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Maternal separation in rodents: a journey from gut to brain and nutritional perspectives

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The developmental period constitutes a critical window of sensitivity to stress. Indeed, early-life adversity increases the risk to develop psychiatric diseases, but also gastrointestinal disorders such as the irritable bowel syndrome at adulthood. In the past decade, there has been huge interest in the gut–brain axis, especially as regards stress-related emotional behaviours. Animal models of early-life adversity, in particular, maternal separation (MS) in rodents, demonstrate lasting deleterious effects on both the gut and the brain. Here, we review the effects of MS on both systems with a focus on stress-related behaviours. In addition, we discuss more recent findings showing the impact of gut-directed interventions, including nutrition with pre- and probiotics, illustrating the role played by gut microbiota in mediating the long-term effects of MS. Overall, preclinical studies suggest that nutritional approaches with pro- and prebiotics may constitute safe and efficient strategies to attenuate the effects of early-life stress on the gut–brain axis. Further research is required to understand the complex mechanisms underlying gut–brain interaction dysfunctions after early-life stress as well as to determine the beneficial impact of gut-directed strategies in a context of early-life adversity in human subjects.

Gut microbiota: Probiotics: Prebiotics: Intestinal permeability

Mounting evidence suggests a pivotal role of gut microbiota in the aetiology of psychiatric symptoms in stress-related diseases such as anxiety disorders and depression[1,2]. The mechanisms underlying this microbiota–gut–brain communication are beginning to be unravelled (see[3–5] for reviews). In particular, certain gut bacteria can have a beneficial effect on mood and emotional behaviour and, as such, have been proposed for potential therapeutic interventions in psychiatry (concept of psychobiotics)[6,7]. The bidirectional interplay between gut and brain is illustrated in population survey studies revealing a strong correlation between anxiety, depression and functional gastrointestinal (GI) disorders. Furthermore, psychological distress can predict later onset of a functional GI disorder and the converse is also true[8].

Early postnatal life is a critical period during which both brain and gut undergo important maturation[9,10]. Moreover, this maturation is greatly influenced by gut microbiota colonisation and diversification during the lactating period. Exposure to stressful events during childhood has been repeatedly associated with increased vulnerability to both psychiatric and GI disorders such as the irritable bowel syndrome (IBS)[11–13]. IBS is defined as a disorder of the gut–brain interaction. According to Rome IV classification, it is characterised by abdominal pain and altered bowel habits[14], but also increased intestinal permeability and gut dysbiosis. Chronic disruption of the mother–infant relationship in rodents, best known as maternal separation (MS), is a useful preclinical tool since it models the co-morbidity between IBS and psychiatric disorders.

Abbreviations: BDNF, brain-derived neurotrophic factor; CRF, corticotrophin releasing hormone; FOS, fructo-oligosaccharides; GOS, galacto-oligosaccharides; GI, gastrointestinal; GF, germ-free; GR, glucocorticoid receptor; HFD, high-fat diet; HPA, hypothalamic–pituitary–adrenal; IBS, irritable bowel syndrome; MS, maternal separation; PFC, prefrontal cortex; PND, post-natal day; PVN, paraventricular nucleus.

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Indeed, it induces a wide range of brain and gut alterations in offspring[15]. In the following, we concisely overview the adverse consequences of MS, which is the most used model of early adversity in gut–brain axis research. We then discuss the effects of gut-directed interventions on the microbiota–gut–brain axis, with a particular focus on stress-related behaviours.

The maternal separation model

Pioneering work from Harlow in non-human primates and Levine, Denenberg, Meaney and Plotsky in rodents has shown that the early environment, in particular the quality of maternal care, shapes emotional behaviour as well as stress responsivity in adult life[16–20]. The work of Hofer also revealed the deleterious impact of early weaning on offspring physiology, including intestinal physiology[21]. Since then, a vast body of literature has documented the effects of early mother–infant separations in rats during the first weeks of life (1–3 weeks). The most common MS paradigm consists in daily 3 h separations between postnatal days (PND) 2 and 14[22]. However, there are other models using different separation durations (3–8 h daily) or an acute 24 h separation[23–26]. MS results in different degrees of perceived stress in dams and pups according to the protocol used (litter isolated in the homecage without the mother or litter isolated in a novel environment; pups individually separated or not; undisturbed control or 'handling' i.e. short separation episode (15 min)). The different models and their respective effects are reviewed in[27–29]. In any case, pups are deprived of maternal care during the separation period. Importantly, the absence of the dam implies that the pups cannot benefit from dams' heat and milk. Temperature issues can be easily corrected by maintaining the room at 28–29°C during separation sessions. However, the lack of milk intake likely contributes to the short and long-term effects of 24 h MS[30,31]. Mother–infant separation-based models have also been developed in other rodents (e.g. guinea pigs and mice) and in primates (rhesus macaques)[32], but the largest literature still involves rats, with mice being more and more used; we will focus on these rodent species in the present review. It appears that mice are less sensitive to early-life stress than rats[33] (see[34] for review). This might be attributable to species specificities in neurodevelopment and maternal care patterns. Another possible reason is that mouse studies more often involve inbred strains (while outbred strains are used in rats) as well as transgenic strains that exhibit different levels of sensitivity to stress[34]. To produce significant behavioural alterations in mice, MS is often combined with others stressors such as unpredictable stress in dams[35,36], early weaning[37] or a combination of perinatal stressors[38].

Maternal separation and emotional vulnerability

Long-term psychoneuroendocrine alterations

Behaviour. The long-term consequences of MS on emotional behaviour have been extensively documented. Available tools to evaluate emotionality are mostly limited to tests with good predictive validity (i.e. sensitive to anxiolytics or antidepressants) such as the elevated plus maze, open-field or light–dark tests for anxiety and the forced swimming test or tail suspension test for depression. These tests have however a poor construct validity contrary to other tests such as sucrose preference or female urine sniffing tests used to assess reward deficiency as index of anhedonia (see[39–41]).

Typically, MS leads to increased anxiety- and depressive-like behaviours. Indeed, animals exposed to MS during early-life display reduced exploration of the open areas in the elevated plus maze, light–dark box and open-field tests compared with non-separated controls[42–72]. Moreover, it has been shown that exposure to a novel stress at adulthood aggravates these anxiety-like behaviours[73,74]. Numerous studies also report increased depressive-like behaviours in the forced swimming test or tail suspension test. Indeed, adult MS rodents show greater immobility time in these tests compared with controls[42,53,58,59,61,62,66,75–91]. MS has been associated with decreased sucrose preference[69,71,75–77,79–81,83,87,92–96] and decreased social behaviour with a conspecific[67,72,93,97–99]. The effects of MS are not limited to the above alterations of emotional behaviours; numerous studies also report that MS exacerbates motivation for alcohol and drugs of abuse (see[100] for review).

Finally, several studies have also shown deleterious effects of MS on cognition (see[101] for review). Briefly, these effects include impaired hippocampal-dependent spatial learning and memory[47,67,79,102–107], altered non-spatial memory[44,105,107–115] and impairments in prefrontal cortex (PFC)-dependent tasks (working memory, extinction, cognitive flexibility)[50,72,107,116–120]. In contrast, amygdala-dependent aversive memory (e.g. fear conditioning) seems to be enhanced by MS[121–127].

Endocrine response and neurobiological correlates. MS exerts long-lasting effects on hypothalamic–pituitary–adrenal (HPA) axis function, leading in most of the studies to endocrine hyper-responsivity to a novel stress[19,47,108,128–135]. Within the central nervous system, this HPA axis hyper-reactivity is associated with an up-regulation of corticotrophin-releasing hormone (CRF) expression in the paraventricular nucleus (PVN) of the hypothalamus and amygdala but also with high CRF concentration and increased CRF receptor density in the locus coeruleus and raphe nucleus[19,130,131,136] (see[137]) as well as altered oxytocin and vasopressin expression (either up- or down-regulated) in the PVN (see[138] for review). MS also decreases glucocorticoid receptor (GR) expression in the hippocampus and PFC[121,139], two main brain areas involved in HPA axis negative feedback. Numerous neurotransmission systems are affected by MS. MS decreases the number of type A γ-aminobutyric acid (GABA-A) receptors in noradrenergic neurons of the locus coeruleus and in the nucleus tractus solitarius[46] and hippocampus[96]. The gabaergic system plays a role in CRF synthesis inhibition in the central amygdala, allowing a buffering of the noradrenergic response to stress. In addition, MS impairs glutamatergic[140–142], serotonergic[48,61,83,143–147],

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dopaminergic[64,145,148–153], opioidergic[152,154] and endocannabinoidergic[66] transmission. In the central nervous system, serotonin is involved in neuronal development[155], emotionality and also pain modulation[156,157]. Among other effects, MS reduces the expression of the serotonin transporter in the raphe nucleus[143]. Interestingly, selective serotonin reuptake inhibitor antidepressants such as paroxetine normalise HPA axis function as well as emotional behaviour in MS rats.

MS induces both functional and structural changes in several brain regions including the PFC, hippocampus, amygdala and nucleus accumbens[150,158–163]. More specifically, impaired synaptic long-term potentiation, dendritic atrophy as well as reduced dendritic spine density have been reported in the medial PFC and hippocampus of adolescent and adult MS rats[63,68,97,104,116,140,153,158,164–171]. By contrast, MS induces dendritic hypertrophy in the amygdala[57]. A recent study reported that mice deficient for motopsin, a serine protease secreted from neuronal cells to induce filopodia, precursor structures of dendritic spines, are resistant to MS-induced increase in anxiety in the open field test[111]. In addition, it has been shown that MS leads to hypomyelination in the medial PFC[72].

MS is also accompanied by decreased expression of neurotrophins such as nerve growth factor and brain-derived neurotrophic factor (BDNF), that are known to play critical roles in dendrite growth and spinogenesis[22,47,74,85,172,173] (see[174] for review). In addition, MS leads to alterations of hippocampal neurogenesis (either decreased or increased) at adulthood[112,175–177]. Interestingly, decreased hippocampal BDNF and neurogenesis are consistent observations in post-mortem brains of depressed subjects and there is mounting evidence that BDNF is involved in emotional vulnerability (see[178] for review).

Peripheral and central inflammation. There is substantial evidence that MS activates inflammatory processes both systemically and within the central nervous system, although the underlying mechanisms remain to be explored. Indeed, increased circulating levels of IL-1β[71] and IL-6[179] have been reported in MS animals. In addition, MS offspring display neuroinflammatory marks such as increased *Tnfa*, *Il-1b* and *Tlr4* expression or increased reactive oxygen species levels and decreased *Il-10* expression in the hippocampus[71,75,87,114], PFC[71] and PVN[180]. Recent studies have shown a decrease in the levels of the astrocytic marker GFAP (glial fibrillary acidic protein) in the PFC of MS animals[91] and the opposite effect in the locus coeruleus of MS females only[181].

Inconsistencies in the maternal separation literature

A number of studies did not replicate the abovementioned findings, reporting no alteration of certain emotional behaviours[33,43–45,50,63,67,73,74,87,89,95,105,112,136,182–192], cognitive function[47,107,120,185,193–197] or HPA axis signalling[48,112] in male or in female MS animals. In addition, others studies reported opposite effects (e.g. lower anxiety or lower depressive-like behaviour)[43,94,96,98,105,110,135,182,198–202].

In some cases, these discrepancies may be attributed to the use of different MS protocols (number of separated pups, separation duration and control group), age of investigation, animal strain and sex, housing conditions (individual or collective cages, light–dark cycle, enrichment), but also other testing protocol issues (e.g. habituation prior testing, brightness, sucrose concentration for the sucrose preference test). Notably, the vast majority of the findings were obtained using males only. However, numerous recent studies report sex-specific behavioural alterations in MS animals.

Nevertheless, differential effects of MS have also been reported in studies using the same MS protocol, age, sex, strain or type of stressor. A recent study suggests that the effects of early adversity (maternal immune activation) depend upon the gut microbiota profile of the dams, in particular the presence of commensal segmented filamentous bacteria (which differs across animal suppliers, i.e. Jackson Laboratories and Taconic Biosciences)[203]. Therefore, the gut microbiota profile may also influence the susceptibility to MS.

Possible early mechanisms at the origin of maternal separation programming

The mechanisms underlying the long-term effects of MS are not fully understood. Multiple, possibly synergistic effects in both dams and pups have been reported (see[204] for review).

Mother–infant communication and maternal care. Maternal care is thought to play an important role in brain maturation and later vulnerability to stress. It has been established that rodent pups vocalise in response to isolation (30–90 Hz ultrasounds)[205,206] and MS has been shown to increase the number of these vocalisations compared with undisturbed pups in several mouse strains[50]. Because these isolation calls elicit retrieval behaviour in the mother, they are thought to serve mother–pup communication and stimulate maternal care towards their pups[207,208]. In the MS model, pups are deprived of maternal care during several consecutive hours, which may constitute a mechanism for the adverse effects of this early-life stress. Indeed, it has been demonstrated that the long-term behavioural effects of acute 24 h MS can be prevented by pup tactile stimulation[31]. Nevertheless, the role of maternal care in the long-term effects of MS remains controversial.

MS also constitutes a potent stressor for the dams. Indeed, it has been reported that this psychological stress induces anxiety and depressive-like behaviours in dams[209–211]. As a matter of fact, several studies suggest that dam's perceived stress plays an important role in the effects of separation in the offspring. Interestingly, MS-induced HPA hyper-response to stress in the offspring can be counteracted by providing a foster litter to the dam while its own litter is being separated[212]. Furthermore, it has been reported that the offspring of dams with an experience of separation with a previous litter exhibit MS-like fear behaviour without direct exposure to the early stress[213].

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Endocrine, immune and neurobiological effects of maternal separation in developing pups. The HPA axis is almost silenced during a short window of early postnatal development (i.e. from PND4 to 14[214–217]). This stress hypo-responsive period is characterised by extremely low basal corticosterone levels in the plasma as well as blunted adrenocorticotropic hormone and corticosterone response to stress. Nevertheless, this stress hypo-responsive period is not absolute, since a potent stressor such as MS is able to induce HPA axis activation[217–220]. It has been proposed that stress and immune activation result in a cross-sensitisation of both systems that possibly creates a self-perpetuating cycle contributing to the emergence of the alterations in animals subjected to early stress. Bacterial translocation into the liver and the spleen has been detected after MS in juvenile PND10 rats[221]. In addition, altered circulating pro-inflammatory IL-1β, IL-6 and TNFα were observed in MS pups[86,114,118,179,222,223]. Furthermore, MS juveniles display increased activated microglia in the PFC and hippocampus[53] and decreased number of astrocytes in the same areas[91,224,225] along with increased *Il-6*, *Il-1b* and *Tnfa* expression compared with controls[65,222]. Increased microglia numbers and activation patterns have also been recently reported in the nucleus of the solitary tract of MS juveniles[91,226]. Interestingly, increased cytokine expression and microglial density have also been reported in the hippocampus of juvenile mice submitted to short MS (15 min) from PND1 to PND21, which led to increased anxiety similar to prolonged MS[227].

Both altered HPA axis activity and neuroinflammation during development have been shown to be deleterious for the immature brain. MS disrupts the normal course of brain development and produces functional and structural alterations including delayed GABA excitatory-to-inhibitory functional switch[110], delayed synaptic maturity[228], decreased spine density[161] and increased neuronal and glial cell death[175,229,230]. Altered expression of neurotrophins such as BDNF and nerve growth factor in separated pups could contribute to these effects[229,231,232]. In addition, MS disturbs the serotonergic system during development. Indeed, reduced expression of the serotonin receptor 5HTr1A in the hippocampus and PFC has been reported in 7-d-old pups [233]. A recent study demonstrates that transient juvenile, but not adult, knockdown of orthodenticle homoeobox 2 in the ventral tegmental area mimics early-life stress by increasing stress susceptibility, whereas its overexpression reverses the effects of early-life stress[234]. Moreover, developmental decrease of the transcription repressor Rest4 (RE-1 silencing transcription factor 4) in the PFC of pups submitted to MS may play a causal role in the long-term effects of MS[67,89]. We recently demonstrated that exposure to a high-fat diet (HFD) during the perinatal period can prevent the long-term MS-associated neurobehavioural alterations, possibly via a protective effect on gene expression in the PFC[67]. Indeed, perinatal HFD prevented the MS-induced alterations of Rest4, Bdnf and 5HTr1A expression in this brain area. A recent work demonstrated that chemogenetic inhibition of MS-induced neuronal hyperactivity in the lateral habenula of mice aged 35 d attenuates depressive-like behaviours[37].

Epigenetic changes in maternal-separation offspring. Epigenetic marks are dynamic and highly sensitive to environmental factors; furthermore they can last in time and even be transferred across generations[235]. As such, they represent a potential mechanism that could underlie the long-term effects of early-life stress[236–239]. Indeed, a number of studies have reported persistent epigenetic marks in the genome of animals submitted to MS (see[240] for review). In particular, changes in DNA methylation of specific regulatory sites in key genes for stress processing such as Crf, Avp, GR or Bdnf in the PVN, hippocampus and PFC of maternally separated animals, have been documented[197,241–245]. It has been shown that administration of a DNA methyltransferase inhibitor prevents the decreased prefrontal Bdnf mRNA expression induced by MS[244]. Moreover, DNA methylation in the offspring has been shown to be associated with the level of maternal care[246]. Nonetheless, the group of Mansuy provided evidence for epigenetically-mediated transmission of behavioural traits induced by early-life stress across generations irrespective of cross fostering[247].

Another major epigenetic process is histone modification, especially acetylation by histone acetyltransferases or deacetylation by histone deacetylases. Histone acetylation patterns as well as histone acetyltransferase and histone deacetylase expressions in the brain are also altered by MS[248]. For instance, MS leads to decreased Bdnf and GR mRNA expressions in the hippocampus, and these effects were accompanied by decreased levels of histone acetylation at their respective promoters[249,250]. Furthermore, a recent study suggests that there is a cross-talk between histone acetylation and DNA methylation[197]. Indeed, treatment with a histone deacetylase inhibitor reversed the MS-induced increased DNA methylation in the GR promoter region.

Finally, the possible role of brain miRNA in mediating the long-term effects of MS has been addressed in a few studies. Uchida and colleagues were the first to report changes in expression of several miRNA in the PFC of MS rats[89]. Another MS study reported an increase in miR-16 in the hippocampus that was negatively correlated with Bdnf expression in the same brain area and also negatively correlated with sucrose preference[77].

Maternal separation as a model of irritable bowel syndrome: impact on the gastrointestinal tract

As mentioned earlier, MS is also widely used as a model of IBS (see[15,251,252] for reviews). In addition to its effects on stress vulnerability, it leads to several GI dysfunctions, in particular increased visceral sensitivity to painful stimuli, and increases the vulnerability to experimental colitis.

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Effects of maternal separation on the enteric nervous system, visceral sensitivity and motility. MS induces dynamic structural and functional changes in the enteric nervous system[253,254]. For instance, MS increases nerve density and synaptogenesis in juveniles, but these effects are no longer present at adulthood[253]. In contrast, the levels of the neuronal marker PGP 9·5 (anti-protein gene product 9·5) in the colon are increased in adult MS animals but not in juveniles. Interestingly, early-life adversity has been shown to affect enteric nervous system development in a sex-dependent manner, with females being more sensitive than males[255]. MS also produces increased intestinal motility in response to stress, as evidenced by reduced total transit time and increased number of faecal pellets[81,256–260]. It has been extensively reported that MS rats display visceral hyperalgesia during colorectal distension[51,67,140,144,146,147,180,220,256,257,259–283]. A recent study demonstrated that MS-induced visceral hypersensitivity is dependent on Paneth cell defects and associated *Escherichia coli* expansion in the gut[284]. MS-induced visceral hypersensitivity is lost in mice deficient for Toll-like receptor 4 (TLR4)[180]. This study suggests that TLR4 signalling in the PVN mediates increased CRF immunostaining and visceral hypersensitivity associated with MS. Interestingly, multiple MS-induced intestinal phenotypes, including visceral hyperalgesia and gut leakiness, can be prevented by CRF receptor antagonist administration[180,259,285–287]. GR antagonists or agonists of the metabotropic glutamate receptor type 7 (mGluR7) also prevent stress-induced visceral hyperalgesia[278,288–290].

The hyper-sensitivity to colorectal distension after MS is larger in females than in males and visceral hyperalgesia is greater when all pups are separated from the dam than when only half of littermates is removed, suggesting that sex and dam's perceived stress play a role in the long-term effects of MS on visceral sensitivity[277]. Indeed, it has been demonstrated that MS-induced visceral hypersensitivity is transferred across generations and that this effect likely depends upon maternal care[291].

Effects of maternal separation on gut microbiota composition. A growing number of studies have reported altered gut microbiota composition in MS animals. However, the use of different species, strains, sex, MS protocols, nature of the sample, microbiota analysis method and age of investigation renders between-studies comparisons difficult, and yet, there is no clear microbial pattern associated with MS.

The first study that has investigated the effects of MS on the gut microbiota was carried out by Bailey and Coe in rhesus monkeys[292]. The authors investigated the stability of gut microbiota 3 d after separation and found a significant decrease in faecal bacteria, in particular from the *Lactobacillus* genus. A few years later, O'Mahony and colleagues reported overall reduced bacterial diversity in MS rats *v.* controls[293]. This finding has been replicated in more recent studies[294,295]. However, another recent study reports no change in diversity[64]. Qualitatively, MS was shown to increase the Firmicutes:Bacteroidetes ratio at the phylum level in some studies[49,286,295,296], but again this finding is not consistent across studies as some report opposite[297] or no effects[295]. A consistent finding, however, is that the effects of MS on microbiota composition vary both qualitatively and quantitatively with respect to the age of investigation. Indeed, several studies comparing at least two time points show completely different patterns[64,295,298,299]. Overall, *Bacteroides* and Lachnospiraceae (*including Clostridium XIVa*) species seem to be consistently altered (either enriched or depleted) across several studies[49,258,295,300]. Interestingly, it has been shown that changes in several bacterial taxa after MS are abrogated by adrenalectomy, suggesting that corticosterone signalling in response to stress is responsible for at least part of its effects on the microbiota[42]. More studies using global 16S-sequencing approaches are needed to better document the effects of MS on gut microbiota and potentially identify candidate species or genera associated with the behavioural effects of MS. Furthermore, considering the importance of sex differences in both stress effects and basal gut microbiota composition, more studies should be conducted in both males and females[38,296].

Effects of maternal separation on the gut mucosa. MS has been associated with alterations in the differentiation and distribution of enteroendocrine cells in the gut epithelium[301] and a defect in Paneth cells[276,284]. Notably, the numbers of enterochromaffin cells in the colon are increased in MS animals compared with controls[264,265,275]. Accordingly, MS animals exhibit substantial increases in the levels of circulating and colonic serotonin (mainly produced by enterochromaffin cells)[144,147,264,265,275,282].

In addition, MS animals were shown to display colonic tissue damage including decreased crypt length and altered number of goblet cells and are more engaged in epithelial cell proliferation[262,286,302–304]. Moreover, MS rats show more colonic damage after dextran sulphate sodium or 2,4,6-trinitrobenzenesulphonic acid-induced colitis than non-stressed animals and as a result, they also lose more weight, indicating that they are more sensitive to experimental colitis[305–307]. There is mounting evidence that MS produces long-term gut paracellular and transcellular hyper-permeability to ions and macromolecules[81,262,272,276,299,302,306,308–311]. Remarkably, stress-induced intestinal hyperpermeability appears to be glucocorticoid-dependent, as it is evoked by the synthetic glucocorticoid dexamethasone and prevented by administration of a GR antagonist, similarly to an inhibitor of the myosin light chain kinase controlling epithelial cytoskeleton contraction[221]. In addition, exposure to a novel stress at adulthood potentiates gut hyperpermeability in maternally separated rats[95,311]. Furthermore, it has been shown that acute MS induces immediate passage of macromolecules across the colonic mucosa and can lead to increased number of bacterial cells penetrating the gut epithelium[221,262,312].

MS also produces several immune alterations in the colon. Indeed, MS animals show an infiltration of immune cells (i.e. polymorphonuclear neutrophils)[262,305] and an

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increase in mucosal mast cell density[81,253,262,271,308]. MS also increases the expression of numerous cytokines including IL-6, IL-1β, TNFα, IFNγ, IL-4, IL-2 and IL-22 in the colonic mucosa[42,64,262,268,276,278,286,303,305,313]. Increased IFNγ and decreased IL-10 expression were prevented by mGluR7 agonist administration[278] in MS animals.

It has been previously shown that MS increases IFNγ and TNF secretion by mesenteric lymph node cells[307]. In addition, increased mRNA expression of TLR3, 4 and 5 has been reported in the colonic mucosa of MS adult rats[314].

Impact of nutrition and microbiota-directed interventions in maternal separation offspring

An early study using the 24 h maternal deprivation paradigm suggested that feeding the pups during separation could prevent its effects on the HPA axis[31]. In the past decade, a growing number of studies have demonstrated that nutrition can modulate the long-term effects of early-life stress on brain and behaviour, although the underlying mechanisms remain unknown. Recent evidence suggests that the direct impact of nutrition on gut physiology and microbiota could counteract the stress-induced disruption of gut homoeostasis and promote a new state of equilibrium.

Nutritional strategies and maternal separation

Choline and vitamins

Several studies demonstrate a preventive effect of dietary choline and other vitamins in animals submitted to MS, and suggest that early nutritional interventions (before adulthood) have the strongest impact. In one study, the maternal diet was enriched with a mixture of essential C_1 metabolism-associated micronutrients containing choline, betaine, methionine, folic acid, zinc, vitamins B_6 and B_{12} during the course of MS. This treatment fully prevented the increased plasma corticosterone levels in MS pups at PND9 and further prevented later alterations of object recognition memory, but not spatial memory in adult MS mice[315]. In another study, dietary choline exposure from weaning to adulthood attenuated object recognition impairments in MS male rats[113]. In contrast, supplementation with a cocktail of methyl donors (choline, betaine, folic acid and vitamin B_{12}) in adult maternally separated female rats failed to reverse the deleterious effect of MS on object recognition memory, but did prevent depressive-like behaviour in the forced swim test[84].

PUFA. Some evidence suggests that *n*-3 PUFA deficiency potentiates the effects of MS. For instance, dietary *n*-3 PUFA deficiency acts in synergy with MS to increase sucrose consumption in adulthood, an effect prevented by desipramine[184,316]. It was further shown that the same dietary intervention also exacerbates MS-induced anxiety in the open-field test[317].

Conversely, it has been reported that supplementation with either *n*-3, folic acid or *n*-acetylcysteine during periadolescence could prevent the MS-induced depressive-like behaviour in the forced swim test, likely through antioxidant effects within the brain[318]. Interestingly, supplementation with a mixture of EPA and DHA from adolescence onwards reverses MS-induced gut-microbiota dysbiosis in adult female rats[297]. However, there was no major effect of the same treatment on anxiety and depressive-like behaviours or cognition in MS animals[319], yet no effect of MS *per se* was observed in this study. Nevertheless, in another study, dietary supplementation with PUFA-rich tuna oil failed to affect long-term visceral hypersensitivity in MS rats, but the diet was only administered after the induction of visceral hypersensitivity by acute stress[320].

High-fat diet. Previous studies have shown that palatable food consumption in adulthood can attenuate the deleterious effects of MS on anxiety and depressive-like behaviours and basal corticosterone levels[62,94].

We reported that the long-term effects of MS on anxiety, social behaviour and stress endocrine response, but also visceral sensitivity, can be prevented by exposing the dams to HFD during gestation and lactation[67]. In addition to this protective effect of perinatal HFD in adult animals, we found similar beneficial effects on the developing brain[161]. Indeed, maternal HFD exposure attenuated the stress-induced changes in mRNA expression of key genes involved in neuronal maturation and structural plasticity in the PFC of PND10 pups. The mechanisms underlying this protective effect of maternal HFD are elusive. We provided evidence that a comfort food effect of HFD in stressed mothers but also a modulation of the gut microbiota and/or gut barrier function by HFD in pups could contribute to its effects on brain and emotional behaviour[67,161].

Microbiota-directed interventions and maternal separation

The gut microbiota is highly sensitive to the environment and alterations of its composition (dysbiosis) have been described under conditions ranging from IBS and obesity to depression and autism[321–324]. In particular, early-life environment, including diet and stressful experience, shapes the gut microbiota towards health and disease later in life[325]. However, the mechanisms underlying the ability of stress to modulate microbiota composition remain to be unravelled. Moreover, it is unclear whether dysbiosis is a causative factor in the aetiology of the abovementioned pathologies. Interestingly, studies using different, but complementary, gut microbiota-directed interventions (germ-free (GF) rodents, antibiotics, faecal microbiota transplantation, probiotics and prebiotics) have demonstrated that gut bacteria can have a beneficial effect on emotional behaviours and, as such, psychobiotics have been proposed for potential therapeutic interventions[6,7].

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Germ-free animals and microbiota transplantation experiments

Germ free. The study of GF (or axenic) animals served as a proof of concept for the role of gut microbiota in the regulation of brain function and behaviour. A large number of studies have explored GF-associated alterations both in the gut and the brain (see[326] for review).

Interestingly, many of the GF phenotypes are normalised by colonisation, although the effects largely depend upon the age of colonisation and the animal species and strain[327–332]. Accordingly, Sudo *et al.* reported the first evidence that colonisation during early development, but not at a later age, could attenuate the increased HPA axis response to stress in GF mice[323]. In line with this study, further showed that locomotor hyperactivity in GF mice could be reversed by colonisation early in life, whereas colonisation at adulthood had no effect[333].

A landmark study by De Palma and colleagues using GF mice exposed to MS revealed that the microbiota is necessary for the long-term effects of MS. Indeed, early-life stress fails to induce long-term endocrine and behavioural alterations in GF mice compared with SPF (specific-pathogen-free) controls[49]. Interestingly, colonisation with the gut microbiota of a conventional SPF control mouse unmasked the effects of early-life stress in GF mice. However, colonisation with the microbiota of an early-stressed animal did not transfer the stress-associated behavioural phenotype in naive GF mice, suggesting that gut bacteria are necessary but not sufficient to mediate the behavioural effects of early-life stress. Although the authors did not measure intestinal permeability, gut leakiness associated with MS could also contribute to the deleterious effects of MS on behaviour. An important limitation of the GF animal model is that the GF status is not specific at all to intestinal microbes. Previous studies suggest that maternal vaginal microbiota also impacts offspring neurodevelopment[334]. Furthermore, GF animals are housed in isolators with limited handling that constitutes a stressful environment and in most of the study the control groups are not housed in similar isolators and thus are not comparable. Together, these studies suggest that gut dysbiosis may be responsible for some, but not all of the MS-associated phenotypes later in life.

Faecal transplantation. The important role of gut microbiota in the regulation of behaviour was further confirmed by demonstrating the successful adoptive transfer of host behavioural phenotype between mice of different strains and with different behavioural profiles (see[335] for review). In animals, faecal transplantation can be achieved by oral gavage of fresh faecal content or by transient co-housing with the donor. The stability of the transplanted microbiota can vary depending upon several factors (strain, sex, age, housing conditions). The first evidence of gut–brain effects following faecal transplantation in animals showed a critical role of gut microbiota in host metabolism and energy balance[336,337]. Since then, accumulating data have demonstrated that faecal transplantation can affect brain and behaviour in rodents. For instance, social deficits in offspring from HFD-fed dams could be reversed by co-housing with offspring from dams fed a regular diet[338], an effect accompanied by restored synaptic plasticity in the brain following social interaction.

Conversely, it has recently been shown that naive rats receiving faecal microbiota from MS donors displayed MS-like intestinal hypermotility[339]. Interestingly, colonisation with the microbiota of IBS patients *v.* healthy controls recapitulated several features of IBS in GF mice, including faster GI transit, intestinal barrier dysfunction, innate immune activation, but also anxiety-like behaviour[340].

The faecal mycobiome of MS rats is altered relative to control animals[341]. Furthermore, fungicide treatment in adult MS rats prevented the visceral hypersensitivity induced by water avoidance stress. Strikingly, transplantation of the microbiota from MS rats could re-establish visceral hypersensitivity in the absence of water avoidance stress, an effect that was absent when the donor microbiota came from fungicide-treated rats. These findings highlight the need of considering exhaustively gut microbiota composition (i.e. bacteria but also viruses and fungi) and of better understanding the complex interactions between stress and gut microbes.

Overall, the potential clinical value of faecal transplantation for the treatment of disorders of the gut–brain axis is promising[342,343] and currently represents an active area of research. To date, the only indication for faecal transplantation in human subjects is the treatment of severe infections with *Clostridium difficile*, resulting in high success rates[344]. In the recent years, two double-blind, placebo-controlled, randomised trials have investigated the impact of faecal microbiota transplantation in IBS patients[345,346]. However, evidence for clinical improvement of GI symptoms and psychiatric symptoms is unclear and has to be further established in larger studies.

Probiotics. The term probiotic, defined as 'a live microbial feed supplement, which beneficially affects the host by improving its intestinal microbial balance' was coined in 1953 by Werner Kollath to contrast with antibiotics[347]. The use of probiotics in animal studies has provided evidence that the gut microbiota posesses psychobiotic properties (i.e. antidepressant and/or anxiolytic-like activity) (see[6,348,349] for reviews).

Probiotic interventions are generally restricted to one or few bacterial species, thereby allowing the association between a given bug and a particular behavioural effect. The most used are members of the *Bifidobacterium* and *Lactobacillus* genera. Beneficial effects of probiotics have been reported in paradigms involving early-life stress. Several studies have shown anti-nociceptive effects of different probiotics (i.e. *Faecalibacterium prausnitzii*; *Bifidobacterium breve* or VSL#3)[263,268,272]. Moreover, the probiotic *Bifidobacterium infantis* chronically administered at adulthood (from PND50 to PND95) was reported to exert antidepressant-like effects in animals exposed to MS[78]. In addition, the increased peripheral levels of the proinflammatory cytokine IL-6 as well as

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the increased CRF mRNA levels in the amygdala in stressed animals were also normalised. Similarly, a lactobacillus strain, *Lactobacillus plantarum PS128*, has antidepressant-like effects in MS mice treated from weaning onwards in both the sucrose preference test and forced-swim test, but has no effect on MS anxiety[350]. Moreover, serum increase in corticosterone (both at baseline and in response to stress), increase in IL-6 and decrease in IL-10 were all reversed by the probiotic. In addition to the beneficial effects of probiotics in adult animals, an increasing body of evidence shows that probiotics supplementation during early-life can have long-term preventive effects. Indeed, it has been shown that a mixture of *Lactobacillus rhamnosus* and *Lactobacillus helveticus* could prevent the elevation in basal plasma corticosterone observed in MS juvenile rats (PND20), in addition to mitigating the associated increased gut permeability[309]. Similar findings have been reported in a mouse model of MS where mice received the probiotic *Bifidobacterium pseudocatenulatum* during the perinatal period[64]. Compared with their placebo-fed stressed counterparts, probiotic-fed mice exposed to early stress showed attenuated HPA axis reactivity and intestinal inflammation at weaning, as well as lower anxiety levels during adolescence. These findings were extended to other probiotic strains belonging to Bifidobacteria and Lactobacilli. Indeed, in juvenile rats, MS-induced hypercorticosteronaemia, intestinal hyper permeability and dysbiosis were all prevented by neonatal treatment with *Bifidobacterium bifidum* G9-1[351]. Pretreatment with *L. fermentum* CECT 5716 was also able to attenuate the effects of a single 4 h-separation episode at PND10 (i.e. hypercorticosteronaemia and intestinal hyperpermeability)[352]. In contrast, although maternal probiotics treatment with *Bifidobacterium animalis* subsp. *lactis* BB-12H and *Propionibacterium jensenii* 702 was shown to prevent the increase in plasma IFNγ in adult MS offspring[319], the same treatment increased plasma IL-6 in juveniles. In line with the latter, Barouei and collaborators showed that the maternal probiotic intervention induces MS-like dysbiosis along with increased levels of circulating corticosterone and adrenocorticotropic hormone in non-stressed developing offspring[298]. A recent study also reports preventive effects of maternal treatment with the probiotic Lacidofil® (*L. rhamnosus* R0011 and *L. helveticus* R0052), *via* the maternal drinking-water during the period of stress, on abnormal mPFC neural fear circuitry development in stressed pups[353]. Interestingly, the effects of neonatal probiotics in MS models are not restricted to stress response and depressive-like behaviours. It has been reported that MS disturbs puberty onset in a sex-dependent manner, but this effect is prevented by probiotic neonatal administration with Lacidofil®[354]. In another study, MS rats transmitted their conditioned aversive memory to the next generation, but this effect was abolished if the F0 fathers or the F1 offspring was supplemented with Lacidofil®[355].

Notably, the majority of these findings were obtained using males only. Since a consistent gender effect has been reported in the prevalence of anxiety and depression, but also IBS, with higher rates in women than men[356], additional preclinical studies using female animals are required. Moreover, the translational potential of these findings is currently limited by methodological and technical issues. It is not clear whether probiotic strains survive under aerobic conditions and are able to efficiently colonise the gut of the recipient. Future studies should systematically assess post-treatment colonisation to draw conclusions. In this regard, comparing heat-killed *v.* live probiotics can also be helpful to better understand their underlying mechanisms of action. Furthermore, there is a need to improve the dosage, treatment duration and route of administration. Indeed, only a few studies in animal models addressed the dose-dependency of the effects of probiotics. It is suggested that multi-strain probiotic combinations may provide greater health benefits, but this hypothesis has not been clearly tested. The systematic comparison of the effects of probiotics with that of a clinical drug such as anxiolytics or antidepressants appears critical to quantify the benefits.

It has been proposed that probiotics might represent an adjuvant therapy in psychiatric disorders including major depressive disorder, although well-designed clinical trials are needed to make clear conclusions[357]. A recent study reported that pregnant women supplemented with *L. rhamnosus* until 6 months postpartum had significantly lower depression and anxiety scores in the postpartum period[358]. To date, antidepressant effects of probiotics have been reported in three double-blind studies conducted in subjects diagnosed with significant anxiety or depression symptoms[359,360] and in major depressive disorder patients[361]. However, based on preclinical data, psychotropic-like effects of probiotics on mood and anxiety in subjects exposed to early-life adversity still need to be confirmed in human trials[362]. One study has explored the effects of probiotic strains *L. rhamnosus* HN001 or *B. animalis* subsp. *lactis* HN019 in 11-year-old children supplemented from fetal life to age 2 years on neurodevelopment, but found no major effect of probiotics[363]; yet the impact on emotional behaviours and especially in early-stressed patients remain unknown.

Prebiotics and symbiotics. Prebiotics are nutrients that can be fermented by microbes in the gut and thus favour the growth of certain microbial communities[364]. In comparison with probiotics, a much smaller number of studies have examined the effects of prebiotics on behaviour (see[365] for review). These include investigations of galacto-oligosaccharides (GOS) and fructo-oligosaccharides (FOS), which are sources of nutrition for *Bifidobacteria* and *Lactobacilli*. The effects of FOS and GOS have been tested in C57BL/6J male mice in basal and chronic stress situations[366]. GOS of FOS alone showed some levels of protective effects but to a much lower extent compared with GOS and FOS. Conversely, human-milk oligosaccharide prebiotics have been reported to impact brain development and cognitive functions[367,368]. Mice supplemented with human-milk oligosaccharides in their diet (2 weeks) were protected against stress-induced hyperanxiety[369]. Apart from

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these effects on emotional behaviours, other studies have reported improved learning and memory performance in animals supplemented with different oligosaccharides including human-milk oligosaccharides[370–373]. Together, these findings suggest that combining several probiotics and/or prebiotics can improve the treatment outcome. For instance, increased intestinal permeability in adolescent MS rats was prevented by a symbiotic diet containing arachidonic acid and DHA, GOS and FOS and *Lactobacillus paracasei NCC2461*[299]. In another study, MS rats were treated with either the prebiotics polydextrose and GOS, the probiotic *L. rhamnosus GG* or the symbiotic combination from weaning onwards[374]. Only the combination of pre- and probiotics was able to normalise anxiety in the open field test, although it impaired corticosterone negative feedback following acute restraint stress. In addition, expression of GABA receptor A2 (*Gabra2*) in the hippocampus was restored only by the combination of pre- and probiotics, whereas expression of GR (*Nr3c1*) was restored by *L. rhamnosus GG* alone.

Conclusion

MS induces a variety of long-term alterations similar to that observed in human subjects with a history of childhood adversity. In this review, we have outlined the specific effects of MS on both the brain and the gut, illustrating the validity of this model with respect to clinical data. In addition, the pivotal role played by gut microbiota in mediating the lasting imprinting by MS is highlighted in numerous studies using microbiota-directed interventions such as probiotics treatments. Preclinical studies suggest that nutritional approaches with pro- and prebiotics may constitute safe and efficient strategies to attenuate the effects of early-life stress on the gut–brain axis. However, it is still not clear whether gut dysbiosis, leakiness or inflammation precede each other and if they are the cause or consequence of stress-induced alterations within the brain. In this respect, studies are needed to understand how chronic neonatal stress disrupts gut–brain homoeostasis during development and which molecular mechanisms underlie the subsequent long-term imprinting. Moreover, despite widespread sex differences in both GI and neuropsychiatric vulnerability, there is still a gap to fill in the literature as regards the issue of sex. Meta-analyses on the impact of probiotics on anxiety and depressive-like symptoms exist, but the vast majority of the studies are conducted in healthy subjects and recent findings demonstrate that the effects of probiotics may differ between stressed and unstressed subjects[375]. Future studies should develop nutritional strategies combining multiple prebiotics and probiotics, in addition to usual pharmacological strategies, to examine their impact at adult age on symptoms associated with early-life adversity using randomised placebo-control trials, with an effort to adapt these strategies according to sex[376]. Furthermore, prebiotics and probiotics effects should also be examined during development in populations exposed to stress. In human trials, it would be particularly valuable to study the potential preventive effects of prebiotics and probiotics after different stress experiences such as early-life traumas, but also parental depression, perinatal infections, premature birth or low parental socioeconomic status. Finally, early-life adversity is associated with poor diet quality at adulthood[377]. In this context, it would be crucial to improve health policies and to implement preventive interventions with nutritional advices in populations exposed to early-life adversity.

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Conflict of Interest

None.

Authorship

M. R. drafted the first version of the manuscript then both authors revised and approved the manuscript.

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