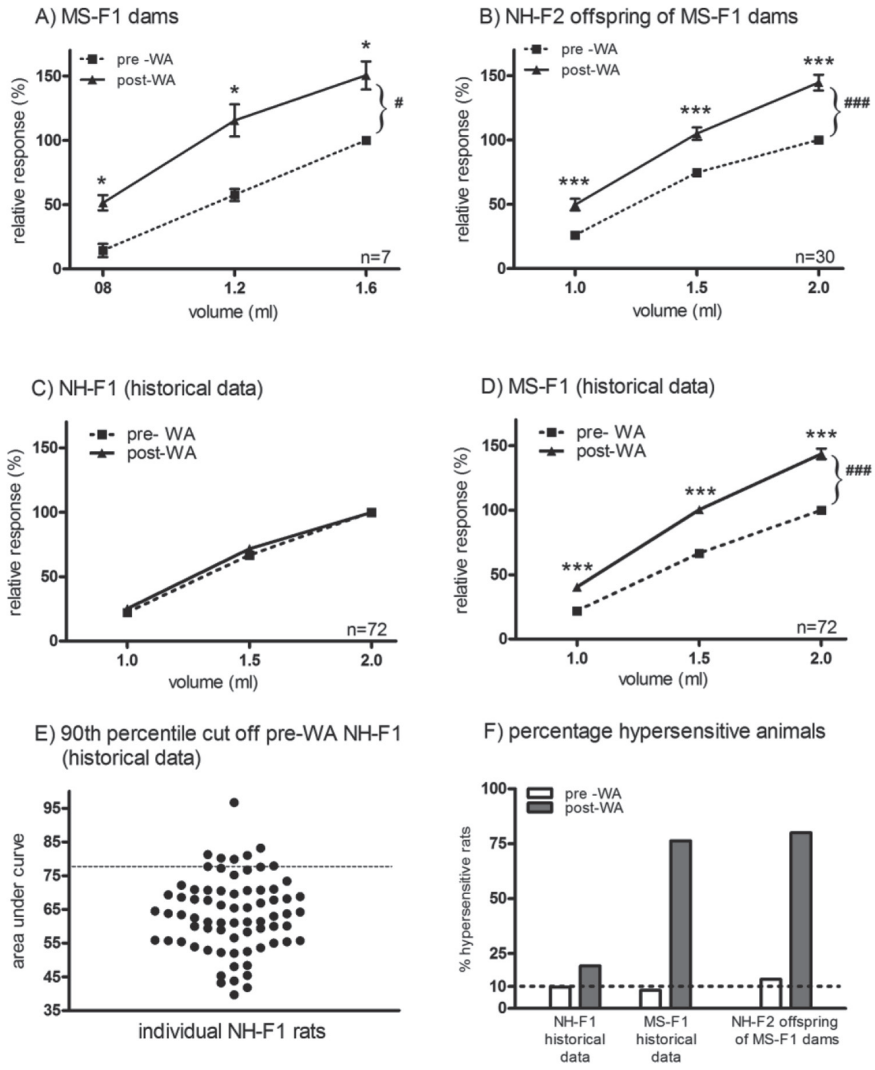


Figure 2. *Effect of WA-stress on visceromotor response to colonic distension, all data in 2A-2D are given as mean ± SEM. Figure 2A depicts enhanced post-WA response to distension in n=7 MS-F1 dams (evaluated after giving birth to NH-F2 offspring shown in 2B); pre-WA vs post-WA AUC, #P<0.05 and increased 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.*

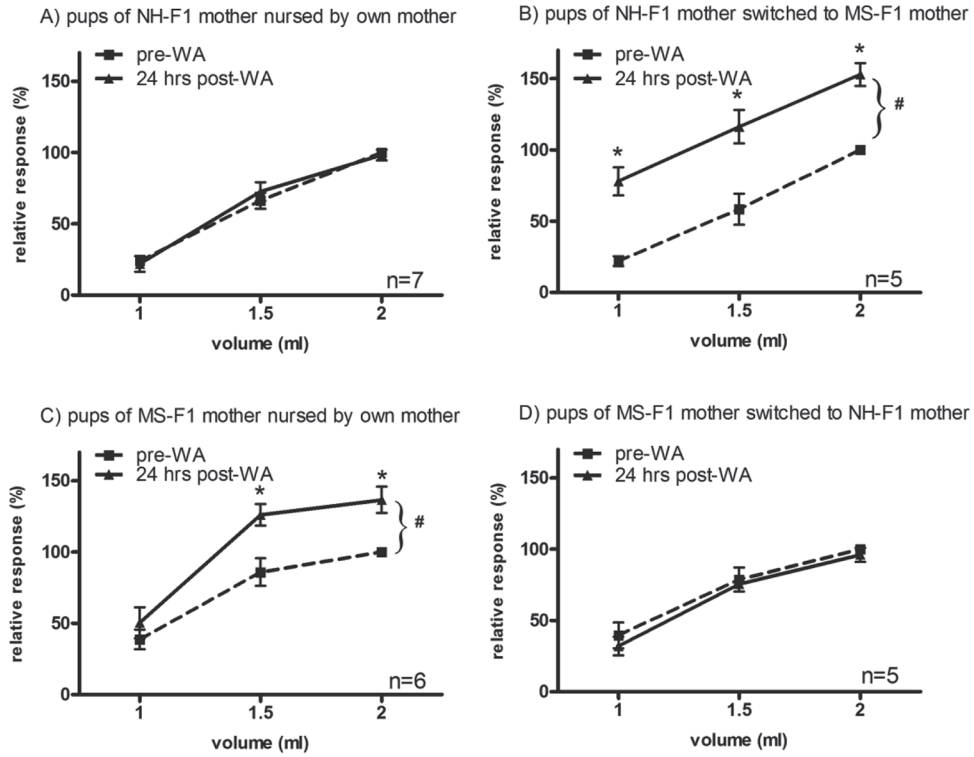


Figure 3. *Volume/relative response data of cross fostering experiments, data are mean ± SEM. (A) Absence of WA-induced visceral hypersensitivity when NH-F2 offspring (n=7) was nursed by own NH-F1 dams. (B) NH-F2 offspring (n=5) switched to MS-F1 dams. WA induced an enhanced response to distension; pre-WA 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.*

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.

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.*

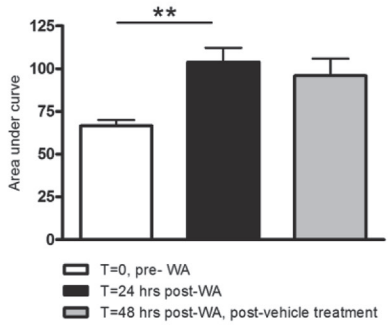
	nurtured by NH-F1 dams		**nurtured by MS-F1 dams**	
	pre-WA	**post-WA**	**pre-WA**	**Post-WA**
offspring of MS-F1 dams	85.5*	78.0*	68.9	119.3*
	75.0	63.3	81.0*	111.3*
	66.0	75.4	64.9	96.2*
	84.5*	62.4	73.2	107.2*
	60.3	69.5	106.2*	131.6*
			71.4	92.8*
offspring of NH-F1 dams	70.3	63.8	70.7	106.5*
	65.9	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		

Mast cell stabilization in male NH-F2 offspring of MS-F1 dams

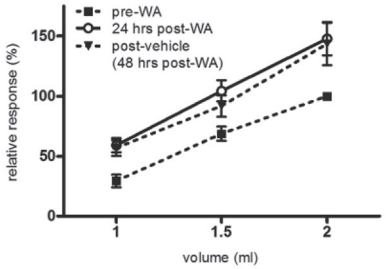
Previous investigations showed that post stress visceral hypersensitivity in MS-F1 Long Evans rats depends on mast cell degranulation.[30] Since NH-F2 offspring of MS-F1 dams also showed post-stress visceral hypersensitivity we evaluated the role of mast cells in this response by post-WA application 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).

A) vehicle treated F2 offspring of MS-F1 dams (AUC)

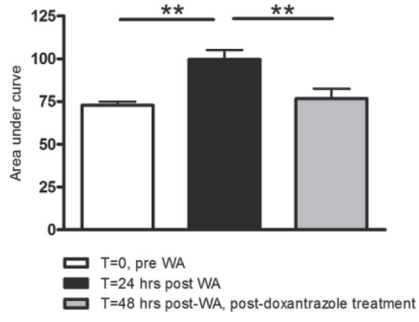


B) vehicle treated F2 offspring of MS-F1 dams (per volume comparisons)

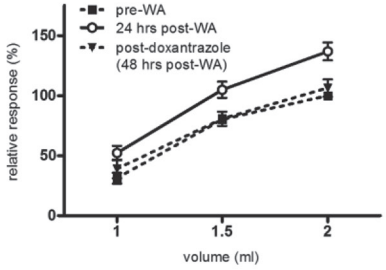


*P<0.05 **P<0.01 ns=not significant	volume		
	1.0 ml	1.5 ml	2.0 ml
pre-WA vs. 24 hrs	**	**	**
24 hrs vs. 48 hrs	n.s.	*	n.s.

C) doxantrazole treated F2 offspring of MS-F1 dams (AUC)



D) doxantrazole treated F2 offspring of MS-F1 dams (per volume comparisons)



*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 and may be ideal to investigate cross-generational effects of (suspected) environmental IBS-triggers. Our data suggest that susceptibility to stress-induced mast cell dependent visceral hypersensitivity in the MS model can be transferred across generations and that transfer depends on maternal care.

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[33] 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[36] but, along with that, 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] These data suggested that, in contrast to the behavior-selection model, maternal

behavior may not be the determining factor in the maternal separation model and, therefore, future investigations should also consider other possibilities. Evidently this does not rule out a role for other epigenetic changes. Since our data indicate that mast cells may play an important role in MS-F1 as well as F2 offspring such changes may involve this cell type.

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.[39] When supernatants of submerged intestinal human biopsies were used, IBS but not normal control supernatants induced histamine-dependent firing of rat mesenteric afferents[40] and serine-protease dependent colonic hyperalgesia in mice.[41] 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 (submitted for publication). 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.[42] 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 filial generation is being targeted.

Although our cross fostering experiments indicated that maternal care, by natural or foster mother, is relevant to the observed phenotype, the exact mode of transmission remains unknown. We speculate that there may be a role for the microbiome. The high incidence of post-infectious IBS triggered an increased interest in the possible role of the gut flora in IBS.[43] 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.[44] O'Mahoney *et al.* showed altered fecal microbiota in maternally separated (i.e. MS-F1) rats when compared to NH rats.[45] 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.[46]

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 4

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

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Neurogastroenterology & Motility, 2012 Mar; 24(3): 274-82

Abstract

Background: Acute stress-induced hypersensitivity to colorectal distention 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 distention, 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]

Materials 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°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

Distention 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) distentions (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 distentions were performed with a latex balloon (Ultracover 8F, International Medical Products, Zutphen, The Netherlands) and carried out as described before.[20] Distentions 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 anesthesia. 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.

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 distentions 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 distentions 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 distentions 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 distentions 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/).

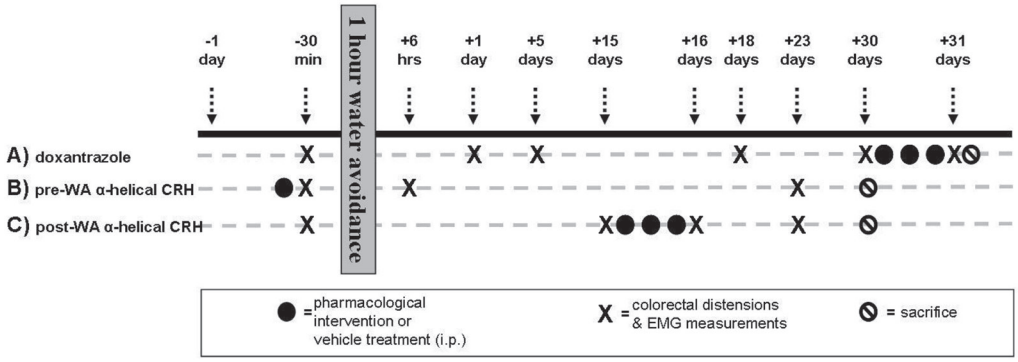

Figure 1. *Schematic representation of pharmacological intervention protocols. Detailed description in Material and Methods paragraph on 'Experimental design of pharmacological intervention studies' A-C.*

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.

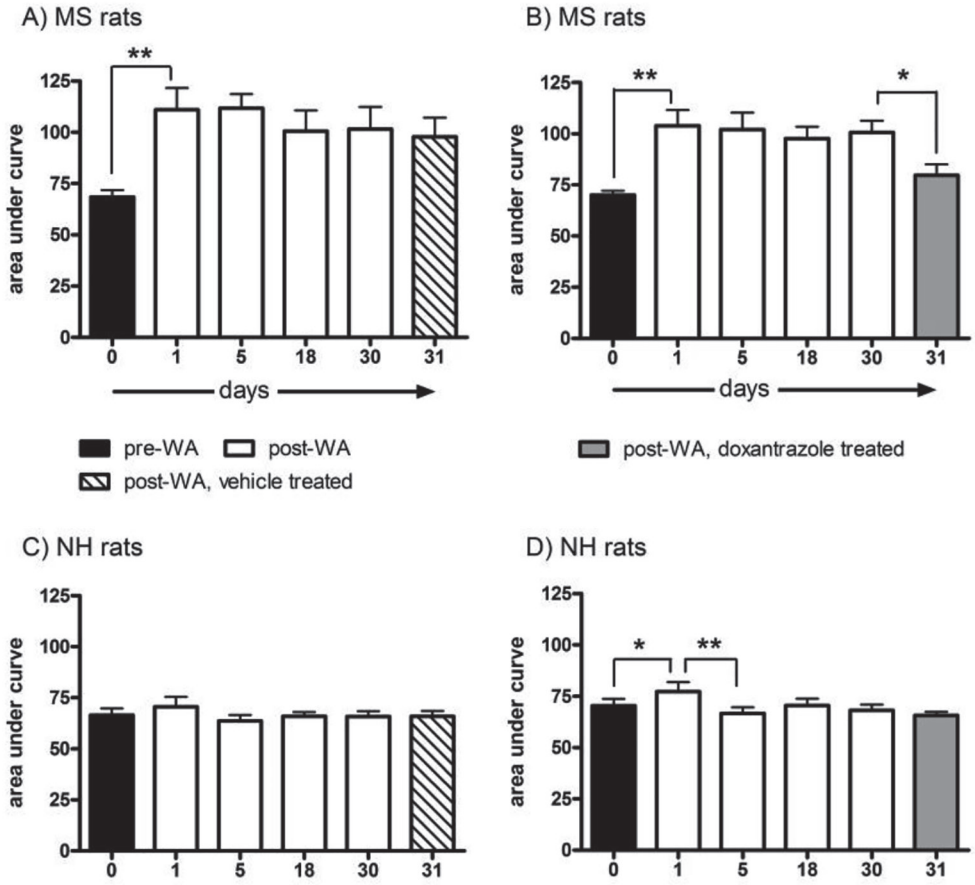

Figure 2. *Post-WA hypersensitivity to distention in maternally-separated rats is long-lasting and can be reversed by mast cell stabilization. In maternally-separated rats (panels A and B), WA induced long-lasting (30 days) hypersensitivity to distention which was reversed by i.p. doxantrazole treatment (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.*

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.

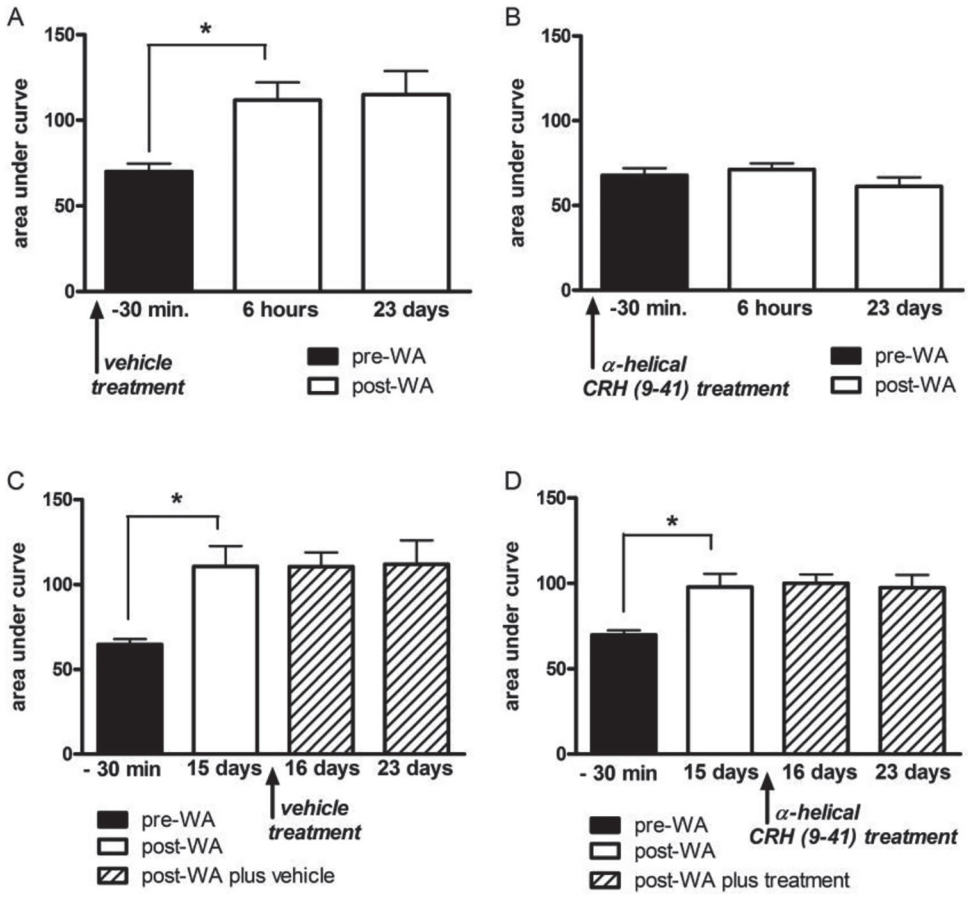

Figure 3. *Pre-WA α-helical-CRF(9-41) administration prevents, but post-WA administration does not reverse, hypersensitivity to distension. Pre-WA administration (panel A) of vehicle alone (i.p.) lead to increased AUC at 6 hours post-WA whereas administration of α-helical-CRF(9-41) inhibited stress-induced 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.*

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.

Statistic evaluation of the relative response data (Supplementary Figure S2) showed enhanced post-WA response for all 3 distension volumes when rats were pre-treated 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.

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 analysed 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.

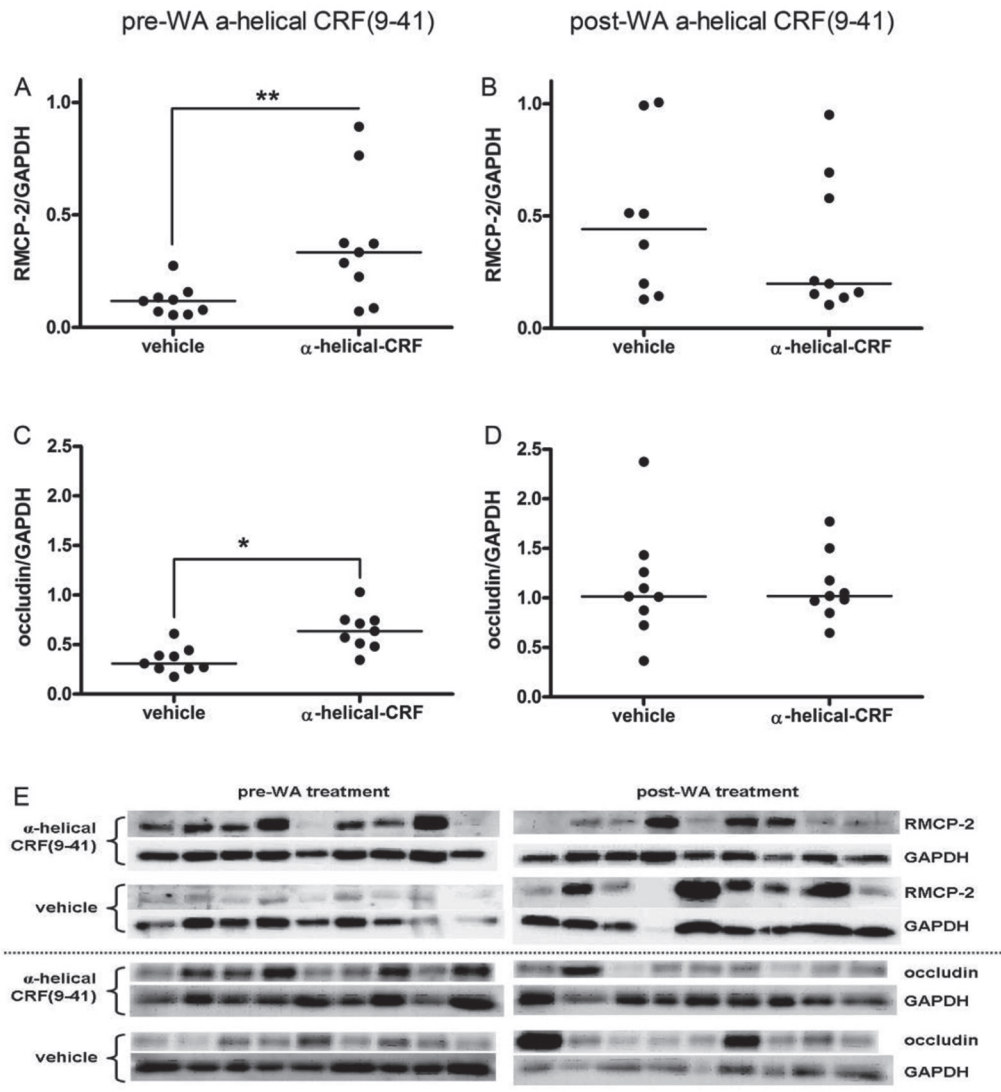

Figure 4. *Pre- but not post-WA α-helical-CRF(9-41) administration leads to differences in mucosal RMCP-2 and occludin protein levels. Distal colonic mucosa collected from the pre- and post-WA α-helical-CRF(9-41) treatment groups was evaluated by Western-blotting. Protein expression levels (relative 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 distention.[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.

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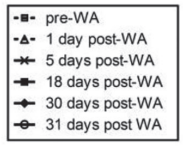
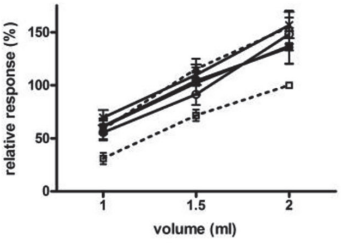


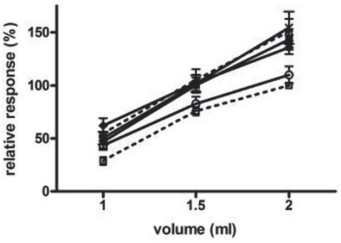
Supplement Figure S1.
vehicle or doxantrazole treatment between day 30 and 31. Left side: relative response to distension. Right side: statistics for individual volumes.

A) vehicle treated MS rats



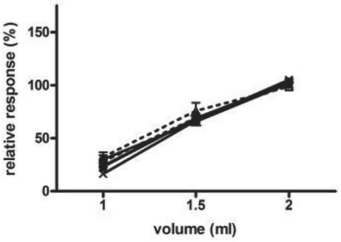
*P<0.05 **P<0.01 ***P<0.001 ns=not significant	volume		
	1.0 ml	1.5 ml	2.0 ml
pre-WA vs. day 1	*	**	**
day 1 vs. 5	ns	ns	ns
day 5 vs. 18	ns	ns	ns
day 18 vs. 30	ns	ns	ns
day 30 vs. 31	ns	ns	ns

B) doxantrazole treated MS rats



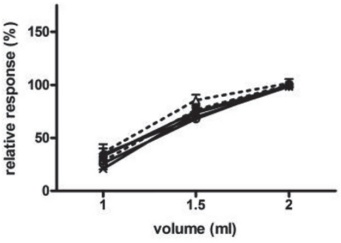
*P<0.05 **P<0.01 ***P<0.001 ns=not significant	volume		
	1.0 ml	1.5 ml	2.0 ml
pre-WA vs. day 1	**	*	**
day 1 vs. 5	ns	ns	ns
day 5 vs. 18	ns	ns	ns
day 18 vs. 30	ns	ns	ns
day 30 vs. 31	*	*	**

C) vehicle treated NH rats



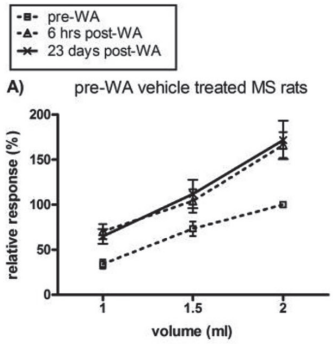
*P<0.05 **P<0.01 ***P<0.001 ns=not significant	volume		
	1.0 ml	1.5 ml	2.0 ml
pre-WA vs. day 1	ns	ns	ns
day 1 vs. 5	*	ns	ns
day 5 vs. 18	*	ns	ns
day 18 vs. 30	ns	ns	ns
day 30 vs. 31	ns	ns	ns

D) doxantrazole treated NH rats



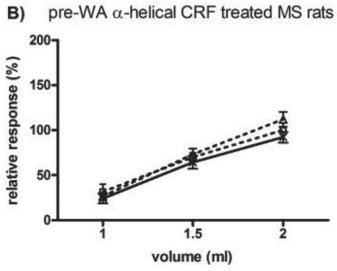
*P<0.05 **P<0.01 ***P<0.001 ns=not significant	volume		
	1.0 ml	1.5 ml	2.0 ml
pre-WA vs. day 1	ns	ns	ns
day 1 vs. 5	*	*	ns
day 5 vs. 18	ns	ns	ns
day 18 vs. 30	ns	ns	ns
day 30 vs. 31	ns	ns	ns

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).*

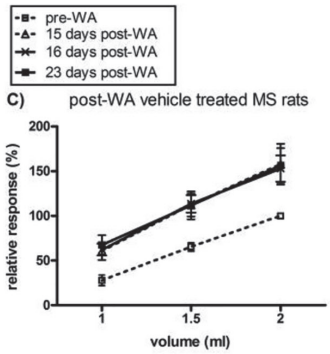


Supplement Figure S2.
pre-WA (A& B) and post-WA (C&D) treatment with vehicle or α-helical CRF. Left side: relative response to distension. Right side: statistics for individual volumes.

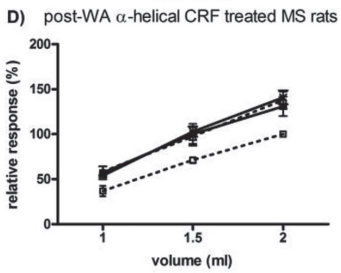
*P<0.05 **P<0.01 ***P<0.001 ns=not significant	volume		
	1.0 ml	1.5 ml	2.0 ml
pre-WA vs. 6 hrs	**	*	**
6 hrs vs. day 23	ns	ns	ns



*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.*

Chapter 5

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

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PLoS One, 2013 Jun 12; 8(6): e66884

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.

Materials and methods

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 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°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 2 mL 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: TGTGAGGGAAT CGACAGGACCC.

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-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/).

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).

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.

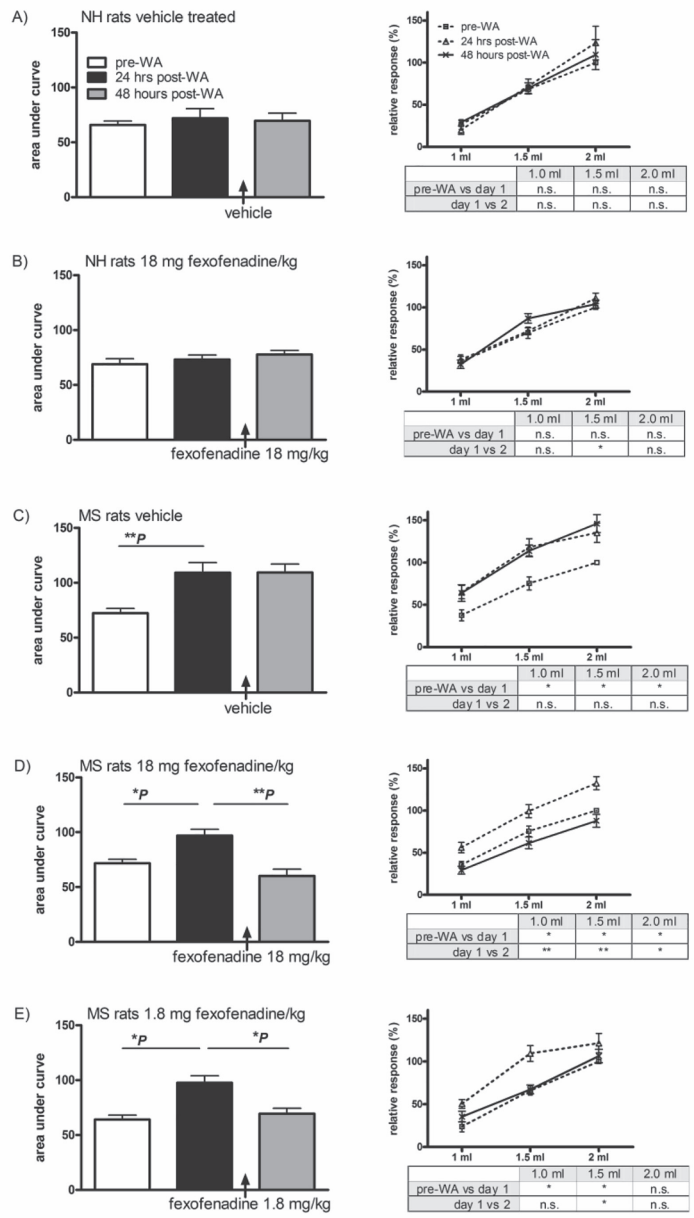

Figure 1. *In vivo post stress fexofenadine treatment. The visceromotor response to distension was measured pre-WA and 24 and 48 hours post-WA in NH and MS rats. Fexofenadine or vehicle was administered 3 times between 24- and 48 hours measurements (cumulative dosages 18 and 1.8 mg/kg). Responses to 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.*

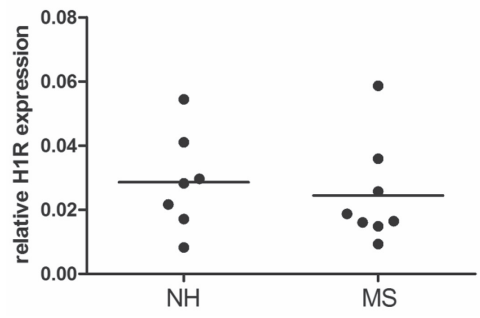

Figure 2. *Relative colonic expression values for the histamine H1 receptor gene. H1R mRNA expression was evaluated relative to the housekeeping gene Ppib in colonic samples of NH and MS rats. Tissue was collected 7 days post vehicle treatment and distensions. There were no significant differences.*

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 3C 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. 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).

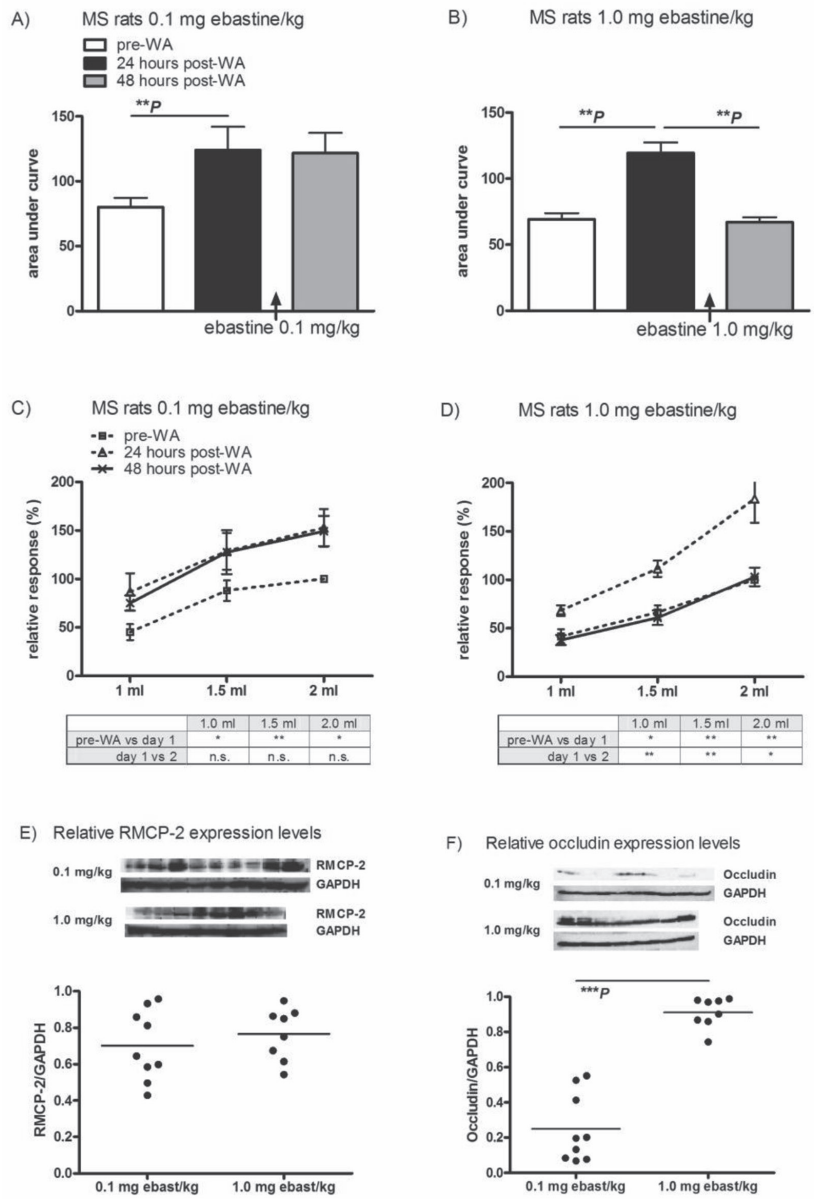

Figure 3. *In vivo post stress ebastine treatment. Sensitivity to distension was measured pre-WA and 24 and 48 hours post-WA in MS rats. Ebastine (cumulative dose 0.1 or 1.0 mg/kg) was administered 3 times between 24- and 48 hours measurements (please refer to Figure 1C for vehicle treatment group). 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.*

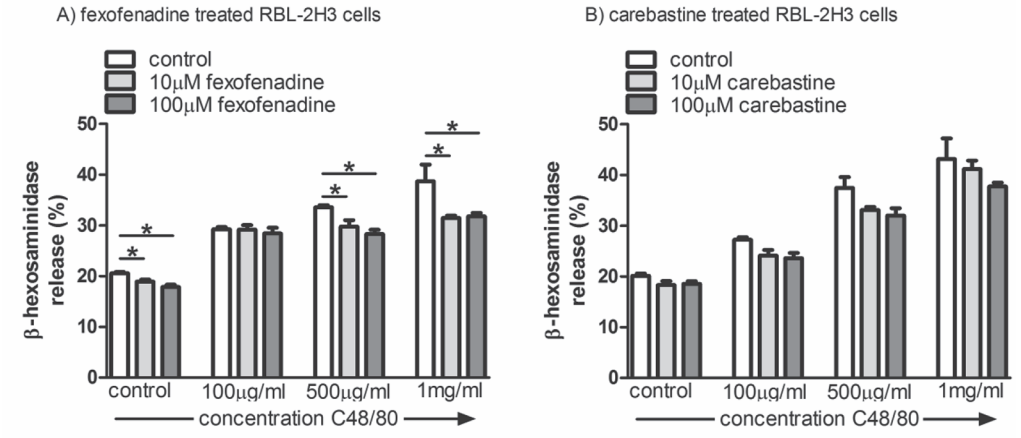

Figure 4. *Modulation of Compound 48/80-induced RBL-2H3 degranulation. Compound 48/80 treatment of RBL-2H3 cells induced a concentration dependent release of β-hexosaminidase. 30 minutes pre-incubation with 10 and 100 μM fexofenadine reduced β-hexosaminidase release in control cells and cells treated 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] could 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 6

Miltefosine treatment reduces visceral hypersensitivity in a rat model for irritable bowel syndrome via multiple mechanisms

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Revision submitted to Scientific Reports, Nature Research

Abstract

Irritable bowel syndrome (IBS) is a heterogenic, functional gastrointestinal disorder of the gut-brain axis characterized by altered bowel habit and abdominal pain. Preclinical and clinical results suggested that, in part of these patients, pain may result from fungal induced release of mast cell derived histamine, subsequent activation of sensory afferent expressed histamine-1 receptors and related sensitization of the nociceptive transient reporter potential channel V1 (TRPV1)-ion channel. TRPV1 gating properties are regulated in lipid rafts. Miltefosine, an approved drug for the treatment of visceral Leishmaniasis, has fungicidal effects and is a known lipid raft modulator. We anticipated that miltefosine may act on different mechanistic levels of fungal-induced abdominal pain and may be repurposed to IBS. In the IBS-like rat model of maternal separation we assessed the visceromotor response to colonic distension as indirect readout for abdominal pain. Miltefosine reversed post stress hypersensitivity to distension (i.e. visceral hypersensitivity) and this was associated with differences in the fungal microbiome (i.e. mycobiome). *In vitro* investigations confirmed fungicidal effects of miltefosine. In addition, miltefosine reduced the effect of TRPV1 activation in TRPV1-transfected cells and prevented TRPV1-dependent visceral hypersensitivity induced by intracolonic-capsaicin in rat. Miltefosine may be an attractive drug to treat abdominal pain in IBS.

Introduction

Abdominal pain is the key contributing factor to severity of IBS and, mainly due to the lack of pathophysiological insight, a major unmet clinical need.[1,2] Visceral hypersensitivity, diagnosed as a decreased threshold of discomfort to colorectal distension, is observed in ~50% of patients and thought to be an underlying mechanism for abdominal pain.[3] We recently showed intestinal mycobiome dysbiosis in hypersensitive IBS patients and addressed the possible importance of this finding in the rat maternal separation model for IBS-like visceral hypersensitivity.[4] Not only did we observe profound mycobiome dysbiosis in maternal separated rats but also provided evidence for the functional relevance of dysbiosis by conducting fecal transfer experiments in fungicide treated animals. In addition, post stress hypersensitivity to colorectal distension was reversed by blocking host recognition of particulate β-glucans. Innate immune cells recognize these fungal cell wall components via the C-type lectin receptor dectin-1 that signals via spleen tyrosine kinase (Syk). The *in vivo* use of soluble β-glucans that antagonize dectin-1 activation and a Syk inhibitor independently inhibited the pain response. Subsequent *ex vivo* experiments then indicated that particulate β-glucans trigger mast cell degranulation. Earlier studies showed the relevance of the mast cell mediator histamine; in the maternal separation model as well as part of the IBS patients, abdominal pain complaints resulted from histamine receptor-1 activation and subsequent sensitization of afferent expressed TRPV1.[5-8]

Taken together, the above data suggest that post stress visceral hypersensitivity results from immune recognition of an aberrant mycobiome via the dectin-1/Syk pathway, leading to mast cell degranulation and subsequent activation of histamine-1 receptors on sensory neurons, which in turn leads to sensitization of TRPV1 and pain signaling. The process of drug development for newly identified targets such as the ones described here is costly and time-consuming. Repurposing of existing drugs with established side effects may partly circumvent these issues.[9] In search for candidate drugs to treat abdominal pain complaints in IBS, we evaluated the FDA approved compound miltefosine. This alkyl-phospholipid was, largely unsuccessful, developed as an antitumor drug but is now approved as an oral treatment of visceral leishmaniasis.[10,11] Miltefosine however, was also shown to have broad-spectrum *in vitro* and *in vivo* fungicidal activity by triggering metacaspase (MCA1)-dependent apoptosis in fungal target cells.[12,13] Thus, *in vivo* administration of this compound may lead to favorable mycobiome modulation in dysbiotic subjects. Miltefosine also inhibited *in vitro* mast cell activation, and oral and topical administration were successfully evaluated in mast cell driven skin conditions.[14-17] Mast cells are modulated in the cytosol by inhibition of Ca^{2+}-dependent protein kinase C (cPKC)[16] and, due to insertion of this phosphatidylcholine analogue, at the plasma membrane were it behaves as a lipid raft modulator.[11,18] Lipid rafts are specialized membrane microdomains that are formed by tightly packed aggregates of phospholipids, glycolipids and cholesterol together with protein receptors, which can be included or excluded depending on their affinity. These rafts act as signal transduction moieties.[19] In trigeminal sensory neurons and TRPV1 transfected cell lines, disruption of raft integrity affected TRPV1 receptor activation by inhibiting opening properties of the cation channel.[20] Although raft disrupting strategies other than

miltefosine were used, these result suggest that miltefosine, in addition to direct targeting of the mycobiome and mast cells, may also interfere with TRPV1 receptor activation to alleviate abdominal pain in IBS.

Here, we evaluated the effect of miltefosine treatment in two different models of visceral hypersensitivity. In the rat maternal separation model, we addressed the possible correlation between miltefosine-induced reversal of post-stress visceral hypersensitivity and fecal myco- and microbiome alterations. Reported fungicidal and bactericidal effects of miltefosine[13,21] were confirmed with *in vitro* agar disk diffusion tests. Finally, we evaluated whether miltefosine reduces the effect of TRPV1 activation in TRPV1-transfected cells, and investigated its effect on *in vivo* TRPV1-dependent visceral hypersensitivity in a rat model of intracolonic capsaicin.

Methods

Animals

Long-Evans rats (Harlan, Horst, The Netherlands) were bred at the animal facility of the Amsterdam UMC, Location AMC (Amsterdam, The Netherlands). All rats used in these investigations were housed under open cage conditions and bred in the same room. All animal 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).

Measurement of the visceromotor response to colonic distension and data analysis

Visceral hypersensitivity in patients is diagnosed as an increased sensitivity to rectal distension, contributing to abnormal perception of pain and discomfort.[3] During distensions, self-rating questionnaires (i.e. visual analogue score) are often used to evaluate pain scores, but these cannot be used in rat. However, colorectal distensions in rats lead to reproducible contractions of abdominal musculature; the so called visceromotor response. Quantification of these contractions by electromyography (EMG) is often used to assess visceral pain.[34] We quantified contractions by radio telemetric EMG. For extensive description and technical details of this technique, please refer to our earlier publications.[4,6,7,23] Data were acquired with AcqKnowledge software (Biopac Systems Inc., Goleta, CA) and then analyzed. Each 20 sec distension period and its preceding 20 sec of baseline recording were extracted from the original raw data, corrected for movement and breathing, rectified and integrated. Absolute datasets were then obtained by subtracting the 20 sec baseline recording from the 20 sec distension result. Similar to our earlier publications, final results were evaluated from normalized data sets, which were calculated from the absolute data by setting the value of the first 2 mL distension at 100%. The area under the curve of these relative responses was calculated for individual rats and used for statistical analyses.

Colonic distension protocol

Distensions were performed at the minimum age of 4 months with a latex balloon (Ultracover 8F, International Medical Products BV, Zutphen, The Netherlands) and carried out as described previously.[4,6,7,23] In short, the balloon catheter was inserted under short isoflurane anesthesia and, after 20 minute recovery, colonic distension was 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 sec distension period, water was quickly removed and an 80 sec resting period was observed.

Post water avoidance (WA) miltefosine treatment in the maternal separation model

In humans, early adverse life events are associated with IBS at adult age and stress is a trigger for visceral hypersensitivity in IBS patients.[35,36] These features of IBS are mimicked in the maternal separation model where Long Evans rats are subjected to neonatal maternal separation, followed by an acute WA-stress at adult age. The combined insults were shown to result in post stress visceral hypersensitivity to colorectal distension that is not observed when nonhandled rats are subjected to WA.[23] For the current experiments, dams were separated from their pups for a period of 3 hours per day from post-natal day 2 to 14. During separation, the dam was placed in a different room while the litter remained in its own cage, placed upon a heat mat to maintain body temperature of the pups. Nonhandled control rats were nursed normally. Pups were weaned at postnatal day 22, and further experiments were carried out at a minimum age of 4 months. Distension protocols were performed pre-WA, 24 hours post-WA and post 7 days treatment. Miltefosine (JADO Technologies, Dresden, Germany) was dissolved in demineralized water and administered once daily by oral gavage, starting 30 minutes after the first post-WA distension protocol. Maternally separated rats received vehicle or 0.1, 1 or 10 mg miltefosine/kg daily, nonhandled rats received vehicle or 10 mg miltefosine/kg daily. For 1 hour WA stress at adult age, rats were positioned on a pedestal attached to the bottom of a Plexiglass tank that was filled with water within 1 cm of the top of the pedestal. A schematic representation of these experiments is given in Figure 1A.

Prophylactic miltefosine treatment in a rat model of intracolonic capsaicin

Investigations in the maternal separation model and IBS patients showed the relevance of the TRPV1 cation channel for visceral hypersensitivity.[7,8] In an earlier investigation, we used the specific TRPV1-antagonist SB-705498 to show that intracolonic capsaicin-induced visceral hypersensitivity is strictly TRPV1 dependent.[22] Here we used the same capsaicin model to address the possible TRPV1 modulating capacity of miltefosine. After performing a baseline distension protocol at day 0, miltefosine (10 mg/kg) or vehicle were administered daily per oral gavage. A second distension protocol was then carried out at day 6. Subsequently, capsaicin (Sigma-Aldrich, St Louis, MO, USA) was administered under short isoflurane anesthesia. First we applied Vaseline (Boots Healthcare, Hilversum, The Netherlands) to the perianal area to avoid stimulation of somatic areas. Next, 100 µL 0.1% capsaicin (dissolved in 10% ethanol, 10% Tween-80, 80% saline) was given through a fine cannula with a rounded tip inserted rectally, 2 cm from the anus. Animals were allowed to recover

for 90 min, after which the last distension protocol was performed. A schematic representation of the intracolonic capsaicin protocol is given in Figure 4D.

DNA extraction

In our previous investigations we compared the fecal mycobiome of nonhandled and maternally separated rats and observed profound differences between groups.[4] In the present investigations we compared the myco- and microbiome of maternally separated rats treated with either vehicle or miltefosine (10 mg/kg daily). DNA was isolated from fecal pellets that were collected directly from the anus on day 8 of the treatment protocol and stored at -80 ºC until use. The DNA isolation procedure was carried out as described earlier.[4]

Sequencing of fungal ITS amplicons and visualization of results

Barcoded fungal internal transcribed spacer regions (ITS) amplicons were generated using a two-step PCR approach identical to our previous investigations.[4] In short, fungal ITS 1 regions were first amplified with the following primers: forward 5'-CTTGGTCATTTAGAGGAAGTAA-3' and reverse 5'- GCTGCGTTCTTCATCGATGC-3. Next a second set of primers was used to generate fungal ITS-1 fragments that included overhanging adapter sequences for compatibility with Nextera XT tagmentation: next-ITS-BITS-F: TCGTCGGCAGCGTCACCTGCGGARGGATCA and nex-ITS-B58S3-R GTCTCGTGGGCTCGGGAGATCCRTTGYTRAAAGTT (adapted from Bokulich & Mills[37]). 5 µl of the previous PCR products were amplified for 10 cycles with an annealing temperature of 49 °C. Reactions were then cleaned and dual barcodes (8 bp) and Illumina sequencing adapters were attached using the Nextera XT Index Kit (Illumina, San Diego, CA) according to manufacturer's protocols. Barcoded amplicons were quantified, normalized to the same concentrations, pooled, and gel purified using the Qiaquick spin kit (Qiagen). Pooled amplicons were 250-bp paired-end sequenced using the MiSeq system (Illumina).

Raw Illumina fastq files were demultiplexed, quality filtered, and analyzed using modules implemented in the Mothur software platform.[38] Unique sequences were taxonomically classified by the RDP-II Naïve Bayesian Classifier[39] using a 60% confidence threshold against the Mothur formatted UNITE Database[40] (Version No. 7). Distances between ITS compositions were visualized by classical clustering based upon the Bray-Curtis dissimilarity index and the UPGMA algorithm and with non-metric multidimensional scaling plots based upon the same dissimilarity index with the bio-statistical program PAST v3.034. One way permutational multivariate analysis of variance (PERMANOVA) was done on the resulting Bray-Curtis Bray Curtis dissimilarities. Raw sequence data generated in this study will be deposited in the European Nucleotide Archive.

Bacterial 16 S sequencing and visualization of results

The hypervariable V4 region of the 16S-rRNA gene of the rat DNA was amplified, sequenced by Illumina MiSeq (Illumina Inc., San Diego, CA, USA) and processed using modules implemented in the Mothur software platform, version 1.31.2 and Btrim.[38, 41] First, reads were checked and quality trimmed (quality threshold 30) by using the 'Btrim' command. Next, read pairs were merged ('make.

contigs'), and merged reads with a length of 240-260 base pairs were aligned ('align.seqs') to a SILVA reference database. Visualization based upon Bray-Curtis dissimilarity was carried out analogous to ITS visualization described above.

Agar disk diffusion assay to address antifungal and antibacterial properties of miltefosine

Agar disk diffusion assays were used to confirm antifungal and antibacterial properties of miltefosine, which was tested at 4 different concentrations (50, 250 and 1000 µM and 10 mM). For the antifungal assay, an aliquot of an overnight culture of *C. albicans* was cultured in Sabouraud broth for 3 hours, diluted in top agar (10^5 counts in 0.6% agarose in Sabouraud) and spread in 20 mL aliquots in petri dishes. After solidification, 3 mm holes were punched and filled with 8 µl miltefosine, vehicle or the fungicide nystatin that was used as positive control. Plates were incubated 24 to 48 hour before the halo of inhibition, visible as a zone of clearing around a punched hole, was measured with a caliper (diameter in mm). For the antibacterial assay, an aliquot of an overnight culture of *B. subtilis* was cultured in 3% tryptic soy broth (TSB) to an OD of 0.800 and then diluted 10 times in 0.3% TBS/1% Agar/0.02% Tween. 15 ml aliquots of this suspension were spread in petri dishes. After solidification, 3mm holes were punched and filled with 8 µl miltefosine, vehicle or a broad spectrum antibiotic mixture containing penicillin and streptomycin (1000 units/ml and 1000 µg/ml respectively) that was used as positive control. After diffusion into the plates (3 hours at 37°C), dishes were covered with a top layer (6% TBS/1% agar) and further incubated for 18 to 24 hours where upon the diameter of the inhibition halo was measured.

Semi quantitative assessment of *in situ* mast cell activation status

Mucosal mast cells were stained following a staining protocol described by Wingren and Enerbäck.[42] As the distension took place in the distal part of the colon, paraffin sections were cut from proximal colon (thickness 4µm) in order to rule out any role for distension-induced mast cell degranulation. Sections were processed for staining and incubated for 6 days in Toluidine Blue in 0.5N HCL (pH=0.5). To semi-quantitatively assess mast cell activation, every 5th section, with a total of 5 sections per rat, was evaluated in a double-blind manner. A total of 50 mast cells were counted per section and each mast cell was categorized as either dark staining intensity, medium staining intensity or light staining intensity (example stainings are shown in Figure 3A). Results of the 5 individual sections were averaged per rat to give a representative outcome. Final results are given as a percentage per category of total mast cells counted.

Assessment of *in vitro* TRPV1 activation and modulation, by fluorimetric measurement of intracellular free calcium

Capsaicin-induced activation of TRPV1 and modulation thereof was monitored with the help of the Indo-1 AM calcium indicator (Invitrogen, Bleiswijk, the Netherlands). In short, TRPV1 recombinant SH-SY5Y human neuroblastoma cells (SH-SY5Y$_{hTRPV1}$, kindly provided by GlaxoSmithKline, Stevenage, UK)[43] were detached with the aid of trypsin free cell dissociation buffer (Gibco, Bleiswijk, The Netherlands), resuspended in HEPES-buffered HBBS and incubated with 10µg/ml Indo-1 AM (30 minutes, 37°C). Next, cells were washed and rested (30 minutes, room temperature) to allow

complete de-esterification of intracellular esters. Hereafter, cells were re-suspended to 10^7 cells/ml, supplemented with 1.2 mM $CaCl_2$ and allowed to adapt to 37°C for 10 minutes. Miltefosine (50, 70, 100 µM) was added 10 minutes before stimulating cells with capsaicin (32 nM, Sigma-Aldrich, St Louis, MO, USA). Similar to our earlier investigations,[7,22] we used the selective TRPV-1 antagonist SB-705494 (5 µM, kind gift of GlaxoSmithKline)[25] as positive control for TRPV1 inhibition. Optimal dosage of capsaicin was first established using wildtype SH-SY5Y and SH-SY5Y_{hTRPV1} cells. Analyses were performed with NOVOstar analyzer (BMG Labtech GmbH, Offenburg, Germany; excitation, 320 nm; emission, 405 nm and 520 nm). Cytosolic free calcium/calcium influx is represented as the change in fluorescence at 405 nm divided by that at 520 nm=Δ405/520.

Statistical analysis

Statistical analysis was performed using GraphPad Prism (version 7.03, Graphpad software, San Diego, USA). All data, excluding myco- and microbiome compositions, were tested for normality using D'Agostino & Pearson normality test. Visceromotor response data were analyzed with the Repeated Measures one-way ANOVA, and tested post-hoc with Sidak's multiple comparisons test. Anti-microbial activity and calcium measurement data were analyzed with one-way ANOVA and Dunnett's multiple comparisons post hoc test. Toluidine Blue staining intensity data were analyzed with the Mann Whitney U test, comparing the percentages of mast cells in each group.

Results

Miltefosine treatment reverses post-stress visceral hypersensitivity in maternal separated rats

To address the possible therapeutic potential of miltefosine, we first evaluated its effects in the rat maternal separation model (experimental setup depicted in Figure 1A). Similar to our previous investigations,[4,6,7,22,23] WA-stress at adult age did not lead to enhanced visceromotor response to colorectal distension in nonhandled rats (Figure 1B, day 0 vs day 1). Moreover, 7 days post-WA treatment with vehicle or miltefosine (10 mg/kg/day) did not change the visceral sensitivity status of these nonhandled animals (day 1 vs day 8). Compared to pre WA, all 4 maternal separation groups showed enhanced visceromotor response to distension after WA-stress (Figure 1C, day 0 vs day 1). This response was reversed after 7 day treatment with 1 mg miltefosine/kg (day 1 vs day 8), but not by vehicle, 0.1 and 10 mg miltefosine/kg. Lack of significant reversal in the 10 mg/kg treatment group may be due to relatively low post-stress visceral sensitivity at day 1.

Miltefosine inhibits *in vitro* growth of Candida albicans and Bacillus subtilis

Fungicidal and bactericidal activity may be relevant to the observed miltefosine-induced reversal of post-stress visceral hypersensitivity in maternal separated rats. Therefore, we carried out radial diffusion assays to confirm antibiotic activity. In *C. albicans* seeded agar, the inhibitory effect of nystatin justified the use of this assay as an anti-fungal readout (Figure 2A). Compared to vehicle (i.e. phosphate-buffered saline; PBS), 250 µM, 1000 µM and 10 mM miltefosine induced a dose

dependent inhibition of fungal growth. *B. subtilis* seeded agar was then used to evaluate possible bactericidal activity. The positive control, a penicillin/streptomycin mixture, as well as 1 mM and 10 mM miltefosine induced significant growth inhibition (Figure 2B). Taken together, these results confirmed earlier reports on the anti-fungal and anti-bacterial activity of miltefosine.[13,21]

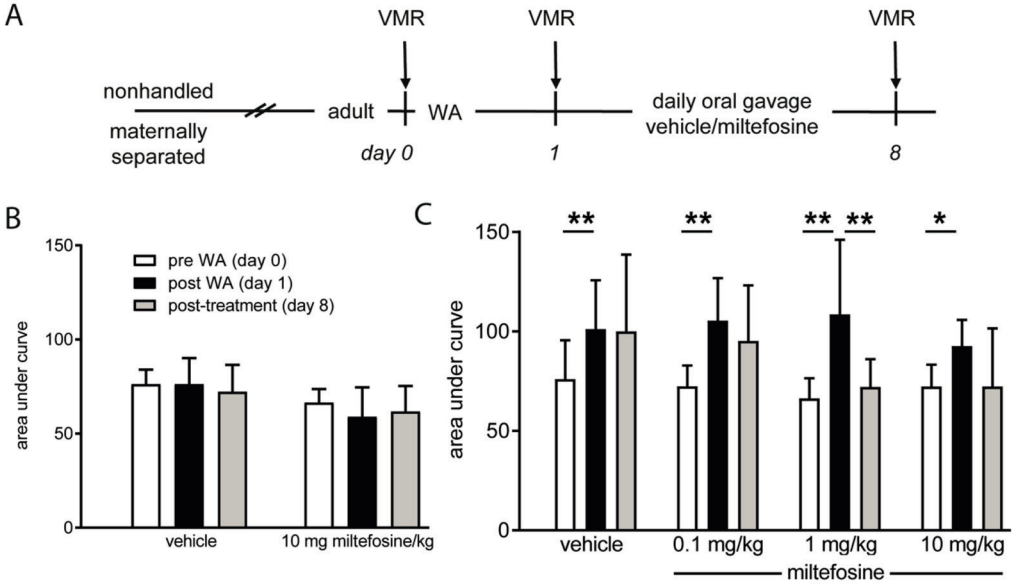

Figure 1. *Miltefosine treatment reversed post water avoidance (WA) visceral hypersensitivity in maternally separated rats. (A) Schematic representation of the experimental set-up; the visceromotor response (VMR) to distension was measured before and 24 hours after WA, and after 7 day miltefosine or vehicle treatment. Data shown in histograms (B) and (C) reflect results of nonhandled and maternal separated rats respectively. Data are given as area under the curve of the relative response to colorectal distension. All data are mean ± SD, n=7–10, *P<0.05 and **P<0.01 (Repeated Measures one-way ANOVA, Sidak's post hoc test).*

Miltefosine treatment modulates the intestinal microbiota in maternal separated rats

The *in vitro* fungicidal and bactericidal properties of miltefosine prompted us to explore whether successful miltefosine treatment in the maternal separation model associated with intestinal myco- and microbiome differences. We performed high-throughput rDNA sequencing of fungal ITS-1 and bacterial ribosomal 16S genes. Amplicons were generated with DNA isolated from fecal pellets of vehicle treated and miltefosine (10 mg/kg/day) treated maternal separated rats, obtained on day 8 post treatment. One DNA sample of the vehicle group did not generate sufficient amount of sequencing reads for mycobiome analysis.

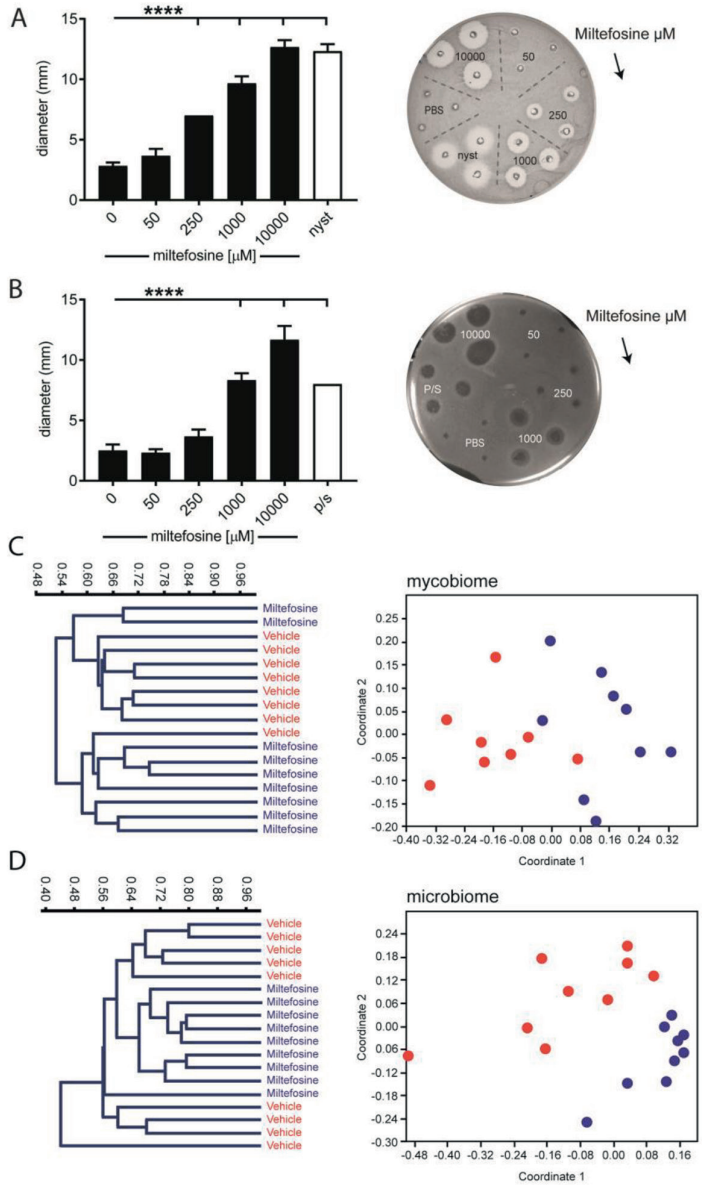

Figure 2. *Miltefosine induced in vitro C. albicans and B. subtilis growth inhibition and in vivo differences in post-treatment myco- and micro-biome composition. Right side photographs show agar disk diffusion assays with C. albicans (A) and B. subtilis (B). Arrows indicate direction of miltefosine concentration series (50, 250, 1000 and 10.000 µM). Controls: phosphate buffered solution (PBS), nystatin (nyst) and penicillin/streptomycin (p/s). Histograms A) and (B) show the average diameter of resulting halo's (mean ± SD, n=3, ****P<0.0001, one-way ANOVA and Dunnett's post hoc test). Visualization of the fecal myco- and microbiome of maternally separated rats subjected to vehicle or miltefosine is shown in (C) and (D) respectively. The Bray-Curtis dissimilarity index was used to generate the left side dendrograms and right side non-metric multidimensional scaling plots.*

Hierarchical clustering based on the Bray-Curtis dissimilarity index and the UPGMA algorithm was performed on classified fungal species. The resulting dendrogram (left panel Figure 2C) showed two main clusters. The upper cluster contained 7 (out of 8) vehicle treated and 2 (out of 9) miltefosine treated maternally separated rats. The lower cluster contained 1 vehicle treated and 7 miltefosine treated maternally separated rats. Similar results were obtained by non-metric multidimensional scaling (right panel Figure 2C).

Spatial patterns obtained with this ordi nation technique revealed two diffuse but separate clusters for vehicle and miltefosine treated rats. One way PERMANOVA multivariate statistics indicated a significant difference between groups *(P*=0.0009, F=6.6). To compare the bacterial microbiome of maternally separated rats treated with either miltefosine or vehicle, we again performed clustering based on the bray Curtis dissimilarity index and UPGMA algorithm. Compared to the mycobiome analysis, the resulting dendrogram (left panel Figure 2D) showed less clear separation into treated and untreated clusters. Nevertheless, non-metric multidimensional scaling (right panel Figure 2D) revealed differential spatial patterns for the two treatment groups. One way PERMANOVA showed significant difference between groups (*P*=0,0001, F=4.1). Collectively, our data suggest that miltefosine treatment modulates both the fecal myco- and microbiome.

Miltefosine affects *in situ* mast cell staining intensity in colonic mucosa

Although the *in vivo* effect of miltefosine may depend on mycobiome and microbiome modulation, a direct effect on mast cells may also be relevant.[14-17] We performed Toluidine Blue stainings on colonic mucosa of vehicle and miltefosine treated maternal separated rats, and assessed differences in mast cell (granule)-staining intensity as indirect measure for *in vivo* mast cell degranulation (example stainings in Figure 3A). Upon miltefosine treatment, we observed higher number of darkly stained mast cells and lower number of mast cells with medium staining intensity (Figure 3B). These data suggest that post-stress mast cell degranulation was partly prevented by miltefosine treatment.

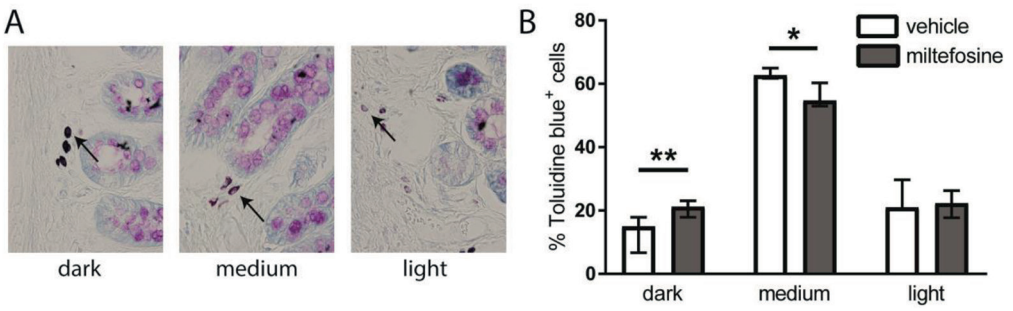

Figure 3. *The % of intensely stained mast cells was higher in miltefosine treated tissues. Arrows in left side photographs (A) indicate representative examples of different mast cell staining intensities obtained with Toluidine Blue. (B) shows % mucosal mast cells per staining intensity when comparing tissue sections of miltefosine and vehicle treated maternal separated rats. Data are in median & range, *P<0.05, **P<0.01 (Mann-Whitney U test).*

Miltefosine affects *in vitro* TRPV1 activation by capsaicin

The TRPV-1 ion channel, sensitized via the histamine-1 receptor, is an essential nociceptor in the rat maternal separation model and IBS patients.[6-8] Because the opening properties of TRPV1 are lipid raft dependent, the analgesic effect of miltefosine may partly result from altered TRPV1 gating.[20,24]

We first addressed this possibility in an *in vitro* model system. Comparing wildtype SH-SY5Y and TRPV1 transfected SH-SY5Y$_{hTRPV1}$ neuroblastoma cells, we showed dose-dependent capsaicin-induced increase in intracellular Ca^{2+} levels in TRPV1 transfected but not in wildtype cells (Figure 4A). Based on this experiment, the 32 nM capsaicin concentration was used in further investigations. To confirm strict TRPV1 dependence of the capsaicin response, SH-SY5Y$_{hTRPV1}$ cells were then pre-incubated with SB-705498.[25] This selective TRPV1 antagonist prevented the capsaicin-induced increase of cytosolic Ca^{2+} (Figure 4B). Next, SH-SY5Y$_{hTRPV1}$ cells were pre-incubated with different concentrations of miltefosine, which dose-dependently decreased capsaicin-induced TRPV1 activation (Figure 4C).

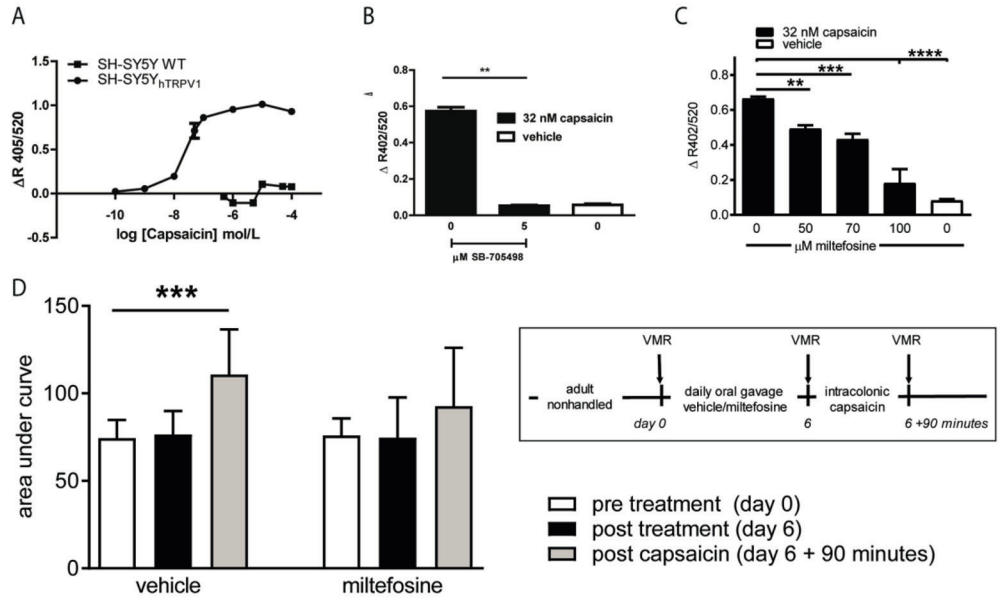

Figure 4. *Miltefosine interfered with in vitro and in vivo capsaicin-induced TRPV1 activation. (A) Intracellular free calcium levels in response to different dosages of capsaicin in wildtype- and TRPV-1 transfected SH-SY5Y human neuroblastoma cells. (B) Capsaicin-induced activation of SH-SY5Y$_{hTRPV1}$ cells in the presence of a specific TRPV1 antagonist (SB-705498). (C) Capsaicin-induced activation of SH-SY5Y$_{hTRPV1}$ cells, pre-incubated with different dosages of miltefosine (mean ± SD, **P<0.01, ***P<0.001, ****P<0.0001, one-way ANOVA, Dunnett's post hoc test). (D) Schematic representation of experiments performed in the intracolonic capsaicin model and results of colorectal distensions in this model. Results are given as area under the curve of the relative response to distension (mean ± SD n=9-10, ***P<0.001, Repeated Measures one-way ANOVA, Sidak's post hoc test).*

In vivo TRPV1 dependent visceral hypersensitivity is prevented by miltefosine treatment

TRPV1 activation is highly relevant in post stress visceral hypersensitivity of the maternal separation model.[7] However, from the results shown in Figure 1, it cannot be dissected whether or not TRPV1 was an *in vivo* target for miltefosine. Earlier, we administered intracolonic capsaicin to nonhandled Long Evans rats and showed that the resulting visceral hypersensitivity is strictly TRPV1 dependent.[22]

Here, we first subjected normal Long Evans rats to a 1 week miltefosine treatment protocol (gavage 10 mg/kg/daily) and then applied 0.1% intracolonic capsaicin. The experimental setup of the experiment is depicted on the right side of Figure 4D. In vehicle treated rats, capsaicin induced an enhanced response to colonic distension that was not observed when rats were pretreated with miltefosine. These findings suggest that miltefosine's *in vivo* mode of action may involve targeting of TRPV1.

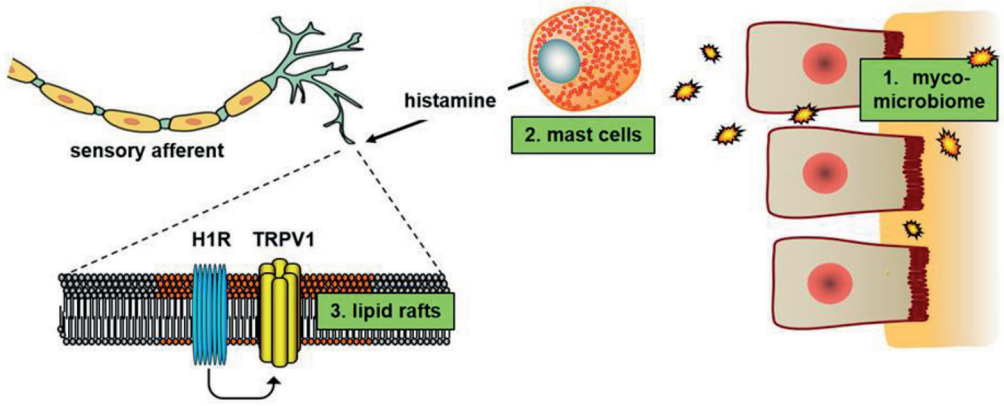

Figure 5. *Miltefosine-targets identified in the maternal separation model. Miltefosine affected the fecal myco- and microbiome (1), mast cell degranulation (2) and the TRPV1 ion channel (3). Effects on the histamine 1 receptor (H1R) may have been relevant as well but were not addressed in the current investigations.*

Discussion

Treatment of IBS is challenging due to the heterogeneous nature of the disorder and, perhaps as a result thereof, lack of truly efficacious therapies. In at least part of the IBS patients, abdominal pain may arise due to immune recognition of an aberrant gut mycobiome. In response, mast cells release histamine and, via the histamine 1 receptor, sensitize TRPV1 on afferent sensory neurons leading to abdominal pain.[4-8] In search for novel treatment options, we identified miltefosine as a candidate drug because it reversed post stress visceral hypersensitivity in the IBS-like rat model of maternal separation. In follow up experiments we showed that it may have targeted several of the different mechanisms leading to fungal-induced visceral hypersensitivity (schematic overview in Figure 5). In line with miltefosine's fungicidal and bactericidal activity, reversal of visceral hypersensitivity associated with altered gut microbiome composition. In addition, miltefosine affected stress-induced degranulation of mucosal mast cells. Finally, this drug inhibited TPRV1 activation in TRPV1 transfected neuroblastoma cells, and prevented *in vivo* capsaicin-induced TRPV1 activation and

resulting visceral hypersensitivity in normal rats. Thus, miltefosine may exert its analgesic effect by acting on 3 different levels of mycobiome-induced visceral hypersensitivity.

Similar to any other animal model, the maternal separation model in rat has its limitations when trying to mimic a complex and enigmatic disorder like IBS. Nevertheless, targets identified in earlier pre-clinical investigations, i.e. mast cells and the histamine 1 receptor, were successfully translated to human.[5-8] Therefore, we used this rat model to assess whether miltefosine should be a drug candidate to alleviate abdominal pain in IBS. Previous investigations showed fungicide-mediated reversal of post stress visceral hypersensitivity in maternal separated rats. Maternal separated and nonhandled Long Evans rats also differed in gut mycobiome composition, and fecal transfer experiments indicated that the observed mycobiome dysbiosis was relevant for visceral hypersensitivity.[4] Thus, compounds capable of inducing mycobiome changes may also affect visceral hypersensitivity. Indeed, miltefosine treatment led to reversal of hypersensitivity while the post treatment mycobiome of miltefosine- and vehicle treated rats differed. Whether these mycobiome associations were causally relevant cannot be deduced from the current data. Yet, the fluconazole/nystatin induced reversal of hypersensitivity that we showed earlier, also associated with compositional changes of the gut mycobiome.[4] In addition, the miltefosine findings are reminiscent of results obtained with a mixture of essential oils from *Mentha* x *piperita* L. and *Carum carvi*. The main components of these oils are menthol and (+)-carvone respectively. Both components were published to have fungicidal activity which we confirmed by agar disk diffusion assays.[26,27] Indeed, when maternal separated rats were treated with the oil combination, reversal of hypersensitivity associated with a shift in mycobiome composition.[28] In parallel with these essential oil results, miltefosine treatment not only led to fungal but also bacterial microbiome changes. Because previous experiments showed an essential role for immune recognition of fungal β-glucans, we suggest that the bacterial microbiome is not the main cause for visceral hypersensitivity in these animals.[4] Alterations of the bacterial microbiome may however also affect the gut mycobiome,[29] and it cannot be excluded that initial bactericidal effects of miltefosine led to secondary but relevant mycobiome changes.

In the maternal separation model, gut mycobiome dysbiosis is essential for the activation of mast cells which eventually leads to visceral hypersensitivity.[4,6,7] Others have shown that miltefosine is capable of inhibiting *in vitro* mast cell activation and successfully used this compound as a therapeutic intervention strategy for mast cell mediated diseases.[14-17] In order to further expand knowledge on possible *in vivo* targets of miltefosine we mainly focused on mechanisms and cell types other than mast cells. We did perform however *in situ* Toluidine Blue stainings that suggested a lower level of degranulation in miltefosine treated rats. Whether this was due to direct targeting of mast cells, or an indirect effect via microbiome modulation cannot be concluded from this limited evaluation. One mechanism via which miltefosine may have affected mast cells directly is via insertion into lipid rafts.[11,16,18] These rafts provide the optimal microenvironment for ligand receptor interactions and subsequent recruitment of cell signaling molecules. Moreover, in case of ion channels, lipid rafts can regulate channel function.[19,30] Using a selective TRPV1 antagonist, we previously showed an important role for this afferent expressed nociceptive cation channel in post stress visceral

hypersensitivity of maternal separated rats.[7] Others provided evidence that interactions between TRPV1 and lipid raft interfaces regulate its gating properties.[20,24] Indeed, our *in vitro* results showed that selective capsaicin induced TRPV1 activation can be inhibited by miltefosine treatment. The latter suggests that miltefosine may have targeted TRPV1 in the maternal separation model as well. Unfortunately however, we are unable to assess the relative contributions, if any, of miltefosine mediated mycobiome-, mast cell- and TRPV1 modulation in the maternal separation setting. Nevertheless, results obtained with the intracolonic capsaicin model confirmed that miltefosine is capable of interfering with *in vivo* TRPV1 activation. Concerning the role of this ion channel it is important to note that histamine 1 receptor ligation leads to sensitization of TRPV1 and subsequent visceral hypersensitivity in IBS patients.[8] Because sensitization depends on intracellular signaling pathways, it can be envisaged that TRPV1 and the histamine 1 receptor translocate to the same lipid rafts for optimal interaction. Although our previous investigations showed the relevance of the histamine 1 receptor in the maternal separation model,[6] we did not address whether miltefosine also interfered with this TRPV1 sensitization mechanism.

Because miltefosine is not specifically targeting TRPV1 containing lipid rafts, signaling pathways not necessarily relevant to abdominal pain may have been affected as well. The cell membrane however, holds many different types of highly dynamic and coexisting rafts with associated proteins[31] and miltefosine microdomain affinity may differ according to dissimilarities in composition. Although this suggests that not all raft assemblies and associated events were targeted to the same extent, unwanted side effects should be considered. During a 4 week, randomized, double-blind, placebo controlled trial, Magerl *et al.* tested the use of orally administered miltefosine in chronic spontaneous urticaria.[14] In this mast cell and histamine dependent skin condition, the urticaria-activity-score levels and number of weals were substantially more reduced in miltefosine treated patients when compared to placebo treatment. The highest daily treatment dose was 150 mg (average patient weight 84.2 kg), which resembles the 1 mg/kg dose used in the maternal separation model. Although no serious adverse events were reported, mild to moderate adverse events, including nausea and vomiting, were frequent in miltefosine and placebo treatment groups. In addition, beneficial effects of miltefosine over placebo were lost within four weeks after discontinuation of treatment. It can be envisaged that also in IBS long-term miltefosine treatment may be needed for a continued effect on TRPV1 dependent pain, but this is not preferable considering the unwanted side effects. Alternatively, miltefosine may be used as a lead compound for the development of drugs effectively modifying TRPV1 responses in the absence of side effects. Yet, this contradicts our original intension of repurposing an existing drug for IBS therapy. Knowing however that miltefosine targets the gut microbiome as well, we suggest that short term treatment should be considered in order to induce a favorable reset of the mycobiome. Our previous results suggested that this can also be achieved with fungicides like fluconazole and nystatin, which are clinically used to treat fungal infections.[4] Fungal resistance against these compounds is however on the rise, and using them for a non-lethal but highly prevalent disorder like IBS may further shorten their clinical life span.[32] Since this would lead to a further increase of infection related deaths, alternative compounds like miltefosine should be considered for IBS therapy. We suggest, that future clinical trials monitor post treatment

symptom improvement and duration thereof, and correlate these results to possible persistence of treatment-induced mycobiome changes.

Abdominal pain in IBS is an unmet clinical need, and development of novel drugs is highly time consuming and costly. The recent identification of novel targets made it possible to evaluate an existing non-selective but FDA approved drug with known safety profile. Treatments with so-called 'dirty drugs' are often avoided. However, off target effects can be used in a meaningful manner, because they enable repurposing of existing therapeutic compounds to other disorders.[9] Moreover, promiscuous drugs might be more effective than single target drugs.[9,33] In an animal model with proven predictive value for IBS, we showed that miltefosine changed the gating properties of the nociceptor TRPV1 and affected mast cell activation and the gut mycobiome. Our results suggest that miltefosine should be evaluated for the treatment of abdominal pain in IBS.

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Chapter 7

Dietary marine n-3 polyunsaturated fatty acids do not affect stress-induced visceral hypersensitivity in a rat maternal separation model

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Journal of Nutrition, 2015 May; 145(5): 915-22

Abstract

Background: Although never evaluated for efficacy, n-3 long-chain polyunsaturated fatty acids (LCPUFAs) are commercially offered as treatment for irritable bowel syndrome (IBS).

Objective: This study was designed to investigate, in a mast cell dependent model for visceral hypersensitivity, whether this pathophysiological mechanism can be reversed by dietary LCPUFA treatment, via peroxisome proliferator-activated receptor (PPAR)γ activation.

Methods: Maternally separated rats were subjected to hypersensitivity inducing acute stress at adult age. Reversal was attempted by protocols with tuna oil supplemented diets (4% soy oil (SO)/3% tuna oil (SO-T3) or 3% SO/7% tuna oil (SO-T7)), compared with control SO diets (7% or 10% SO respectively) 4 weeks after stress. PPARγ agonist rosiglitazone was evaluated in a 1-week preventive protocol (30 $mg.kg^{-1}.day^{-1}$). Erythrocytes were assessed to confirm LCPUFA uptake, and tissue expression of lipoprotein lipase and glycerol kinase as indicators of PPARγ activation. Colonic mast cell degranulation was evaluated by toluidine blue staining. *In vitro*, HMC-1 cells were pre-treated with rosiglitazone, eicosapentaenoic acid or docosahexaenoic acid, stimulated with phorbol 12-myristate 13-acetate (PMA) and Calcium Ionophore or compound 48/80 and evaluated for TNF-α and β-hexosaminidase release.

Results: Stress led to visceral hypersensitivity in all groups. Hypersensitivity was not reversed by SO-T3 or control treatment (pre-stress vs 24 hours post-stress vs. post-treatment area under curve; 76 ± 4 vs 128 ± 12 (P<0.05) vs 115 ± 14 and 82 ± 5 vs 127 ± 16 (P<0.01) vs 113 ± 19 respectively). Comparison of SO-T7 with its control showed similar results (74 ± 6 vs 103 ± 13 (P<0.05) vs . 115 ± 17 and 66 ± 3 vs 103 ± 10 (P<0.05) vs 117 ± 11 respectively). Erythrocytes showed significant LCPUFA uptake in the absence of colonic PPARγ activation. Rosiglitazone induced increased PPARγ target gene expression, but did not prevent hypersensitivity. Mast cell degranulation never differed between groups. Rosiglitazone, and LCPUFAs significantly reduced PMA/CI-induced TNF-α release but not degranulation of HMC-1 cells.

Conclusion: Dietary LCPUFAs did not reverse stress-induced visceral hypersensitivity in MS rats. Although further research is needed, claims concerning LCPUFAs as a treatment option in IBS cannot be confirmed at this point and should be regarded with caution.

Introduction

Dietary supplementation of long-chain n-3 polyunsaturated fatty acids (LCPUFAs) has been demonstrated to elicit potential health effects, ranging from decreased blood pressure in hypertension and cardiovascular disease to a decrease of inflammation in rheumatoid arthritis, inflammatory bowel disease, and asthma.[1] While n-3 fatty acids are thought to act in an anti-inflammatory manner, n-6 fatty acids are generally considered pro-inflammatory. The latter is in particular attributed to the role of n-6 arachidonic acid (AA) and its pro-inflammatory metabolites.[2] In humans consuming a typical Western diet, the membrane phospholipids of blood inflammatory cells contain significant amounts of AA (usually 10–20% of fatty acids), while the amounts of n-3 eicosapentaenoic acid (EPA) (usually 0.5–1% of fatty acids) and docosahexaenoic acid (DHA) (usually 2–4%) are much lower.[3-6] This shift towards n-6 PUFAs is thought to play a role in a variety of diseases,[7] and has recently been shown in female patients suffering from the Irritable Bowel Syndrome (IBS).[8] Presently, beneficial effects of LCPUFAs in IBS treatment are often discussed on patient internet forums and, despite the absence of scientific evidence for efficacy, LCPUFA preparations are sold online as a treatment option for this disorder (internet search: 'omega, IBS').

IBS is a gastrointestinal disorder that affects approximately 11% of the global population.[9] Patients experience abdominal pain or discomfort associated with changes in bowel habits without a structural explanation.[10] Although prevalence and socioeconomic impact of IBS are high, the pipeline for novel drugs, especially those targeting abdominal pain, is limited. Visceral hypersensitivity, measured by an enhanced response to colonic distension, is considered an important pathophysiological mechanism to explain abdominal pain. Indications are that stress-induced mast cell degranulation is an important trigger for visceral hypersensitivity.[11] Admittedly, most of these data were obtained in animal models and their validity for IBS is sometimes questioned.[12] However, in patient biopsies, mast cells were shown to be in closer proximity to colonic nerve endings, and this was shown to correlate with severity and frequency of abdominal pain.[13] Further, supernatants from patient-derived mucosal biopsies were shown to release mast cell mediators capable of activating intestinal nerves in *ex vivo* settings.[14,15] In a clinical trial, we have shown that treatment with the mast cell stabilizer and Histamine-1-Receptor (H1R) antagonist ketotifen successfully increased the threshold of discomfort (meaning lowered sensitivity to colonic distension) and decreased other symptoms in hypersensitive IBS patients.[16] Based on these trial results, we next evaluated the peripherally restricted H1R antagonists ebastine and fexofenadine in the rat maternal separation (MS) model for IBS. Both antihistaminics reversed post-stress visceral hypersensitivity in rats,[17] and more recently, van Wanrooij *et al.* translated these results into a successful double-blind randomized control trial.[18] In concert, these results suggest that the activation of colonic mast cells and subsequent activation of sensory afferents by mast cell mediators plays an important role in visceral hypersensitivity and IBS symptom generation.

Although current knowledge of the effects of n-3 LCPUFAs on mast cell activation is limited, dietary supplementation of n-3 LCPUFAs has proven beneficial in several other diseases in which mast

cells are thought to be relevant, e.g., rheumatoid arthritis, asthma, and allergic disorders.[1-3,7,19,20] In addition, in *in vitro* experiments, treatment with different n-3 LCPUFAs, among which are the marine n-3 EPA and DHA, reduced both the production and release of a variety of mast cell mediators.[21-23] Immune modulatory actions of n-3 LCPUFAs are elicited via several mechanisms, including competition with AA for the 2-acyl position of membrane phospholipids, thereby influencing concentrations of metabolite and/or hormone that regulate behavior of cells. In addition, n-3 LCPUFAs activate the nuclear peroxisome proliferator-activated receptor (PPAR)-γ, which negatively regulates inflammatory cytokine production by interfering with the activation of transcription factors.[1] Importantly, PPARγ agonists also reduce the production and release of histamine by mast cells,[24,25] and, in the rat MS model, we showed reversal of post-stress visceral hypersensitivity with two different histamine-1-receptor antagonists.[17] These data indicate that dietary n-3 LCPUFA supplementation may prove beneficial, possibly via PPARγ-mediated mast cell modulation, in IBS. Given the current internet attention and premature commercialization of the LCPUFA hypothesis in relation to IBS, preclinical studies addressing this hypothesis are eagerly awaited. Thus, we investigated whether dietary supplementation with marine n-3 LCPUFAs, in the form of tuna oil, can reverse post-stress hypersensitivity to colonic distension in the maternal separation model.

Material and methods

Ethics statement

All procedures were conducted in accordance with the institutional guidelines and were 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 bred and housed at the animal facility of the Academic Medical Center (Amsterdam, The Netherlands) under conditions of controlled light (06:00–18.00 h), temperature (20–22°C) and humidity (45%). Water and food were available *ad libitum*.

Maternal separation

In earlier papers,[26-28] we demonstrated that Long-Evans rats, when predisposed by MS, showed an enhanced response to colorectal distension upon acute stress (i.e., water avoidance; WA) later in life. In contrast, when non-handled rats were subjected to the same acute stressor at adult age, they did not become hypersensitive to distension. In the present investigation, we only evaluated MS animals. On postnatal day 2, female pups were removed from the litter and dams were separated from remaining pups for 3 hours daily for a 12-day period (postnatal day 2 to 14) as described earlier.[28]

Measurement of the visceromotor response to colonic distension and data analysis

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. During distensions, pain scores in patients are often evaluated by self-rating questionnaires (i.e., visual analog score) that cannot be assessed in rats. However, colorectal distension in rats leads to reproducible contractions of abdominal musculature, the so-called visceromotor response (VMR), and the quantification of these contractions by electromyography (EMG) is often used to assess visceral pain. In the present investigation, we assessed abdominal EMG signals during distension protocols (1, 1.5, and 2 mL) by radiotelemetry. For details on colonic distensions, telemetric-tracing methodology, and data analysis, please refer to earlier papers.[17,26-28]

Experimental protocol and diet composition

The soy oil source of cow's milk protein-free AIN-93G diet[29] (Research Diet Services, Wijk bij Duurstede, The Netherlands) was partially replaced by tuna oil (provided by Bioriginals, den Bommel, The Netherlands), the fatty acid composition of which is shown in Table 1.

Table 1. *FA composition of tuna oil batch used to partially replace the soy oil source of the AIN-93G diet in SO-T3 and SO-T7 diets (SO-T3, 4% soy oil and 3% tuna oil; SO-T7, 3% soy oil and 7% tuna oil. Abbreviations: DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; MUFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids; SFA, saturated fatty acids.*

Tuna Oil Fatty Acid Composition	
fatty acids	**Value (g/100 g fatty acid)**
SFA	27.8
MUFA	20.4
PUFA	39.4
n-6 PUFAs	5.6
n-3 PUFAs	33.8
EPA	6.2
DHA	24.7

Rats (n=10) were fed a 4% soy oil/3% tuna oil (SO-T3) diet and rats in the control group (n=8) were fed a 7% soy oil (SO) diet. A third group of rats (n=9) received a 3% soy oil/7% tuna oil (SO-T7) diet and its control rats (n=8) were fed a 10% SO diet. In the latter two groups, an increase in the total fat percentage was needed to meet the requirements for an adequate amount and balance of the essential fatty acids ALA and linoleic acid;[29] an appropriate amount of cornstarch was replaced by extra fat. Diets were stored at -20°C and refreshed weekly. Rats of all groups were fed with tuna-oil-enriched or control diet post-WA only. Prior to dietary LCPUFA, intervention rats were fed with standard CRM-expanded diet (CRM-E; Special Diet Services, Technilab BMI, Someren, The Netherlands). Proximate composition of the CRM-E diet was moisture 10%, crude oil 3.4%,

crude protein 18.6%, crude fiber 4.1%, ash 5.8%, and nitrogen-free extract 57.6%. Distensions and concurrent EMG recordings were performed 30 min prior to WA-stress, 24 hours post-WA and on day 2, 8, and 28 or 29 post-WA. During WA, rats were positioned on a pedestal surrounded by water for 1 hour.[28]

Because symptoms are already present in IBS patients, we usually prefer post-WA treatment, aiming to reduce visceral hypersensitivity. However, when testing the possibility of activating PPARγ to modulate visceral hypersensitivity, we chose a pre-stress treatment with a pharmacological PPARγ agonist to reach maximal efficacy. Rosiglitazone (30 $mg \cdot kg^{-1} \cdot day^{-1}$) (Sigma-Aldrich, Zwijndrecht, The Netherlands) was added to ground CRM-E chow diet, which was then converted into pellets. For control rats, pellets were made from non-supplemented CRM-E chow. Rats were fed these diets for a 7-day period pre-WA. In this experiment, distensions and concurrent EMG recordings were performed prior to dietary treatment, 30 min prior to WA-stress, and 24 hours post-WA.

Erythrocyte fatty acid profile

Immediately after rats were killed humanely by CO_2 inhalation, blood was collected by heart punction in lithium-heparin-coated blood tubes. Plasma and cells were separated by centrifugation. Erythrocytes were stored at -80°C until analysis. Lipids were extracted as described by Bligh and Dyer.[30] The relative membrane fatty acid composition was analyzed by gas chromatography as described previously.[31]

Histochemistry

Mucosal mast cells were stained following a staining protocol described by Wingren and Enerbäck.[32] Distal colon paraffin sections (thickness: 4 µm) were incubated for 6 days in toluidine blue in 0.5 M HCl (pH=0.5). To semi-quantitatively assess mast cell activation, every fifth section, with a total of five sections per rat, was evaluated in a blinded manner. A total of 50 mast cells were counted per section, and each mast cell was categorized as either dark staining intensity, medium staining intensity, or light staining intensity, with lightly stained mast cells indicating degranulation and intensely stained cells representing non-degranulated mast cells.

RT-qPCR

Total RNA was isolated from white adipose tissue or the distal colon of rats treated with rosiglitazone and vehicle, or the SO-T7 and its control SO group, respectively, by using TriPure (Roche Diagnostics, Almere, The Netherlands) and following the manufacturer's protocol. Following DNAse treatment, cDNA was obtained using the RevertAid First Strand cDNA Synthesis Kit (Fermentas, Burlington, Ontario, Canada) Quantitative PCR was performed with SYBR Green in a LightCycler480 system (Roche) using a default 60°C program. Primer pairs used are detailed in Supplemental Table 1.

***In vitro* mast cell TNF-α release and colonic TNF-α, Il-1β, and Il-6 levels**

PPARγ activation is known to interfere with NFκB signaling and TNF-α is one of the genes regulated by NFκB activation.[33,34] Therefore, and since mast cells are a major source of intestinal TNF-α,[35] in order

to evaluate the effects of EPA, DHA, and rosiglitazone on mast cell cytokine release, we used TNF-α as the prototype NFκB-dependent cytokine. We evaluated TNF-α in HMC-1 cell culture supernatants. HMC-1 cells (human mast cell line, kindly provided by Dr. Butterfield[36]) were pre-treated with rosiglitazone, DHA, or EPA, which were all dissolved in DMSO, for 30 minutes. Concentrations used ranged from 0 to100 μM for rosiglitazone and 0 to 50 μM for DHA and EPA where indicated. Controls were pre-treated with equal volumes of DMSO alone (1% final concentration). Next, cells were stimulated with a combination of PMA (25 $ng \cdot ml^{-1}$) and calcium ionophore A23187 (CI, 0.5 μM) for 4 hours. The different EPA and DHA experimental conditions were tested in triplicate, rosiglitazone conditions in sixplicate. Supernatant was analyzed by using a human TNF-α ELISA (R&D Systems, Abingdon, United Kingdom). In addition, rat distal colons were homogenized in Green Berger Lysis buffer with added protease inhibitor cocktail (Sigma-Aldrich, Zwijndrecht, Nederland) by using Tissue Lyzer (Qiagen, Venlo, Netherlands). Cytokine levels were determined by rat TNF-α, Il-1β, and Il-6 ELISAs (R&D Systems, Abingdon, United Kingdom).

In vitro mast cell degranulation

As a readout for mast cell degranulation, we used the β-hexosaminidase assay that is often used as a marker enzyme for histamine-containing granules (described earlier in[23]). In short, HMC-1 cells were pre-treated with different concentrations of rosiglitazone (0–100 μM), DHA (0–50 μM), or EPA (0–50 μM), and then stimulated with compound 48/80 (1 $mg \cdot ml^{-1}$; Sigma-Aldrich, Zwijndrecht, the Netherlands). The different EPA and DHA experimental conditions were tested in sixplicate, rosiglitazone conditions in nineplicate. Upon centrifugation, supernatants were collected and remaining cell pellets were lysed with Triton X-100. Supernatants and cell pellets were then incubated with 4-methylumbelliferyl glucosaminidase, and release of fluorescent 4-methylumbelliferone was measured as the readout for β-hexosaminidase activity (release calculated as a percentage of total cellular content).

Statistical analysis

Analysis was performed using SPSS for Windows (version 16.0; SPSS Inc., Chicago, IL, USA). The normality and homogeneity distribution of data were assessed with Bartlett´s test. Data concerning the visceromotor response to colonic distension and *in vitro* β-hexosaminidase and TNF-α release by HMC-1 cells were subjected to log transformation and analyzed statistically using two-way repeated measures analysis of variance (ANOVA), followed by Bonferroni's multi-comparison *post hoc* test. Data regarding RT-qPCR, toluidine blue mast cell staining, *in vivo* cytokine levels, and fatty acids were analyzed using the Mann-Whitney U test for independent samples. In all cases, differences were considered to be significant when $P \leq 0.05$. Data in all figures are presented untransformed as the mean ± SEM.

Results

Effects of dietary n-3 LCPUFAs on visceral hypersensitivity and mucosal mast cell activation

WA stress significantly increased the visceromotor response to colorectal distension in all experimental and control groups (Figure 1A and C). Similar to SO control diets, dietary treatment with the SO-T3 (Figure 1A) or the SO-T7 diet (Figure 1C) did not reverse post-WA hypersensitivity. Colonic tissue sections were used to establish mast cell activation status in a semi-quantitative manner by evaluating toluidine blue staining intensity of mucosal mast cells. When comparing tuna oil diets with respective control diets, we observed no differences between the percentages of cells scored in the three staining intensity categories (Figure1B and D).

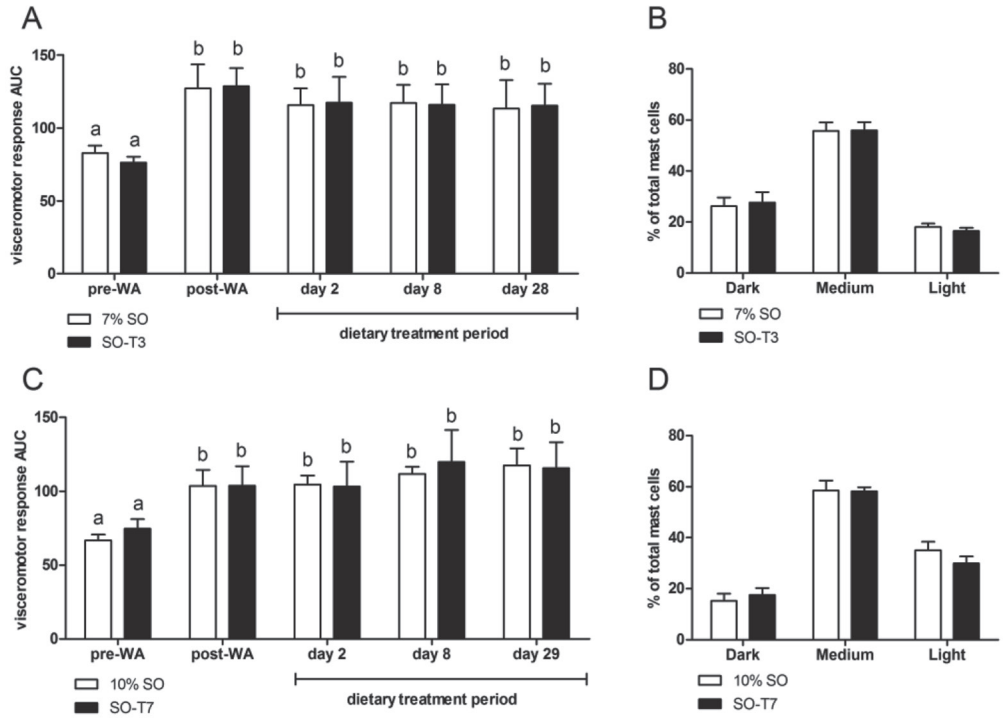

Figure 1. *Visceromotor response and mast cell degranulation status of rats treated with SO-T3 or SO-T7 or their respective control SO diets. VMR response data for SO-T3 and 7% SO control diets (A) and SO-T7 and 10% SO control diets (C) are presented as mean AUC ± SEM, n=8–10 rats. Means in the same treatment group labeled without a common letter differ: P<0.05. Different toluidine blue staining intensities of mucosal mast cells in distal colon are presented as percentage (%) of total stained cells. Comparisons were made between rats fed with SO-T3 (B) and SO-T7 diets (D) and their appropriate control groups. Abbreviations: AUC, area under curve; MS, maternally separated; SO, soy oil; SO-T3, 4% soy oil/3% tuna oil; SO-T7, 3% soy oil/7% tuna oil; VMR, visceromotor response; WA, water avoidance.*

LCPUFAs induce changes in erythrocyte fatty acid profile

Compared to those of rats fed with the control diet, the relative content of EPA (C20:5) and DHA (C22:6) in the erythrocyte membrane was significantly increased in rats fed with SO-T3 or SO-T7 diets (Figure 2A, B, D, and E). Importantly, in rats fed SO-T7, the relative content of AA (C20:4) in the erythrocyte membrane was significantly decreased (Figure 2F).

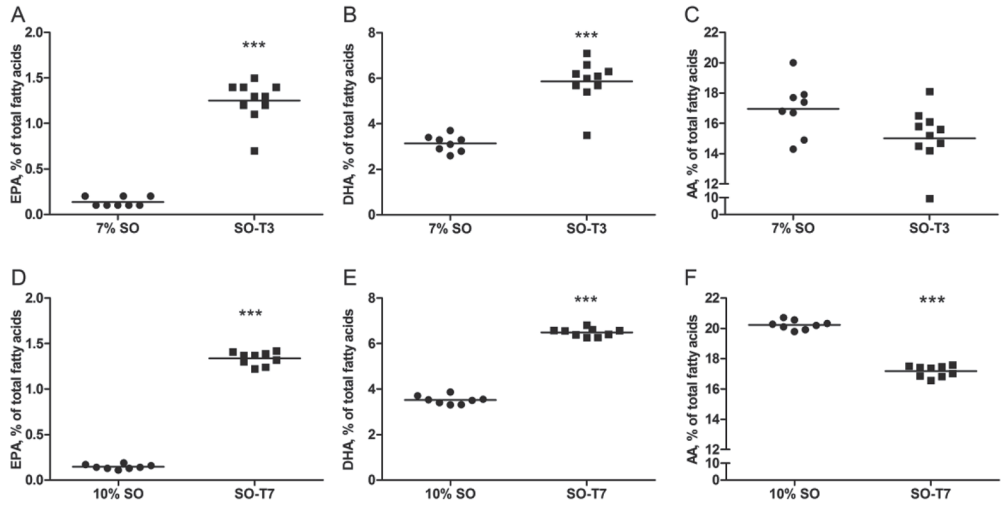

Figure 2. *Dietary LCPUFA-mediated changes in rat erythrocyte membrane fatty acid composition. Relative content of EPA (A and D), DHA (B and E), and AA (C and F) in erythrocyte membranes of rats fed SO-T3 (A, B, C), SO-T7 (D, E, F), or control SO diets. Data are presented as mean % EPA, DHA, or AA of total fatty acids ± SEM, n=8–10 rats, ***different from control diet, P<0.001. Abbreviatons: AA, arachidonic acid; DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; LCPUFA, long-chain n-3 polyunsaturated fatty acids; SO, soy oil; SO-T3, 4% soy oil/3% tuna oil; SO-T7, 3% soy oil/7% tuna oil.*

Effects of rosiglitazone-mediated PPARγ activation on visceral hypersensitivity and mucosal mast cell activation

Comparison of pre-treatment and pre-WA measurements did not show significant rosiglitazone-induced changes in baseline sensitivity to distension. Subsequent WA stress significantly increased the visceromotor response in rats fed with the rosiglitazone-supplemented diet, as well as control rats (Figure 3A). In accordance with these sensitivity results, treatment with rosiglitazone did not affect mucosal mast cell activation (Figure 3B).

Dietary LCPUFA and rosiglitazone induce mRNA expression of PPARγ target genes

We investigated the expression of lipoprotein lipase and glycerol kinase, two target genes of PPARγ that are involved in lipid metabolism. mRNA expression of both genes was significantly increased in

abdominal fat tissue of rats treated with rosiglitazone when compared to control animals (Figure 3C and D), but not in colonic tissue of rats fed with the SO-T7 diet (Figure 3E and F).

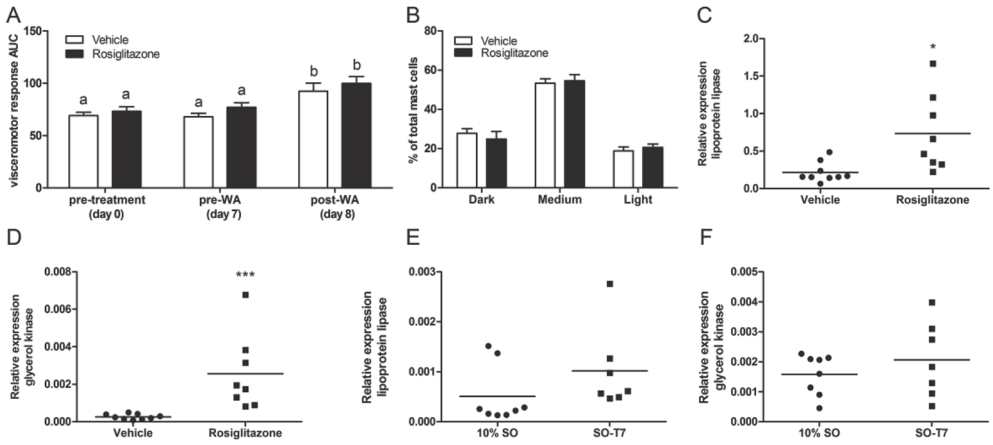

Figure 3. *Visceromotor response and mast cell degranulation status of rosiglitazone-treated rats plus PPARγ target gene expression in rosiglitazone-treated and SO-T7-treated rats. (A) VMR data of rosiglitazone-treated rats are presented as mean AUC ± SEM, n=9–10, Means in the same treatment group labeled without a common letter differ: P<0.05. (B) Toluidine blue staining intensities of mucosal mast cells in distal colon of rosiglitazone-treated rats are presented as percentage (%) of total stained cells ± SEM, n=9. Relative mRNA expression of PPARγ target genes lipoprotein lipase (C) and glycerol kinase (D) measured in rat adipose tissue of rosiglitazone-treated rats, and lipoprotein lipase (E) and glycerol kinase (F) in colonic tissue of SO-T7-treated rats; relative mRNA expression data presented as mean ± SEM, n=7–9, asterisks indicate differences compared with vehicle treatment *P<0.05, ***P<0.001. Abbreviations: AUC, area under curve; MS, maternally separated; PPARγ, peroxisome proliferator-activated receptor-γ; SO, soy oil; SO-T7, 3% soy oil/7% tuna oil; VMR, visceromotor response; WA, water avoidance.*

In vivo release of TNF-α, Il-1β, and Il-6 in the colon

When comparing colon homogenates of rats fed with control 10% SO diet to those fed with the SO-T7 diet, we observed no significant differences in TNF-α, Il-1β, or Il-6 levels (see Supplemental Figure 1).

In vitro modulation of mast cell mediator release by EPA, DHA, and rosiglitazone

Although rosiglitazone did not affect degranulation as measured by release of β-hexosaminidase (Figure 4A), ≥25 μM rosiglitazone (Figure 4D) did significantly inhibit release of TNF-α. Rosiglitazone treatment did not affect basal release of β-hexosaminidase or TNF-α in unstimulated cells. Treatment with different concentrations of EPA and DHA did not affect release of β-hexosaminidase in unstimulated and 48/80 stimulated cells (Figure 4B and C). Release of TNF-α, however, was

significantly reduced in DHA-treated PMA/CI stimulated cells (Figure 4F). Basal TNF-α release did not change in EPA- or DHA-treated cells.

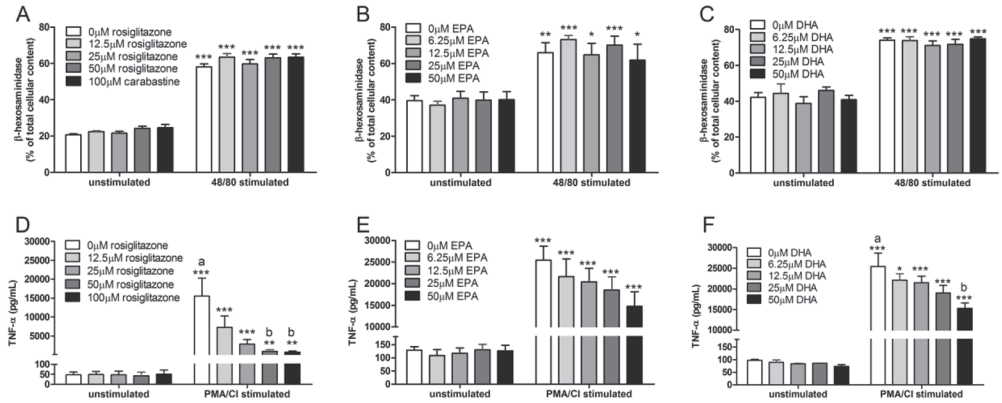

Figure 4. *In vitro effects of rosiglitazone, EPA, and DHA on a human mast cell line. Compound 48/80 induced β-hexosaminidase release by HMC-1 cells pre-incubated with rosiglitazone (A), EPA (B), and DHA (C). PMA/CI induced TNF-α release by HMC-1 cells pre-incubated with rosiglitazone (D), EPA (E), and DHA (F). Data is presented as mean ± SEM, n=3–9 per condition. Asterisks indicate differences compared with unstimulated controls; *P<0.05, **P<0.01, ***P<0.001. Means within a group labeled without a common letter differ (a–b P<0.01). Abbreviations: CI, calcium ionophore; DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; HMC-1, human mast cell line-1; PMA, phorbol 12-myristate 13-acetate.*

Discussion

Internet-based IBS patient groups and forums often discuss the use of LCPUFAs as a treatment option, and LCPUFA preparations are sold online for this purpose. However, although (pre)-clinical evidence exists for treatment efficacy in other disorders/diseases, such evidence is completely lacking for IBS. Since visceral hypersensitivity is a hallmark trait in the majority of patients and is thought to be a pathophysiological mechanism, we evaluated the use of marine LCPUFAs to reverse post-stress visceral hypersensitivity, possibly via mast cell modulation, in the rat maternal separation model. Our results suggest that LCPUFAs do not reverse existing stress-induced visceral hypersensitivity in this model.

Our previous animal experiments and a successful clinical trial with the mast cell stabilizer ketotifen indicated that mast cells may be an attractive target in IBS therapy,[16,17,26] and others have shown that LCPUFAs are capable of modulating mast cell mediator release.[21-23] In search for nutritional routes to modulate mast cell activation, and fueled by internet attention, we evaluated the use of marine n-3 LCPUFAs in our rat model. However, in contrast to earlier experiments in which we

successfully used the mast cell stabilizer doxantrazole[37] and the histamine-1-receptor antagonist ebastine[17] to induce reversal of post-stress visceral hypersensitivity in maternal separated rats, such reversal was not achieved by LCPUFA treatment. As mentioned earlier, one of the mechanisms by which n-3 LCPUFAs might elicit their beneficial effect is by competing with AA for the 2-acyl position within the phospholipid membrane, and thus interfering with the production of pro-inflammatory AA metabolites. Hence, an imbalance towards n-6 fatty acids and their metabolites is thought to play a role in a variety of inflammatory states. Importantly, an n-6/n-3 fatty acid imbalance was also shown in female IBS patients[8] and the maternal separation model.[38] In patients, the observed imbalance correlated with increases in the AA metabolites PGE_2 and LTB_4. As PGE_2 and LTB_4 are biologically active at very low concentrations, profound downstream effects can be induced by even small changes in AA levels.[39] Thus, similar to other diseases,[19,20] neutralizing the n-6/n-3 fatty acid balance might beneficially affect IBS symptoms. In our experiments, incorporation of EPA and DHA into the phospholipid membrane was assessed by monitoring the fatty acid profile of erythrocyte membranes. Although not specifically evaluated here, it is known that other cell types, including those of the immune system, also incorporate these fatty acids into their cell membranes.[3] Our data showed a profound increase in membrane DHA and EPA levels at the expense of AA, but without affecting post-stress hypersensitivity to colorectal distension. These results are in line with the study of Clarke *et al.*, in which plasma AA levels did not correlate with symptom severity in IBS patients.[8] Combined with the present data on LCPUFA supplementation, it may be concluded that increased AA levels are not a probable cause for IBS symptoms.

Another possible mechanism via which LCPUFAs reportedly modulate mast cells is via PPARγ agonistic activity.[1,24,25] Consequently, our negative *in vivo* results on visceral hypersensitivity reversal may suggest that the LCPUFA treatment protocol insufficiently activated PPARγ. Therefore, we used the pharmacological PPARγ agonist rosiglitazone as a positive control. Indeed, evaluation of the PPARγ target genes lipoprotein lipase and glycerol kinase indicated effective *in vivo* PPARγ activation by rosiglitazone that was not observed in the high-dose LCPUFA experiment. Nevertheless, rosiglitazone also did not affect visceral hypersensitivity in our model. Celinski *et al.* showed that the same rosiglitazone dosage (30 mg rosiglitazone·kg^{-1}·day^{-1}) was adequate to suppress inflammation and inflammatory cytokine response in DSS colitis.[40] Thus, we suggest that the inability of rosiglitazone to prevent the stress-induced increase in sensitivity was unlikely to be due to insufficient dosing. In relation to the tuna oil dosage scheme used in the present study, it should be mentioned that we previously used 6% tuna oil and 4% soy oil to successfully prevent allergic sensitization to cow's milk protein in mice.[41] Although in the present experiments we used the same batch of tuna oil, an important difference with the current protocol was the preventive approach in the former experiments. Mice were fed the LCPUFA-rich diet starting two-weeks prior to- and during the cow's milk sensitization protocol. Since IBS patients are hypersensitive to begin with, our aim in the present investigations was a treatment strategy for reversal of symptoms instead of prevention. This may have influenced the outcome of our *in vivo* investigations. On the other hand, we did use a 'prevention protocol' to test the efficacy of rosiglitazone and this strategy was also ineffective.

The inability of LCPUFAs and rosiglitazone to affect visceral hypersensitivity suggests that mast cell degranulation was not impaired by these treatment protocols and this was confirmed by toluidine blue staining of mucosal mast cells. This observation was also supported by our *in vitro* experiments, where rosiglitazone, EPA, and DHA showed no effect on compound 48/80-induced degranulation of HMC-1 mast cells. Although these negative results confirm our earlier observations on IgE-mediated degranulation of LAD2 cells,[23] they also add to the existing controversy. Some studies report suppressive effects of n-3 PUFAs on mast cell mediators and cytokine release,[22,23,42] whereas others report augmenting effects.[43,44] Since our data on erythrocyte n-3 LCPUFA levels confirm proper uptake of dietary LCPUFAs, the lack of effect on mast cell-dependent visceral hypersensitivity suggests that their *in vivo* effect on degranulation is limited. In contrast to the negative results on degranulation, we did observe *in vitro* inhibitory effects on TNF-α release, with moderate modulation by EPA and DHA, and strong inhibition by rosiglitazone. Because mast cells are a major source of TNF-α in the intestine,[35] we expected to confirm these inhibitory effects *in vivo*. However, when comparing TNF-α content of colon homogenates, there was no significant difference between vehicle and rosiglitazone treatment groups, suggesting that rosiglitazone did not alter TNF-α production *in vivo*. However, by using whole-colon homogenates, relatively small differences may have been rendered undetectable. Irrespective of this, at present there are no indications that TNF-α plays a role in this animal model.

On the internet, LCPUFAs are offered as a possible treatment option for IBS, but (pre)-clinical studies on efficacy are lacking. This animal study is the first to evaluate the possible effect of LCPUFAs on an important pathophysiological mechanism of this disorder. In summary, it shows that neither the increased intake of n-3 LCPUFAs nor the pharmacological activation of PPARγ can modulate post-stress visceral hypersensitivity in maternally separated rats. These results should not be regarded as definitive proof that LCPUFA supplementation in IBS will be ineffective, but more extensive investigations are required to strengthen that conclusion. Our results do, however, indicate that current claims should be regarded with caution.

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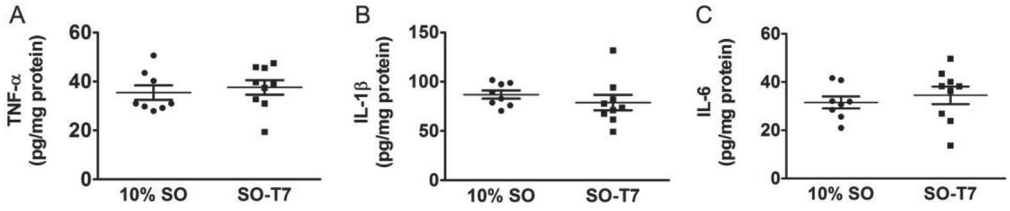
Online supporting material

Supplemental Table 1. *Sequence of primer pairs used to assess relative mRNA expression of PPARγ target genes in rat adipose tissue and colon by RT-qPCR.*

Primer pair name	Sense	Antisense
Lipoprotein lipase	AAGGTCAGAGCCAAGAGAAGCA	CCAGAAAAGTGAATCTTGACTTGGT
Glycerol kinase	GGGTTGGTGTCTGGAGTCTTG	GATTTCACTTTCTTCAGCATTGA
B2M	CGTGATCTTTCTGGTGCTTGTC	TTCTGAATGGCAAGCACGAC
Ppib	TGAGCATGGCCAATGCAGGC	TCACATCCTTCAGGGGCTTGTCC



Supplemental Figure 1. *TNF-α, Il-1β, and Il-6 levels in colonic tissues of rats treated with SO-T7 or control SO diet Colon homogenates of rats fed with control 10% SO diet or SO-T7 diet were compared for cytokine expression levels (TNF-α, Il-1β, and Il-6). Data presented as pg cytokine/mg total protein ± SEM, n=8–9 rats. Abbreviations: SO, soy oil; SO-T7, tuna oil/soy oil 7%/3%.*

Chapter 8

Summarizing discussion and future perspectives

In the current thesis we aimed to gain more insight in the pathophysiology of IBS and investigated different therapeutical strategies to modulate stress-induced visceral hypersensitivity. The irritable bowel syndrome (IBS) is a functional gastrointestinal disorder that is diagnosed by symptom-based criteria, according to Rome IV. The Rome IV criteria define IBS as a disorder in which recurrent abdominal pain (on average at least 1 day per week in the last three months) is associated with two or more of the following criteria; 1) related to defecation, 2) associated with a change in the frequency of stool, 3) associated with a change in the appearance of stool. Symptom onset should occur at least 6 months prior to diagnosis.[1] These manifestations affect patients' quality of life substantially. Despite the prevalence and impact of IBS, the exact etiology is not yet known. However, increased sensitivity to colorectal distension (also called visceral hypersensitivity) is present in the majority of IBS patients (33-65%) and is considered a possible pathophysiological mechanism.[2-5] Although the causes underlying this altered sensory motor function are only partially understood, stress is a known trigger for visceral hypersensitivity in IBS[6-8] and the activation of mast cells is implicated as a relevant relay event in stress-induced visceral hypersensitivity. As a response to stress, neuropeptides such as CRH are released from peripheral nerve terminals and eosinophils.[9,10] CRH has a major function in regulating the stress response by activation of the hypothalamic-pituitary-adrenal (HPA) axis and the sympathetic nervous system.[11] CRH has been shown to induce both mast cell degranulation and piecemeal degranulation,[12,13] the latter of which indicating the selective release of specific granule contents in membrane vesicles, rather than the rapid granule release that occurs by regular degranulation. Interestingly, another neuropeptide released during stress, substance P, was shown to influence mast cell function by upregulating TLR expression levels, while downregulating expression levels of the high affinity IgE receptor, thereby altering its receptor profile and subsequently mast cell function.[14] Several of the mediators released after mast cell activation, such as histamine, serotonin, TNF-α, NGF and tryptase, can stimulate nociceptors (such as TRPV-1) expressed on afferent sensory nerves.[15] Rather than acting on these nociceptors directly, mast cell-derived mediators are thought to sensitize these nociceptors by acting on co-expressed receptors (PAR2 being the best studied of which), lowering the threshold for activation.[15] This could explain increased pain perception to colorectal distension seen in IBS.

Traumatic events during childhood, which among others include emotional neglect and unsupportive parent-child interactions, are often associated with IBS.[16] In line with this, twin studies suggested that, although they both contribute, environmental factors have greater influence than genetic components.[17] Animal models reflecting this trigger may, although they can never fully reflect the complex human situation, contribute to a better understanding of the pathophysiology of IBS. One of the best studied models is the maternal separation model, in which neonatal pups rat are daily separated from their mother for a time period of three hours for 12 consecutive days (postnatal day 2−14). Adult rats that have undergone maternal separation display an IBS-like phenotype characterized by reduced intestinal barrier function and visceral hypersensitivity. The onset of the observed visceral hypersensitivity is dependent on the rat strain; Long Evans rats display visceral hypersensitivity only after undergoing an episode of acute stress at adult age whilst Wistar rats develop visceral hypersensitivity without being subjected to stress. In our group we choose to

work with Long Evans because also in IBS-patients acute stress is an important trigger for the onset of symptoms.[8] The clustering of IBS in families indicates that, when environmental factors are essential, each generation has either been exposed tot this factor independent of the previous generation, or the environmentally-induced phenotype is transferred form one generation to the next. In **Chapter 2** we investigated the second option by using the maternal separation model. We demonstrated that susceptibility to stress induced visceral hypersensitivity in maternally separated rats (F1) could be transferred to the next generation (F2) without the need to expose the F2 to the separation protocol. In other words, non-handled offspring of maternally separated female rats, developed visceral hypersensitivity when subjected to stress at adult age. Our cross fostering experiments indicated that the F2 phenotype fully depends on the phenotype of the foster mother. In IBS the transgenerational impact and familial clustering is thought to be (in part) explained by epigenetics.[18] Also in our model the observed changes in the F2 phenotype are more likely the result of soft-inheritance by epigenetic modifications than of Mendelian-inheritance, as our cross-fostering experiment shows rapid adaptation to the foster-mother phenotype. Although we did not exclude possible separation-induced genetic mutations in F1 offspring, it is known that Mendelian-inheritance of mutations is a slow process that needs many generations, where inheritance by epigenetic modifications is suited to quickly adapt to environmental changes.[19]

Previously, Meaney *et al.* investigated the effects of maternal separation on stress reactivity, where they found pituitary-adrenal responses to acute stress and decreased glucocorticoid receptor binding in the hippocampus amongst others.[20] In our experiments we failed to show differences in hippocampal glucocorticoid-receptor protein levels. Our results are in line with Daniels *et al.* who failed to detect differences in methylation status of the glucocorticoid receptor promoter between maternally separated and non-handled rats.[21] Combined, these data may suggest that, although maternal care is relevant, other factors than maternal behaviour might determine the IBS-like phenotype. In line with this, after observing altered gut mycobiome composition in IBS patients, our group assessed the possible role of fungi in the maternal separation model. Interestingly, we found that fungicide treatment of hypersensitive rats was able to reverse the enhanced response to colonic distension. Moreover, subsequent fecal transplantations of maternal separation mycobiome were able to restore visceral hypersensitivity to fungicide-treated maternally separated rats.[22] Although we did not examine F2 mycobiome, taken together these experiments might suggest that maternally separated rats develop an altered mycobiome, which could then be passed on to following generations. Importantly, we did investigate the role of mast cell activation in F2 rats and showed that doxantrazole treatment was able to effectively reverse existing established visceral hypersensitivity in non-handled offspring of maternally separated rats, suggesting that visceral hypersensitivity is mediated by mast cells irrespective of the generation studied.

One way of intervening with stress-induced and mast cell dependent visceral hypersensitivity is by blocking the initial degranulation trigger(s). One of the major hormones released during stress is corticotrophin-releasing hormone (CRH). In experiments using animal models pre-stress administration of CRH-receptor antagonists prevented mast cell degranulation and subsequent

visceral hypersensitivity,[23,24] therefore others have implicated an important triggering function for CRH in stress-induced mast cell degranulation in IBS. It has to be taken into account, however, that these studies aimed on preventing the stress-induced hypersensitivity, whereas the reversal of which might be more relevant to patients. Therefore in **Chapter 3**, we studied the ability of CRF receptor antagonism to prevent and/or reverse the stress-induced IBS-like phenotype. The non-selective CRF receptor antagonist α-helical CRF was administered prior to acute stress to test the effects on the initial mast cell activation and concomitant visceral hypersensitivity Long Evans rats. In agreement with previous publications, we found that CRF was essential in the initiation of stress-induced visceral hypersensitivity. However, post-stress α-helical CRF treatment failed to reduce established hypersensitivity. Nevertheless, we were able to reverse long-term established post-stress visceral hypersensitivity by treatment with doxantrazole. These results suggest that, although mast cells are relevant in acute as well as in prolonged post-stress hypersensitivity, triggers for mast cell degranulation may differ in time. Because IBS patients, in contrast to these rats, are most likely exposed to repetitive stress episodes, their intestinal mucosal mast cells may be subject to continues CRF triggers for mast cell degranulation. However, clinical trials in which CRF-receptor antagonists showed no patient benefit support our hypothesis of different triggers for mast cell activation in IBS.[25] Therefore, therapeutic interventions targeting CRF-receptors alone may be insufficient. Other options, such as the use of mast cell stabilizers, interference with mast cell mediators, or targeting triggers for mast cell activation such as fungal antigens may prove more beneficial.

Based on these considerations a double-blind placebo-controlled trial with the mast cell stabilizer/histamine (H)-1 receptor antagonist ketotifen was performed.[26] In this trial treatment with ketotifen reduced threshold of discomfort, decreased abdominal pain and other IBS-symptoms and improved health related quality of life of IBS patients. Importantly, treatment did not alter mast cell numbers or mediator release measured in rectal biopsies, suggesting that the beneficial effect of ketotifen did not depend on mast cell stabilization but on histamine-1 receptor antagonism instead. Because ketotifen is known to cause central side effects it may be beneficial to evaluate other, more potent, H1-receptor antagonists that will not penetrate into brain. In **Chapter 4** we evaluated two peripherally restricted receptor-antagonists (fexofenadine and ebastine) in a post-stress situation in the maternal separation model and found that WA-stress induced enhanced sensitivity to colorectal distension and both compounds effectively reversed this state of hypersensitivity. When the mast cell stabilising effects of these compounds were tested *in vitro*, only a small reduction of mast cell mediator release was found, which is probably due to autocrine signalling, indicating that the effects observed *in vivo* in the maternal separation model are due to antagonizing H1-receptors rather than stabilizing mast cells. Our results suggest that H1-receptors are a promising target in IBS. Indeed, recently the effects of 12-week treatment with the H1 receptor antagonist ebastine were evaluated in a clinical trial in IBS patients.[27] Abdominal pain scores were decreased significantly by ebastine when compared to placebo treatment, while global symptom relief was increased significantly. This study not only showed histamine-induced sensitization of TRPV-1 channels as a pathophysiological mechanism underlying visceral hypersensitivity in IBS, they also confirmed our suggestion for selective pharmacological targeting of H1 receptors as treatment option for IBS patients.

As previously mentioned, CRH is implicated to initiate visceral hypersensitivity in an acute setting, but does not trigger the ongoing mast cell activation underlying established visceral hypersensitivity. Our group recently showed intestinal mycobiome dysbiosis in both hypersensitive IBS patients as well as in maternally separated rats.[22] Importantly, this mybobiome dysbiosis was proven relevant for an IBS-like phenotype by fecal transfer experiments. In addition, post stress hypersensitivity to colorectal distension was reversed by fungicide treatment as well as by blocking host recognition of particulate β-glucans.[22] These data suggest that post-stress visceral hypersensitivity results from mast cell recognition of an aberrant mycobiome through activation of the Dectin-1/SYK pathway. Subsequent degranulation leads to the release of histamine that activates HR-1 receptors on sensory neurons which in turn leads to sensitization of TRPV1 and pain signaling. Antagonizing TRPV-1 was previously shown to effectively reverse established visceral hypersensitivity in the maternal separation model.[28] Importantly, lipid rafts have been shown to regulate the activation of the TRPV-1 pathway. Modulating lipid rafts might therefore be a plausible target to treat abdominal pain in IBS. In search for novel treatment options, in **Chapter 5** we investigated miltefosine as a candidate drug as it was not only shown to behave as a prototype lipid raft modulator[29] but also has a broad-spectrum *in vitro* and *in vivo* fungicidal activity.[30,31] Treatment led to reversal of post-stress visceral hypersensitivity in maternal separated rats. We showed that miltefosine may have exerted its analgesic effects by acting on 3 different levels; a) by modulation of the gut mycobiome, b) by reducing mast cell degranulation and c) by inhibiting TRPV-1 activation. The latter two assumedly via lipid raft modulation. We were, however, unable to assess relative contributions of miltefosine-mediated mycobiome-, mast cell-, and TRPV-1 modulation in the maternal separation model. Yet, results obtained with the intracolonic capsaicin model confirm the ability of miltefosine to interfere with *in vivo* TRPV-1 activation. As miltefosine is not specifically targeting TRPV-1 containing lipid rafts, signaling pathways other than those relevant to abdominal pain may have been affected as well. The cell membrane holds many different types of highly dynamic and coexisting rafts with associated proteins[32] and miltefosine microdomain affinity may differ according to compositional dissimilarities, which suggests that not all raft assemblies were targeted to the same extend. Nevertheless, unwanted side effects should be considered. The oral use of miltefosine in chronic spontaneous urticarial was tested in a 4 week, randomized, double-blind, placebo controlled trial by Magerl *et al.*[33] In this study the highest dose resembles the one used in the maternal separation model. Although no serious adverse events were reported, mild to moderate side effects (including nausea and vomiting) were frequents in both miltefosine and placebo treated groups. Furthermore, beneficial effects of miltefosine over placebo were lost within 4 weeks after discontinuation of treatment. Also in IBS long term miltefosine treatment may be needed for a lasting effect on TRPV-1-dependent pain, which is not preferable considering the unwanted side effects. Knowing however that miltefosine targets the gut microbiome as well, a short-term treatment could be considered in order to induce a favorable reset of the mycobiome. Although our previous results suggest that the same effect can be achieved by treatment with fungicides,[22] resistance against these compounds is on the rise and using them for a non-lethal but highly prevalent disorder like IBS may further shorten their clinical life span.[34] We suggest that alternative compounds, like miltefosine, should be considered for clinical trials with IBS patients where post treatment symptom improvement and

the duration of which is monitored and correlated to possible persistence of treatment-induced mycobiome changes.

Dietary interventions are an attractive treatment option for IBS because of low risk of side effects and relatively low cost. As rafts are lipid based, their composition, structure and function are susceptible to manipulation by dietary components. In our search for nutritional routes to intervene with mast cell activation, we evaluated the effects of marine omega 3 (n-3) polyunsaturated fatty acids (PUFAs) in our rat maternal separation model in **Chapter 6**, as n-3 PUFAs have been shown to alter raft fatty acid composition.[35] Interestingly, besides acting as a lipid raft modulator, n-3 PUFAs are shown capable of modulating mast cell mediator release, possibly via their PPARγ agonistic activity.[36,37] As a positive control for PPARγ activation, we used the pharmacological PPARγ agonist rosiglitazone. Different from our expectations, neither the increased intake of n-3 LCPUFA, nor the pharmacological activation of PPARγ was able to modulate mast cell degranulation and subsequent visceral hypersensitivity in the rat maternal separation model. These findings are confirmed by our *in vitro* experiments in which we show that although both strategies have suppressive effects on the release of TNF-α by mast cells, they fail to modulate mast cell degranulation. The effects of n-3 PUFAs on mast cells remains controversial, as some studies report suppressive effects of n-3 PUFA on mast cell mediator[38,39] and cytokine release,[40] whereas others report augmenting effects.[41,42] Since our *in vivo* data on erythrocyte n-3 LCPUFA levels confirm proper uptake of dietary LCPUFA, the lack of effect on mast cell-dependent visceral hypersensitivity suggests that their *in vivo* effect on degranulation is indeed limited.

We did however find suppressive effects of on the *in vitro* release of TNF-α by mast cells, with moderate effects by n-3 PUFAs and strong inhibition after rosiglitazone treatment. PPARγ activation modulates the gene transcription pathway of TNF-α.[43,44] Therefore, with successful PPARγ activation after rosiglitazone treatment *in vivo*, we would expect lower levels of TNF-α in tissue homogenates after rosiglitazone treatment. As mast cells are a major source of TNF-α in the intestine,[45] we evaluated TNF-α levels in colon homogenates. However, we did not find any rosiglitazone related differences in colonic TNF-α levels. This may suggest that rosiglitazone failed to alter TNF-α production *in vivo*, or relatively small differences may have rendered undetectable. Importantly, mast cell mediator and cytokine release profiles depend on the identity of the activation trigger. Therefore it might be possible that, when triggered by fungal antigens, TNF-α is not part of the mast cell mediator release profile. Further investigations on mast cell triggers and subsequent mediator profile should point out whether changes in mast cell TNF-α levels are to be expected, but until now strong evidence for an important role of TNF-α in this model or IBS is lacking.

In our experiments PUFA-enriched diets were given at adult age after acute stress, aiming to reverse established visceral hypersensitivity. Interestingly, Pusceddu *et al.* showed normalization of early-life stress-induced disruption of rat gut microbes.[46] In this model, gut microbiota analysis of adult maternally separated female rats showed altered microbiome composition, which was restored when rats were treated with EPA/DHA at the age of 5 weeks old. An effect that the authors suggest

is related to PUFAs anti-inflammatory activity. In this experiment the effects EPA/DHA on visceral hypersensitivity were not investigated. Nevertheless, we previously showed that modulation of gut mycobiome by miltefosine or fungicide treatment beneficially affects visceral hypersensitivity in our maternal separation model.[22] It would therefore be interesting to check gut microbe composition of PUFA-treated rats in our experiment to see whether we find the same changes. In addition, knowing that Pusceddu *et al.* found effects with treatment at 5 weeks of age,[46] a different time point of treatment, perhaps in the neonatal phase, might proof beneficial.

This thesis advances our understanding of the underlying pathophysiology of stress-induced visceral hypersensitivity and subsequently identifies possible new therapeutical targets. We showed that, although mast cells are relevant in acute as well as in prolonged post-stress hypersensitivity, triggers for mast cell degranulation may differ in time, with CRH triggering mast cell activation in the acute phase while an aberrant gut mycobiome is likely responsible for visceral hypersensitivity in the prolonged phase. Although the maternal separation model in rat has its limitations when trying to mimic a complex and enigmatic disorder like IBS, our data seem to reflect the human situation as antagonizing the CRF receptor showed no patient benefit,[25] while targets such as mast cells and the histamine 1 receptor, were successfully translated to human.[27] Therefore we consider this model as one with proven predictive value for IBS.

From a patient point of view, reversal of established symptoms, instead of preventing the onset of symptoms is more relevant. Therefore, when aiming to treat current IBS patients, therapies should be aimed at targeting the triggers for (ongoing) mast cell activation in this phase of the disease. In our rat maternal separation model, fungal antigens, or the recognition of which, seem a plausible candidate. To strengthen this hypothesis, the effects of modulating gut mycobiome need to be established in more than one animal model for visceral hypersensitivity or, perhaps most predictive for IBS, in maternal separated rats with a humanized (patient *vs* healthy volunteer) mycobiome. The next step would be to test fungicide treatment in a trial with IBS patients. Relevant fungi could then be assessed pre- and post-treatment by mycobiome analysis and correlated to patient pain scores.

IBS clusters in families and environmental factors are considered essential to this clustering. In our experiments in rat, maternal separation led to stress-induced visceral hypersensitivity at adult age. This susceptibility was transferred across generations without need for further separation protocols. This may suggest that also in IBS such vertical transfer is relevant and, therefore, can be intervened with when the mechanism of transfer is identified. This would mean an important shift from treatment to prevention-directed research efforts. Because we recently showed the relevance of maternal separation induced mycobiome changes, we suggest that mother-to-child mycobiome transfer in rat should be investigated as one possible cause. If so, transfer prevention strategies such as prepartum maternal mycobiome modulation can be addressed in this model. Although we are still al long way from effectively treating IBS patients, in this thesis we do provide possible new targets for future therapies aimed at either modulating mast cell activation i.e. by lipid raft modulation, or by targeting the initial trigger(s).

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Appendices

Nederlandse samenvatting

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Nederlandse samenvatting

Het prikkelbaar darm syndroom (IBS) is een van de meest voorkomende gastro-intestinale aandoeningen in de Westerse wereld, met een prevalentie van 9−23% van de totale bevolking. Kenmerkende symptomen voor IBS zijn (chronische) buikpijnklachten, een opgeblazen gevoel en een veranderde stoelgang zonder dat hier een duidelijke oorzaak voor wordt gevonden. Hoewel IBS geen levensbedreigende ziekte is, zorgt het voor een aanzienlijke verlaging in de kwaliteit van leven. De behandeling van IBS is vaak teleurstellend, met name doordat effectieve therapieën vaak gepaard gaan met ernstige bijwerkingen die het doel overstijgen, waaronder misselijkheid en leverschade. De ontwikkeling van nieuwe medicatie is lastig omdat we nog weinig weten over het precieze onderliggende mechanisme van deze ziekte. Bij het merendeel van de patiënten met IBS is sprake van een verhoogde gevoeligheid voor prikkels in de darm, ook wel viscerale hypersensitiviteit genoemd. Deze viscerale hypersensitiviteit wordt gemeten door een distensie van de darm uit te voeren, welke door gezonde mensen als niet-pijnlijk wordt ervaren, maar voor IBS patiënten erg pijnlijk of vervelend is en wordt gezien als een belangrijk mechanisme in het ontstaan van IBS-gerelateerde klachten.

Verschillende klinische studies wijzen uit dat stress, en dan met name stress in de kindertijd (zoals een verstoorde band tussen ouder en kind, verwaarlozing, lichamelijk en sexueel misbruik), samenhangt met de ontwikkeling van IBS. Stress in de kinderjaren wordt zelfs geassocieerd met een abnormale communicatie tussen hersenen en darmstelsel en geeft daarbij een mogelijke verklaring voor de link tussen stress en pijnklachten in IBS. Hoewel zij natuurlijk nooit de complexe situatie van de menselijke setting kunnen vervangen, geven diermodellen waarin stress in de kindertijd wordt nagebootst belangrijke informatie om het ontstaan van IBS beter te begrijpen en om targets waarop medicatie kan aangrijpen te identificeren. Onze onderzoeksgroep maakt gebruik van het ‘ maternal separation’ rat model, waarin neonatale rattenpups 12 dagen lang 3 uur per dag van hun moeder gescheiden worden en waarin zo een verstoorde ouder-kind relatie wordt nagebootst. Wanneer deze ratten op volwassen leeftijd stress ondergaan ontwikkelen zij de viscerale hypersensitiviteit welke kenmerkend is IBS. Belangrijk om te melden is nog dat ook in patiënten acute stress gerelateerd is aan het ontstaan en oplaaien van pijnklachten.

IBS komt vaak bij meerdere leden van dezelfde familie voor. De vraag is dan of dit komt doordat zij dezelfde genetische achtergrond hebben, of doordat zij dezelfde, mogelijk oorzakelijke, omgevings-factoren delen. Uit onderzoek met tweelingen is gebleken dat omgevingsfactoren waarschijnlijk belangrijker zijn dan genetica. Toch clustert IBS in families; dit zou verklaard kunnen worden doordat meerdere generaties aan dezelfde factoren worden blootgesteld of doordat omgevings-gerelateerde veranderingen in het fenotype aan de volgende generatie worden doorgegeven. In **hoofdstuk 3** hebben wij de tweede optie onderzocht in ons maternal separation model. Hierin hebben wij aangetoond dat de door stress geïnduceerde viscerale hypersensitiviteit van maternaal gesepareerde ratten (F1) naar de volgende generatie (F2) overgedragen kan worden, zonder dat de

pups aan het separatie protocol worden blootgesteld. ‘Cross fostering’ experimenten, waarin pups van controle moeder ratten worden omgewisseld met de pups van maternaal gesepareerde ratten, tonen aan dat de pups het fenotype van de foster pleeg moeder overnemen. Dit geeft aan dat het clusteren binnen families verklaard zou kunnen worden door een niet-genetische overdracht van het fenotype van de ene generatie naar de volgende. In dit experiment hebben wij niet gekeken naar de verschillende factoren die hierbij belangrijk zouden kunnen zijn, denk hierbij bijvoorbeeld aan verschillen in darmflora. Wel hebben we gekeken naar de rol van mest cellen, een type immuun cel waarvan is aangetoond dat de activatie hiervan een belangrijke rol speelt in de door stress-geïnduceerde viscerale hypersensitiviteit. Met behulp van doxantrazole, een mest cel stabilisator, hebben wij aangetoond dat net als in de F1 generatie de viscerale hypersensitiviteit van F2 ratten afhankelijk is van de activatie van mest cellen.

Wanneer een mest cel wordt geactiveerd komen granulen met onder andere histamine vrij in de omgeving, een proces dat degranulatie genoemd wordt. Verscheidene van die vrijgekomen stoffen grijpen aan op receptoren aanwezig op sensorische zenuwuiteinden en kunnen zo bijdragen aan de verhoogde gevoeligheid voor pijn in IBS. Het blokkeren van de trigger voor mest cel activatie is een manier op mest cel-afhankelijke hypersensitiviteit te behandelen. Een van de belangrijkste hormonen die vrijkomen tijdens stress is corticotropin-releasing hormone (CRH). Eerder onderzoek laat zien dat wanneer een CRH-receptor antagonist voorafgaande aan het ondergaan van stress in een diermodel wordt toegediend, activatie van mest cellen en de daarop volgende viscerale hypersensitiviteit niet plaatsvindt. Hoewel dit mooie resultaten zijn, is het belangrijk op te merken dat voor IBS patiënten het terugbrengen van bestaande klachten meer relevant is dan het voorkomen van de ziekte. Daarom hebben wij in **hoofdstuk 4** onderzocht of het antagoneren van de CRH receptor ook in staat is om bestaande door stress-geïnduceerde viscerale hypersensitiviteit te doen omkeren. Bij een pre-stress toediening van de CRH-receptor antagonist α-helical CRF in ons maternal separation model bevestigden wij de resultaten van eerdere onderzoeken; CRH blijkt essentieel in de initiatie van stress-geïnduceerde viscerale hypersensitiviteit. Echter, bij behandelding na de stress bleek α-helical CRF niet in staat om bestaande viscerale hypersensitiviteit terug te brengen. Deze resultaten suggereren dat, hoewel mest cellen in zowel de acute fase, waarin klachten ontstaan, als in de late fase, waarin klachten worden ‘onderhouden’, essentieel zijn, de trigger voor mest cel activatie in beide fasen verschillend is. Medicatie gericht op het blokkeren van CRH receptoren alleen zal daarom waarschijnlijk onvoldoende zijn. Meer winst zou gehaald kunnen worden uit behandeling gericht op het stabiliseren van mest cellen of het interfereren van de acties van de mediatoren die worden vrijgelaten bij mest cel activatie.

Met dit in gedachte is in onze groep middels een trial in IBS patiënten het effect van de histamine-1 receptor antagonist ketotifen getest. In deze trial verlaagde behandeling met ketotifen pijnklachten en andere IBS-gerelateerde symptomen en werd de kwaliteit van leven verhoogd. Echter, passeert ketotifen de bloed-brein barrière en behandeling gaat daardoor gepaard met bijwerkingen. Om deze reden hebben wij in **hoofdstuk 5** het effect van 2 in het perifere systeem blijvende HR-1 receptor antagonisten (fexofenadine en ebastine) getest in ons maternal separation model. Wij laten zien dat

post-stress behandeling met deze middelen bestaande viscerale hypersensitiviteit doet omkeren en suggereren hierbij dat het blokkeren van de HR-1 receptor een veelbelovende tactiek is om pijn gerelateerde IBS symptomen te bestrijden. Recent is het effect van ebastine in IBS patiënten getest, waar een behandelingsduur van 12 weken een significante symptoom verlichting en verlaging van pijnscores met zich mee bracht.

Zoals eerder genoemd, is CRH enkel betrokken bij de activatie van mest cellen in de acute fase en lijkt een andere trigger verantwoordelijk voor de continue activatie van mest cellen die ervoor zorgt dat (pijn)klachten voortduren. Onlangs heeft onze groep aangetoond dat het mycobiome (de samenstelling van schimmels) in de darm veranderd is in zowel hypersensitieve IBS patiënten als in maternaal gesepareerde ratten. Het belang van deze mycobiome dysbiose in relatie tot viscerale hypersensitiviteit is aangetoond met feces transplantatie experimenten en in onderzoek naar het effect van behandeling met fungiciden. Gecombineerd met ons eerdere onderzoek waarin we het belang van het TRPV-1 ion kanaal aanwezig op sensorische neuronen aantonen, suggereren deze resultaten dat post-stress viscerale hypersensitiviteit het resultaat is van mest cel activatie door herkenning van een veranderd mycobiome. Hierop volgende degranulatie leidt dan vervolgens tot het vrijkomen van histamine die de HR-1 receptoren activeert op sensorische neuronen en daarbij TRPV-1 kanalen sensitizeert en pijn signalering activeert. De activatie van TRPV-1 kanalen wordt gereguleerd door lipid rafts, kleine platformen in het celmembraan waarin bepaalde cellulaire processen afgebakend kunnen plaatsvinden. Het moduleren van zo'n lipid raft zou dus een mooie strategie kunnen zijn om viscerale hypersensitiviteit aan te pakken. In **hoofdstuk 6** testen wij het effect van miltefosine, een goed-gekeurd medicijn dat momenteel wordt gebruikt voor de behandeling van Leishmanias. Miltefosine is niet alleen in staat om lipid rafts te moduleren, het heeft ook fungicide werken en zou dus op meerdere punten kunnen aangrijpen. En inderdaad, post-stress behandeling met miltefosine brengt de door stress geïnduceerde viscerale hypersensitiviteit effectief terug, waarschijnlijk op 3 manieren; a) door modulatie van het mycobiome van de darm, b) door mest cel degranulatie te verminderen en c) door de activatie van TRPV-1 te remmen. De laatste 2 opties waarschijnlijk door het moduleren van lipid rafts. Omdat miltefosine niet specifiek aangrijpt op de lipid rafts die TRPV-1 omvatten, moet men rekening houden met ongewenste bijwerkingen. Bij een behandelingsduur van 4 weken miltefosine in patiënten met een andere aandoening werden milde tot matige bijwerkingen gerapporteerd, waaronder misselijkheid en braken. Bij behandeling bij IBS zal ook een lange behandelingstermijn nodig zijn en daarom zal miltefosine zelf niet aantrekkelijk zijn. Wij raden daarom aan om alternatieve middelen, met een specifieker bereik dan miltefosine te overwegen voor klinische trials met IBS patiënten.

Omdat veel behandelingsstrategieën toch gepaard gaan met bijwerkingen, die in het geval van IBS vaak ernstiger zijn dan de ziekte zelf zou een interventie gericht op een veranderd dieet een uitkomst kunnen zijn. En omdat lipid rafts uit lipiden bestaan is hun compositie, structuur en functie gevoelig voor manipulatie door componenten in de voeding, waaronder omega 3 (n-3) meervoudig onverzadigde vetzuren (PUFAs). Buiten dat zij lipid rafts kunnen moduleren, is ook aangetoond dat n-3 PUFAs het vrijkomen van mest cell mediatoren kunnen remmen, waarschijnlijk via activatie

van de PPARγ receptor. In **hoofdstuk 7** hebben wij gekeken naar het effect van n-3 PUFA verrijkte voeding op de viscerale hypersensitiviteit. Als positieve controle voor PPARγ activatie hebben wij ook gekeken naar het effect van behandeling met de PPARγ agonist rosiglitazone. Anders dan onze verwachtingen waren zowel de rosiglitazone als het met PUFA verrijkte dieet niet in staat om de hypersensitiviteit in het maternal separation model te moduleren. Ook in onze *in vitro* experimenten waren wij niet in staat om mest cell degranulatie te moduleren.

Samenvattend wordt in dit proefschrift onderzoek gedaan naar de achterliggende mechanismen van mest cel activatie in door stress geïnduceerde viscerale hypersensitiviteit en worden mogelijke targets waarop medicatie zou kunnen aangrijpen om buikpijnklachten in IBS te verlagen getest. Wij laten zien dat, hoewel mest cellen in zowel de acute als de late fase essentieel zijn, de triggers voor de activatie van mest cellen in de tijd veranderen; waar CRH mest cellen activeert tijdens stress, is een veranderd mycobiome waarschijnlijk verantwoordelijk voor activatie in de latere fase. Het maternal separation model blijft natuurlijk een diermodel en zal nooit de complexe humane situatie compleet kunnen nabootsen. Toch lijken onze resultaten de humane situatie goed weer te geven aangezien het blokkeren van de CRH receptor ook in IBS patiënten geen gunstig effect had, terwijl targets als mest cellen en de HR-1 receptor met succes vertaald zijn naar de mens. Andere targets in dit proefschrift, waaronder lipid rafts en (de herkenning van) een veranderd mycobiome zouden dan ook mooie strategieën kunnen zijn om toekomstige therapieën op te richten.

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PhD Portfolio

	Year	ECTS
General courses		
Practical Biostatistics	2009	1,1
Scientific writing	2009	1,5
Specific courses		
Laboratory Animals	2008	3,9
Anatomy of the house mouse	2009	1,1
Drug development Simulation (TI Pharma)	2009	1
National meetings		
PhD retreat ACM	2012	1
PhD retreat ACM	2011	1
International meetings		
Digestive Disease Week (oral presentation)		1,5
United European Gastroenterology Federation (UEGW) (oral presentation)	2011	1
International Mast cell and Basophil Meeting (poster)	2010	1
Intestinal Proteases; Opportunity for Drug discovery (IPODD) annual meeting (oral and poster presentation)	2009	1,5
Intestinal Proteases; Opportunity for Drug discovery (IPODD) annual meeting (oral and poster presentation)	2010	1,5
Local meetings		
Journal clubs		4
Weekly department seminars		4
Teaching		
Supervising biomedical sciences student, research internship		2

Dankwoord

Nu is het natuurlijk de truc om het kort te houden, iets waar ik niet heel goed in ben, en iedereen die een bijdrage heeft geleverd persoonlijk te benoemen. Dit laatste is eigenlijk onmogelijk en ga ik ook niet proberen. Desalniettemin is mijn dank groot voor iedereen die op welke manier dan ook een bijdrage heeft geleverd aan het tot stand komen van dit proefschrift.

Allereerst wil ik graag mijn co-promotor, Dr. R.M. van den Wijgaard bedanken. Beste René, na al die jaren toch nog een boekje! Wie had dat gedacht.. En dat is mede dankzij jouw hulp, tijd en inzet! Ik weet nog goed dat je op mijn eerste dag zei; “we gaan er iets moois van maken!” Nu zal ik eerlijk zijn dat ik je niet altijd geloofd heb, maar gelukkig kreeg je gelijk en hebben we er, ondanks de negatieve resultaten van het colitis model, toch nog wat moois van gemaakt! Je gedrevenheid zal ik nooit vergeten. Bedankt voor je ideeen, feedback, inzet en hulp, ik heb veel van je geleerd!

Mijn promotor, Prof. Dr. W.J.de Jonge. Beste Wouter, ook jij hebt je hard gemaakt voor mijn promotie. Veel dank hiervoor! Je bent natuurlijk pas in de laatste fase mijn promotor geworden, maar bent ook tijdens mijn promotie onderzoek betrokken geweest. Bedankt voor al je suggesties en ideeen. Ik kon altijd laagdrempelig bij je terecht en dat heb ik zeer gewaardeerd! Bedankt voor alles!

Prof. DR. G.E. Boeckxstaens. Beste Guy, mijn promotietraject begon met jou als mijn promotor. Ondanks dat je toen al in Belgie zat, heb ik toch veel van je geleerd. Je wetenschappelijke kennis en gedrevenheid heb ik altijd zeer gewaardeerd, maar ook de leuke gesprekken bij de TI Pharma meetings. Dankjewel!

En dan Olaf, wat heb ik veel van jou geleerd! Het was altijd gezellig om met je samen te werken! Het ‘dierwerk’ was niet mijn favoriete bezigheid, maar met jou was het allemaal een stuk ‘aangenamer’. Ochtenden vol grappen en leuke verhalen over volleybal, maar ook advies over het aio zijn en over de experimenten. Vanaf het begin nam jij me een soort van onder je hoede en dat stel ik zeer op prijs. Bedankt voor alles; de leuke tijd, je luisterend oor, samen terug in de trein of auto na (promotie)feestjes van collega’s, adviezen en natuurlijk ook voor al het werk dat je hebt verricht! Ook zonder jou zou dit boekje er niet zijn!

Oana, my collegue and friend. You made me laugh so hard sometimes! (Remember your moustache comment ;-)). We clicked from day 1, both inside the lab and outside. I will never forget our ‘minivacation’ together in New Orleans before we went to the DDW. Thank you for al the joy you brought to the ‘kippenhok’ and for all the laughter and interesting stories!

Mijn collega aio's. Susan en Esmerij, met jullie was het altijd gezellig! Laurens, altijd in voor een drankje en een praatje! Sjoerd, mijn mest cel maatje! Ik heb met plezier met je samengewerkt! Shobbit, José, Ronald, Jenny en Luca, friends inside the lab and outside. Thanks for all the gezellige dinners, vacations and borrels. We had a lot of fun together! José, thanks for taking us to your home in Portugal. It was an experience I will never forget! Ronald, bedankt voor je luisterend oor, jij hebt wat dat betreft veel te verduren gehad ;). Breg, Tamira, Wout, Pim, Simone, Charlotte, bedankt voor de gezellige tijden op het lab, de DDW en op de borrels! Aan alle Tytgat collega's, bedankt voor alle gezellige en leerzame meetings, uitjes en borrels!

Mijn lieve vrienden en vriendinnen, Bianca, Jeroen, Juul, Marieke, Marjolein en Marloes, jullie hebben alle misère en stress moeten aanhoren en ik heb me altijd door jullie gesteund gevoeld. Ik ben dankbaar voor jullie vriendschap en alle gezellige tijden en leuke dingen die wij samen beleefd hebben en er komen er nog veeeeel meer!

Lieve Jolanda, jij hebt je altijd ingeleefd in mijn promotietraject en hebt me geprobeerd te motiveren om het toch succevol af te ronden. En met success! Bedankt voor je steun en alle gezelligheid! Ik vind het een eer dat je mijn paranimf bent!

Dear Léa, collegue and friend! We had so much fun together! Thank you for everything! You took us to your family in France, which was amazing! I miss our Saturday brunches in our neighbourhood. I am prood to call you my paranimph and hope we will have some more of those brunches in the future!

Mijn lieve familie en schoonfamilie. Ik mag mezelf gelukkig prijzen met zoveel lieve mensen om mij heen. Bedankt voor jullie interesse en steun!

Mijn lieve ouders en broer. Ik ben zo blij dat jullie mijn ouders zijn. Jullie staan letterlijk altijd voor me klaar, jullie liefde, steun en vertrouwen zijn hebben mij gemaakt tot wie ik nu ben. Lieve papa, ons grapje is altijd 'ik heb het allemaal van m'n vader geleerd'. Een grapje, maar ik heb wel echt veel van je geleerd, van jullie allebei. Lieve mama, wat heb jij veel meegemaakt in de laatste jaren. Ik heb ongelooflijk veel respect voor hoe je alles hebt ondergaan. Ik ben trots dat jij mijn moeder bent! Lieve Laurens, al vanaf dag 1 ben ik trots dat jij mijn broertje bent en dat ben ik nog steeds! Ik hoop dat mijn kinderen net zo blij met elkaar zijn als ik met jou ben.

Lieve Edwin, wat een geluk dat wij elkaar na al die jaren weer ontmoet hebben! En dat gewoon toevallig die ene avond in de Swing! Mijn oude 'buurjongen' bleek mijn grote liefde te zijn en kijk eens waar we nu zijn met twee fantastische kinderen! Ik hou van je en ben supertrots op wat wij samen bereikt hebben. Je bent pas in de laatste fase van mijn promotie betrokken, maar wel belangrijk geweest in een aantal grote beslissingen en ik ben heel blij dat ik dit met jou aan mijn zijde kan afronden!

En dan mijn lieve, kleine oogappeltjes. Jullie zijn het beste wat mij is overkomen, ik ben supertrots en dankbaar dat ik jullie moeder mag zijn! Lieve Yanieck, nog geen 3 jaar oud en zo bijzonder! Ik ben ongelooflijk trots op wie jij bent, elke dag weer! Ik heb me zo schuldig gevoeld dat ik na de komst van jouw zusje ook nog mijn tijd aan de promotie moest besteden en niet aan jou. Wat dat betreft is de timing helemaal verkeerd. Gelukkig ben je ongelooflijk lief en heb je ook gezellig boven gespeeld terwijl ik aan het typen was. Maar je mist mama wel, en dat is terecht. Straks is het voorbij en kan ik me weer helemaal op jou (en je papa en zusje) richten en gaan we weer allemaal leuke dingen met elkaar doen! Lieve Amélie, wat lig je lief in mijn armen te slapen nu ik dit schrijf. Nog maar 2 weken oud en nu al zo speciaal! Dat je zo'n lieve, rustige en tevreden baby bent helpt wel enorm in deze fase! Ik ben enorm trots op mijn mooie, kleine meisje en ben heel benieuwd hoe jij je gaat ontwikkelen!