Pathophysiology of stress-induced visceral hypersensitivity

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

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ABSTRACT

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

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

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

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

The irritable bowel syndrome (IBS) is a functional bowel disorder characterized by abdominal pain or discomfort associated with defecation or a change in bowel habit. 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. 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. Although a genetic aetiology for IBS was also put forward because IBS tends to cluster in families, 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. Risk-factors such as infection, diet, childhood affluence, illness behaviour of parents, physical and sexual abuse and adverse parent-child interactions 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. 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. 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. In the present study we tested the hypothesis that susceptibility to stress-induced visceral
hypothesis 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\textsuperscript{26} and possibly IBS patients,\textsuperscript{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.\textsuperscript{25} Insertion of the catheter was performed under brief isoflurane anaesthesia. Distensions started after a 20 minute recovery period. They were performed at the minimum age of 3 months (weight > 300 grams) and achieved by inflation of graded volumes of water (male rats: 1.0, 1.5 and 2.0 mL, female rats: 0.8, 1.2 and 1.6 mL). Length and diameter of the balloon during a 2 mL maximum volume distension were 18 and 15 mm respectively. For each volume a 20-s distension period followed by an 80-s resting period was exercised. For acute stress at adult age (WA stress) rats were positioned on a pedestal surrounded by water for a time-period of one hour. Sensitivity to distension was evaluated pre- and 24 hours post-WA.

**Measurement of the visceromotor response to colonic distension and data analysis.** Distension of the colon induces contractions of abdominal musculature: the visceromotor response. Quantification by means of electromyography (EMG) reflects visceral sensitivity. We previously validated a radio-telemetry technique in freely moving rats to record these signals\textsuperscript{25} and further details on techniques and data analysis system have been published extensively.\textsuperscript{25,28-30} Data analysis was performed by extracting, from the raw EMG data file, each 20-s distension period and its preceding 20-s of baseline recording. After correction for movement and breathing, data were rectified and integrated. Absolute
data sets were then obtained by subtracting the 20-s baseline recording from the 20-s distension result. Similar to our earlier publications, final results are given as normalized data sets, which were calculated from the absolute data by setting the 2mL value of the first (pre-stress) distension at 100%. Subsequently, area under the curve (AUC) of relative responses was calculated for individual rats and used to 1) establish whether, based upon a predefined cut off value, individual rats were hypersensitive to distension, and 2) to calculate average AUC within treatment groups. Relative response data were also used to evaluate possible changes on a per volume basis. Data are always depicted as mean +/- SEM.

**Historical data and definition of AUC cut off value.** Although not all MS rats will become hypersensitive to distension upon WA, we always included all evaluated animals when presenting averaged results in previous publications. 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, data were obtained in male rats only, female pups were always eliminated from the nest on post natal day 2 (please refer to figure 1A for schematic representation of groups). The arbitrary AUC cut off value was defined by calculating the upper 10% pre-WA AUC (distension-vs-response) of the 72 NH rats. Subsequently, all individual rats (NH and MS) with an AUC above cut off were considered hypersensitive to distension.

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

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

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

**Mast cell stabilization experiments.** Our previous investigations showed that stress-induced hypersensitivity to distension in male MS-F1 Long Evans rats depends on mast cell degranulation. When administered pre-WA, the mast cell stabilizer doxantrazole prevented stress-induced visceral hypersensitivity whereas post-WA administration reversed increased sensitivity. Here we determined whether WA-induced hypersensitivity in adult male NH-F2 offspring of MS-F1 dams can also be reversed by mast cell stabilization. In short, after establishing pre-WA sensitivity status, the visceromotor response was measured again at T= +24 hours and T= +48 hours post-WA. In between these two post-WA time-points (at T= +24½, 32 and 47½) rats (n=9) received i.p. doxantrazole (10 mg kg⁻¹, gift of Agnès Francois, Institut Gustave Roussy, Villejuif, France) dissolved in 0.5%
NaHCO\textsubscript{3}/0.9\% saline pH 7.5, or vehicle alone (n=9). The experimenter (OW) was blinded to the different treatment groups that were only disclosed after interpretation of the individual tracings. See figure 1D for schematic representation of the experiment.

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

**RESULTS**

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

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

**Cut off value and % hypersensitive rats in different groups** The volume/relative response data of 72 NH-F1 and 72 MS-F1 male rats measured in previous studies are depicted in Figure 2C and D respectively. Pre-WA vs post-WA comparisons of AUC as well as per volume differences show significantly increased post-WA responses in MS-F1 rats (pre-WA vs post-WA AUC; 63.8 ± 1.4 vs 96.3 ± 2.8, \( ###P<0.001 \) and pre-WA vs post-WA per volume comparisons of 1.0, 1.5 and 2.0 ml all \( ***P<0.001 \)). Figure 2E shows the individual pre-WA AUC data for the 72 NH rats. The 90 percentile AUC of their pre-WA response was 77.8. This number was then used as an arbitrary cut-of-value to define hyper- or normo-sensitivity status in individual rats (AUC>77.8 defined as hypersensitive). Based on this, figure 2F depicts the percentage of hypersensitive animals in the different groups. By definition, 10% of the 72 NH rats showed pre-WA hypersensitivity, this number increased to 19.4% post-WA. In contrast, in the 72 MS-F1 rats 8.3% was hypersensitive pre-WA and this increased to...
76.4% post-WA. Similar results were obtained for the 30 NH-F2 rats; 13.3% was hypersensitive pre-WA and 80% post-WA.

Figure 2. Effect of WA-stress on visceromotor response to colonic distension, all data in 2A-2D are given as mean +/- SEM. Figure 2A) depicts enhanced post-WA response to distension in n=7 MS-F1 dams (evaluated after giving birth to NH-F2 offspring shown in 2B); pre-WA vs post-WA AUC, *P<0.05 and increased post-WA response in all 3 per volume comparisons (*P<0.05). Figure 2B) shows enhanced post-WA response to distension in 30 NH-F2 male offspring of MS-F1 dams; pre-WA vs post-WA AUC, ###P<0.001 and increased post-WA response in all 3 per volume comparisons (**P<0.01). 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<0.01). Figure 2E) shows the individual pre-WA AUC data for the 72 NH-F1 rats. The 90 percentile pre-WA AUC value was calculated (77.8) and used as an arbitrary cut off value for further evaluations. In Figure 2F) the AUC cut off value was used to define the % hypersensitive rats in different investigated groups. In the NH-F1 group WA stress only induced a moderate increase in the number of hypersensitive rats. In contrast, strongly increased numbers were observed in post-WA MS-F1 and NH-F2 groups.
Cross fostering experiment In these cross fostering experiments none of the F2 offspring was subjected to the maternal separation protocol. Average volume/relative-response data of the 4 different groups are given in figure 3. When nursed by their natural NH-F1 mothers, WA was unable to induce an enhanced response to distension in NH-F2 offspring (n=7, Figure 3A). In contrast, when pups of NH-F1 dams were nurtured by MS-F1 foster mothers there was a significant increase in post-WA AUC (pre-WA vs 24 hours post-WA AUC; 59.7±5.6 vs 115.8±7.9, #P<0.05, n=5) as well as increased responses based on per volume comparisons (*P<0.05 for all 3 distension volumes, Figure 3B). Figures 3C confirms that, when raised by their natural mother, pups of MS-F1 dams become hypersensitive to distension upon WA; pre-WA vs post-WA AUC; 77.6±6.1 vs 109.7±5.9, #P<0.05 (n=6) and *P<0.05 for 1.5 and 2.0 ml distension volumes. When, on the other hand pups of MS-F1 dams are switched to NH-F1 foster mothers, the enhanced post-WA response to distension does no longer occur (Figure 3D, n=5).

The individual sensitivity status of cross fostered rats is depicted in Table 1. Again, rats with volume/relative response AUC>77.9 were considered to be hypersensitive to distension (marked with an asterisk). When nurtured by NH-F1 dams, all but two rats (one delivered by a MS-F1 dam and one by a NH-F1 dam) were normo-sensitive upon WA. In contrast, all animals reared by MS-F1 dams showed post-WA visceral hypersensitivity.
Figure 3. Volume/relative response data of cross fostering experiments, data are mean ± SEM. A) Absence of WA-induced visceral hypersensitivity when NH-F2 offspring (n=7) was nursed by own NH-F1 dams. B) NH-F2 offspring (n=5) switched to MS-F1 dams. WA induced an enhanced response to distension; pre-WA vs 24 hours post-WA AUC; *P<0.05 and **P<0.05 for pre-WA vs post-WA comparisons of all 3 distension volumes. C) MS-F2 offspring (n=6) nursed by own MS-F1 dams. WA induced an enhanced response to distension; pre-WA vs post-WA AUC; *P<0.05 and **P<0.05 for 1.5 and 2.0 ml distension volumes. D) Absence of post-WA hypersensitivity to distension when MS-F2 offspring (n=5) was switched to NH-F1 dams.

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

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

Previous investigations showed that post stress visceral hypersensitivity in MS-F1 Long Evans rats depends on mast cell degranulation. 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).

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

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

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

Epigenetic modifications are often put forward as an ideal mechanism to facilitate soft inheritance; they refer to functionally relevant modifications to the genome that do not involve a change in the nucleotide sequence. In a recent opinion article by Dinan et al. it was suggested that the absence of a clear genetic phenotype for IBS is supportive of the view that the disorder is epigenetic in nature and that epigenetics may help explain familial clustering and transgenerational impact of IBS.\(^35\) Methylation of cytosine residues of DNA is among the most investigated epigenetic effects. Since MS rats were shown to have increased HPA-axis responsiveness\(^36\) it is tempting to compare our ‘transfer-
results’ with those obtained in another transfer model were methylation effects seem to play an important role. In this model Meaney and colleagues studied naturally occurring variations in rat maternal care. Not only were extremes in maternal care transmitted across generations\textsuperscript{33} but also enhanced HPA-axis responsiveness. The latter was linked to hyper-methylation of the hippocampal glucocorticoid-receptor promoter.\textsuperscript{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.\textsuperscript{38} Our own data on GCR protein levels confirm equal hippocampal expression in MS and NH rats (supporting information S1). These data suggest that, in contrast to the behavior-selection model, behavior may not be the determining factor in the maternal separation model. Indeed, Macrì et al. convincingly showed that, in contrast to the behavior-selection model, active maternal care is not decreased in MS vs NH rats.\textsuperscript{39,40} However, other non-behavioral factors associated with maternal care should not be ruled out as an explanation for results observed in our cross fostering experiments. In relation to this it is known that even brief maternal separations induce increased corticosterone release in dams and indications are, albeit in a different setting, that enhanced corticosterone plasma concentrations can be transmitted to offspring via lactation.\textsuperscript{41} Whether this holds true for the MS model remains to be investigated.

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

Negative results on the epigenetic modulation of hippocampal GCR in MS do not rule out a role for epigenetic changes in other target tissue or cells. In relation to this, several lines of evidence suggest that mast cells play an important role in IBS. Barbara et al. not only showed enhanced numbers of degranulated mucosal mast cells, but mast cell proximity to nerves also correlated with severity and frequency of abdominal pain/discomfort. When supernatants of submerged intestinal human biopsies were used, IBS but not normal control supernatants induced histamine-dependent firing of rat mesenteric afferents and serine-protease dependent colonic hyperalgesia in mice. 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.

In MS-F1 Long Evans rats we previously showed that pre-WA administration of the mast cell stabilizer doxantrazole prevented and post-WA administration reversed 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. 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.

In these experiments female rats were treated with intra-muscular β-endorphin on day 19 of pregnancy. At 7 weeks of age, peritoneal mast cells obtained from the F1-offspring contained significantly more histamine than offspring of control dams and similar results were obtained in their non-treated F2 progeny. Irrespective of the underlying mechanisms, our data provide further evidence that mast stabilizers may be an interesting therapeutic approach for IBS no matter which generation is being targeted.

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


Transfer across generations


38 Daniels WM, Fairbairn LR, van TG et al. Maternal separation alters nerve growth factor and corticosterone levels but not the DNA methylation status of the exon 1(7) glucocorticoid receptor promoter region. Metab Brain Dis. 2009; 24: 615-627.


45 Crouzet L, Gaultier E, Del'homme C et al. The hypersensitivity to colonic distension of IBS patients can be transferred to rats through their fecal microbiota. Neurogastroenterol.Motil. 2013.


