

Early Life Stress Alters Behavior, Immunity, and Microbiota in Rats: Implications for Irritable Bowel Syndrome and Psychiatric Illnesses

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Background: Adverse early life events are associated with a maladaptive stress response system and might increase the vulnerability to disease in later life. Several disorders have been associated with early life stress, ranging from depression to irritable bowel syndrome. This makes the identification of the neurobiological substrates that are affected by adverse experiences in early life invaluable.

Methods: The purpose of this study was to assess the effect of early life stress on the brain-gut axis. Male rat pups were stressed by separating them from their mothers for 3 hours daily between postnatal days 2–12. The control group was left undisturbed with their mothers. Behavior, immune response, stress sensitivity, visceral sensation, and fecal microbiota were analyzed.

Results: The early life stress increased the number of fecal boli in response to a novel stress. Plasma corticosterone was increased in the maternally separated animals. An increase in the systemic immune response was noted in the stressed animals after an *in vitro* lipopolysaccharide challenge. Increased visceral sensation was seen in the stressed group. There was an alteration of the fecal microbiota when compared with the control group.

Conclusions: These results show that this form of early life stress results in an altered brain-gut axis and is therefore an important model for investigating potential mechanistic insights into stress-related disorders including depression and IBS.

Key Words: Animal model, brain-gut axis, early life stress, IBS, maternal separation, psychiatric illness

An inability to adequately cope with stress has been implicated as an important factor in the onset and exacerbation of a wide range of disorders, from depression (1–4) to the irritable bowel syndrome (IBS) (5–7).

The early environment has a great impact on the development of behavioral and hormonal responses to stress (8–11). Consequently, events interrupting this development, such as adverse early life events, are associated with a maladaptive stress response system and might increase vulnerability to disease (12–14).

A mother's care influences both the physiological and the psychological development of the child (15). Inadequate maternal care has been linked to developmental, emotional, and social deficits in human infants (16,17) and in the rat (18).

Maternal separation is a well-established model of early life stress (19), resulting in profound and long-lasting changes in the development of the central nervous system (CNS) (20,21), including the systems that regulate stress responsiveness (22).

Maternal separation is a powerful stressor that results in permanent behavioral alterations associated with changes of the hypothalamic-pituitary-adrenal (HPA) axis (23,24). The neonatal period of a rat is characterized by a stress hypo-responsive

period (25). This hypo-response is counteracted by applying a harsh stressor such as maternal separation. Maternal separation is associated with increased corticotrophin releasing hormone (CRH) content in hypothalamic neurones and increased release in response to stress in adulthood (26).

The bidirectional communication between the brain and the gut including neural, immune, and endocrine pathways is referred to as the brain-gut axis (27–29). This axis can be affected by a variety of stressors whether it is neuroimmunological or neuroendocrinological (27). Given the fact that adverse early life events affect the adult stress response, these in turn can influence the manner in which the brain-gut axis responds to certain stressors (27).

Because there is a high comorbidity between functional bowel and stress-related psychiatric disorders (30,31), it is of interest to investigate whether the symptoms of both disorders can be recapitulated in an animal model of early life stress. Therefore, the aim of the following study was to assess the effect of early life stress in the form of maternal separation on the brain-gut axis in respect to behavior, immune responses, endocrine parameters, visceral pain, and gut microbiota. We have shown in previous studies that maternal separation results in an altered central serotonin system (32).

Methods and Materials

Animals

Two cohorts of 2x22 Sprague Dawley rat pups were used in these studies. The pups were housed with their mothers in plastic cages (15 × 22 × 9 cm). The animal room remained temperature-controlled (20 ± 1°C) and on 12-hour light/dark cycle (lights on at 7:00 AM).

There were two groups: maternally separated (MS) (*n* = 11) and non-separated (NS) (*n* = 11). This study was powered to detect differences at the .05 level. The ethical committee of University College Cork approved the experimental procedure.

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Separation Procedure

Adopted from Neumann *et al.* (33): on postnatal day (PND) 2 the MS animals were removed from their home cages and placed into plastic cages maintained at 30°–33°C, in a separate room. Separations lasted for 3 hours/day until PND 12 from 9:00 AM to 12:00 PM. The NS group subjects were left undisturbed with their mothers.

Novel Stress

At 7–8 weeks the behaviorally naïve rats were tested for responsiveness to a novel stress, the open field (circular white arena 90 cm in diameter with 40-cm-high walls and brightly lit [900 lux]). The animals were placed in this arena for the four 5-min trials. The fecal pellets were counted and stored in 96% ethanol.

Colorectal Distension

A separate group of animals were used to assess the effect of MS on visceral sensation. The separation procedure and the controls were maintained: MS animals ($n = 11$), NS ($n = 11$). Each animal was lightly anaesthetized with isoflurane, and a latex balloon (6 cm in length) was inserted into the colon, 1 cm from the anus. The animals recovered for 10 min before the colorectal distension (CRD). The balloon was distended from 0 to 80 mm Hg over 8 min, during which 2 parameters were measured: 1) the threshold pressure (mm Hg) that evokes a visually identifiable visceral pain behavior, and 2) the cumulative number of visceral pain behaviors. Postures defined as visceral pain-related behaviors were stretching, abdominal retractions, and/or abdominal withdrawal reflex. The animals were tested in a random fashion, and the experimenter was blind to the individual groups.

Endocrine and Immune Measurements

Five days after the novel stress, animals were killed from 9:00 AM until 12:00 PM in a random order by decapitation.

The trunk blood was collected in ethylenediaminetetraacetic acid (EDTA)-coated tubes. A sample of whole blood was set aside for stimulation. The rest of the whole blood was centrifuged at maximal speed for 15 min at 4°C. The plasma was removed.

Whole Blood Stimulation

Twenty microliters of sample was diluted with 1800 μ L of Dulbecco's Modified Eagle's Medium containing lipopolysaccharide at a final concentration of 1 μ g/mL. This was repeated for each sample without lipopolysaccharide also to account for the un-stimulated control. The whole blood was incubated (5% carbon dioxide) for 48 hours at 37°C, after which the supernatant was drawn off.

Flow Cytometry

The BD Biosciences Pharmingen, (San Diego, California) cytometric bead array rat soluble protein flex sets were used for the analysis of the cytokine concentration in the whole blood. The pro-inflammatory cytokines interleukin (IL)-6, tumor necrosis factor (TNF)- α , interferon (IFN)- γ , and the anti-inflammatory cytokines IL-10 and IL-4 were analyzed. The range of standards was 0–20,000 pg/mL.

Corticosterone Immunoassay

The plasma corticosterone concentrations were analyzed with an immunoassay (R&D systems, Oxfordshire, United Kingdom). The sensitivity of this assay is < 27.0 pg/mL.

Extraction of Bacterial DNA From Fecal Samples

The DNA was extracted from 250 mg of stool material with a FastDNA SPIN kit (Q-BIO gene, Irvine, California).

Polymerase Chain Reaction of Partial 16S Ribosomal RNA Gene Fragments and Analysis by Denaturing Gradient Gel Electrophoresis

All DNA samples were amplified with the primers 968FGC (5'-CGC CCG GGG CGC GCC CCG GGC GGG GCG GGG GCA CGG GGG GAA CGC GAA GAA CCT TAC-3') and 1401R (5'-CGG TGT GTA CAA GAC CC-3') to target the V6-8 region of the 16S ribosomal RNA gene.

Denaturing Gradient Gel Electrophoresis (DGGE) was performed with a DCODE universal mutation detection system run at 60°C, 85V for 16 h.

Profile Analysis of 16S Ribosomal RNA Gene DGGE Patterns

The DGGE profiles were analyzed with the Gel Compar II (Applied Maths, Sint-Martens-Latem, Belgium). Similarities between samples were determined by calculating similarity indices on the basis of the Dice's similarity coefficient and the un-weighted pair group method with arithmetic averages (UPGMA). Two identical profiles create a value of 100%, whereas two completely different profiles result in a value of 0%.

Statistical Analysis

All data were normally distributed according to Gaussian distribution analysis. All data are expressed as mean \pm SEM. Student *t* tests were conducted in all *in vivo* studies except for the cumulative number of visceral pain-related behaviors, where a random coefficient power analysis was used. A two-way analysis of variance (ANOVA) was used to analyze the CRD and the open field data globally. Dendrograms of DGGE banding profiles were constructed to visualize any clustering patterns evident and to generate similarity matrixes for numerical and subsequent statistical analysis. All similarity results given are the Dice's and UPGMA percentage similarities, because this method (band based) and Pearson correlation coefficient (curve based) were in agreement (data not shown). A Student *t* test was used to analyze the data.

A *p* value of < .05 was considered significant.

Results

Novel Stress/Open Field

The average number of fecal pellets produced by the MS group was significantly greater ($3.1 \pm .5$ vs. $1.7 \pm .3$) compared with the NS group [$t(22) = 2.45, p < .05$].

CRD

In the MS group there was a decrease in the threshold ($p < .05$) (Figure 1A), compared with control animals. Moreover, there was also an increase in the number of cumulative pain behaviors ($p < .05$) (Figure 1B). A two-way ANOVA revealed an interaction between pressure and maternal separation [$p < .001, F(7,160) = 4.18$]. There was also an effect of pressure [$F(7,160) = 29.12$] and treatment [$F(1,160) = 45.51$].

Corticosterone Concentrations

The corticosterone levels in the plasma of the MS group was significantly greater than that of NS animals [$t(20) = 3.0, p < .01$] (Figure 2).

Immunology

The concentration of TNF- α in the stimulated whole blood in the MS group was significantly higher compared with NS animals [$t(20) = 2.8, p < .05$] as was IFN- γ [$t(20) = 2.61, p < .05$]. There was no significant difference between the groups with regard to the other cytokines (see Table 1).

Microbiota

The % similarity of the DGGE profiles of fecal pellets from the NS group was $75.2 \pm 16.8\%$, whereas that of the MS group that received the placebo was $56.9 \pm 21\%$, indicating that MS animals differed significantly from their NS counterparts ($p < .05$).

Discussion

The MS animals displayed diverse phenotypic changes, which is indicative of comorbid anxiety/stress and functional bowel disorders. These occur at a multifunctional level with behavioral alterations suggestive of increased anxiety and visceral hypersensitivity, physiological alterations including elevated HPA-axis function, and increased systemic immune responses; finally, perturbations in gut microbiota were also observed. Together, these data indicate that early life stress induces persistent

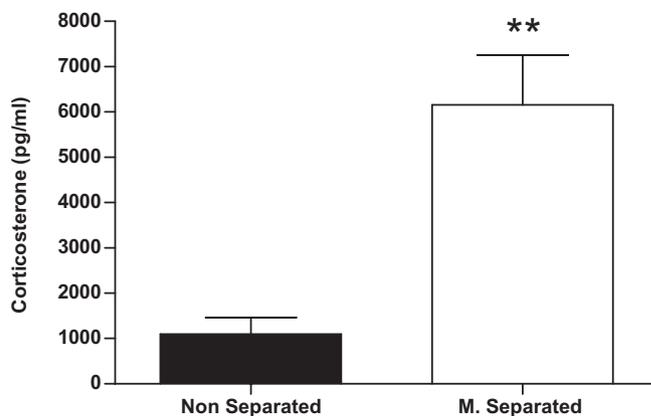


Figure 2. The average concentration of plasma corticosterone from each group. $**p < .01$.

changes that contribute to symptoms of IBS and psychiatric disorders in adulthood.

The studies presented here offer a multifaceted view of the maternal separation model and its impact on brain-gut axis. Our studies clearly demonstrate that such a model can be robustly generated and is ideally positioned to allow further mechanistic studies and ultimately discover novel therapeutic targets.

This study clearly demonstrates, as others have (12), that there is an altered stress system in adulthood after early life stress. It has been noted in not only depression but also IBS that early life stress plays a role in the onset (34), and adult stressors play a role in the exacerbation (35,36). We feel that the maternal separation model we employ is a useful study tool for disorders associated with early life stress and construct and face validity as an animal model of IBS and psychiatric disorders.

Anxiety-like behavior in rodents is typically associated with a decrease in movement and lack of exploration in the open field (37). The MS animals displayed this behavior in response to the novel stress (Supplement 1). Because stress-induced increases in colonic motility have been reported in rodents (38), we sought to determine whether differences in stress responsiveness in the adult rat are also reflected in differences in stress-induced colonic motility. Thus, we quantified the fecal pellet output in response to the open field-stress and demonstrated that the MS animals have an increase fecal pellet output. This observation is indicative of an altered brain-gut axis as stress, through central corticotropin-releasing factor-1 receptors signals the autonomic nervous system, particularly the parasympathetic, to increase

Table 1. Concentrations of Pro- and Anti-Inflammatory Cytokines in the Stimulated and Unstimulated Whole Blood of the Two Groups

Cytokine	Unstimulated (pg/mL)		Stimulated (pg/mL)	
	NS	MS	NS	MS
TNF- α	43.5 \pm 11.4	24.9 \pm 4.6	63.5 \pm 9.9	102.6 \pm 9.6 ^a
IL-6	22.4 \pm 7.4	16.8 \pm 5.2	21.9 \pm 7.4	34.4 \pm 4.7
IFN- γ	13.0 \pm 1.9	14.5 \pm 1.5	8.9 \pm 1.6	13.9 \pm 1.1 ^a
IL-4	5.3 \pm .5	5.3 \pm .7	4.2 \pm .4	5.1 \pm .7
IL-10	48.8 \pm 13.4	30.9 \pm 7.5	80.3 \pm 19.5	97.8 \pm 17.2

Maternal separation (MS) induced an alteration in the immune response after a challenge with lipopolysaccharide. There was an increase in the concentration of the pro-inflammatory cytokines tumor necrosis factor (TNF)- α and interferon (IFN)- γ in the stimulated whole blood samples of the maternally separated group compared with the control group (NS).

^a $p < .01$ MS vs. NS; $n = 11$ MS and $n = 11$ NS.

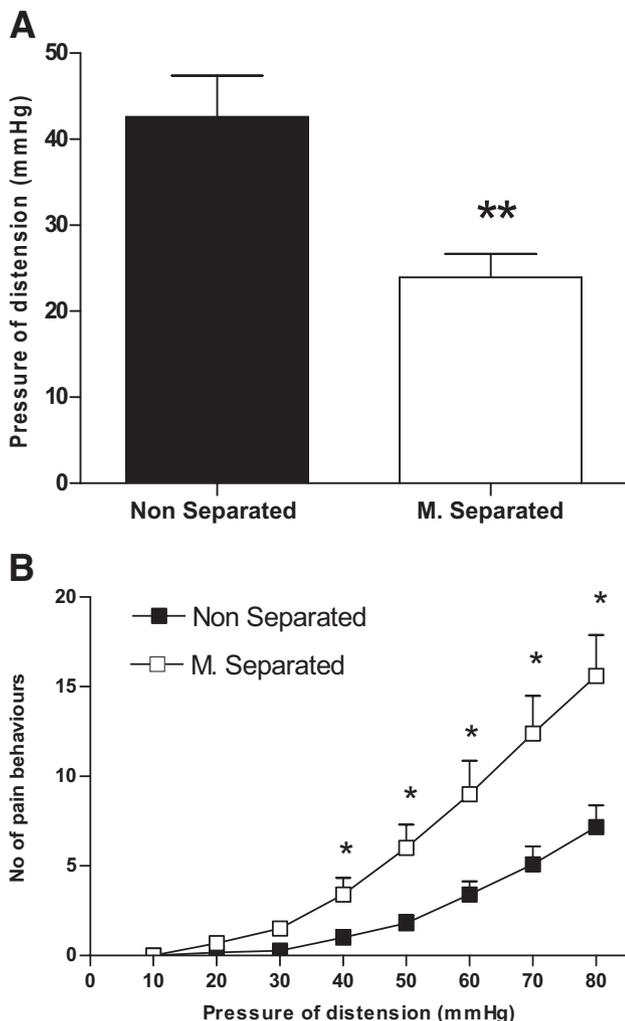


Figure 1. (A) The average threshold value (pressure at which the animal shows the first pain behavior) for each group. $**p < .01$. (B) The cumulative number of pain behaviors of each group. $*p < .05$.

colonic motility (38,39). Therefore, it is possible to conclude that early adverse experiences such as maternal separation lead to an abnormal behavioral stress response in adulthood.

In this study the MS rats displayed an enhanced visceral hypersensitivity. Research has shown that stress, in particular that experienced in early life, triggers long-term changes in visceral functions and sensitivity (40,41). Functional brain imaging studies of IBS patients have shown altered central pain modulatory circuitry demonstrating the significant role that the central nuclei play in the development and maintenance of visceral hyperalgesia (42). Therefore, early life stress might play a role in the sensitization of these nuclei and be implicated in visceral hypersensitivity. Interestingly, our data demonstrate that early-life stress-induced visceral hypersensitivity is manifested without the need of an acute adult stressor, which has previously been shown to be a prerequisite of the phenotype (43). Although reasons for such differences are not overtly apparent, it further increases the utility of the model as we employ it.

We demonstrated that, consistent with other studies (44), the MS rats had higher plasma levels of corticosterone than the NS animals. This alteration of the HPA axis might be due to the interruption of the precise developmental pattern of the axis by maternal separation. Maternal separation initiates a chain of events that results in an increase in plasma corticosterone levels (45). The sensitivity of the glucocorticoid feedback is decreased due to downregulation of glucocorticoid and mineralocorticoid receptor gene expression in the CNS, particularly the hippocampal region CA1 and the paraventricular nucleus (46). Maternal care plays a significant role in the development of the HPA axis, although other factors can contribute also (47).

Sudo *et al.* (29) showed that exposure to microbes at an early developmental stage is required for a fully functional HPA axis. Our current data use an alternative approach but confirm that there is a link between stress physiology and gut microbiota. Here, stress during early life caused marked population-based alterations in the fecal microbiota of MS animals. The diversity of the bacteria within each group was measured, and the MS animals seemed to have a disrupted microbiota, which might be due to an increase in coliforms, as can happen after stress (48). Although we did not specifically analyze the different types of bacteria within the gut, our analysis clearly demonstrates that there is a markedly altered fecal microbiota compared with the uniform content in NS control subjects. Future elaborated metagenomic approaches might help delineate the specificities of such changes, as has recently been observed in animal models of obesity (49).

The immune system is a central coordinator of brain-gut axis function. Thus we analyzed whether there was shift to a pro-inflammatory phenotype in rats subjected to maternal separation. The MS animals had a significantly increased immune response compared with control subjects. There was a significant increase in the pro-inflammatory cytokine TNF- α and IFN- γ and a trend toward an increase in IL-6. This effect was also noted in mice after a neonatal stress (50). Pro-inflammatory cytokines, especially IL-6, are potent activators of the HPA axis (50). Persistent activation of this axis might lead to a downregulation of the glucocorticoid receptors that are involved in the negative feedback controlling the HPA axis. This would result in an overactive HPA axis and a failure to suppress an inflammatory response. This might be the case in our study, because there was an elevation of corticosterone as well as an increase in inflammatory cytokines in the MS animals.

This increase in inflammation was somewhat specific, be-

cause it was not seen when the Peyer patches and the mesenteric lymph nodes (MLN) were analyzed (Supplement 1), although there was a trend toward a decrease in both pro- and anti-inflammatory cytokines in the MLN of the MS animals.

In conclusion, we have established that early life stress can induce multiple changes across the brain-gut axis that might contribute to the susceptibility to develop stress-related disorders such as IBS and psychiatric disorders in adulthood. Moreover, it confirms that the natural history of both functional bowel and psychiatric disorders might overlap and contribute to shared symptomatology. We do appreciate that there are differences in the pathologies/etiologies of IBS and depression/anxiety; yet there is considerable comorbidity and overlap (6). Having a robust animal model with multiple alterations across the regulation of the brain-gut axis will have important implications for development of novel therapeutic strategies to combat stress-related and psychiatric and gastrointestinal disorders.

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Supplementary material cited in this article is available online.

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