Role of the gut-brain axis in early stress-induced emotional vulnerability

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ROLE OF THE GUT-BRAIN AXIS IN EARLY-STRESS-INDUCED EMOTIONAL VULNERABILITY

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Titre : Implication de l'axe intestin-cerveau dans la vulnérabilité émotionnelle associée au stress précoce

Résumé : Les maladies psychiatriques présentent de fortes comorbidités avec des désordres gastrointestinaux, ce qui suggère l'existence de bases physiopathologiques communes. Une littérature abondante démontre que l'adversité précoce (infection, stress) augmente la vulnérabilité aux désordres psychiatriques à l'âge adulte. Chez le rongeur, le modèle de séparation maternelle induit chez la descendance adulte des comportements hyperanxieux associés à une hypersensibilité au stress, ainsi que des dysfonctionnements de la sphère gastrointestinale. De plus, des études récentes rapportent une hyperperméabilité de la barrière intestinale chez les ratons soumis au stress de séparation, un effet conduisant potentiellement à une dysbiose et une perturbation de la communication intestin-cerveau. Le but de ma thèse était donc d'étudier le rôle de l'axe intestin-cerveau dans la mise en place des effets à long terme du stress précoce. Nos travaux récents ont montré que certains effets à long-terme de la séparation maternelle peuvent être atténués par l'exposition des mères à un régime hyperlipidique. Dans un premier temps, nous avons testé les effets du régime hyperlipidique maternel sur le cerveau et l'intestin de ratons soumis à la séparation maternelle. Nos résultats montrent que le régime maternel hyperlipidique protège de l'augmentation de la permeabilité intestinale induite par le stress. Nous avons ensuite testé le rôle causal de la perméabilité intestinale sur les comportements émotionnels à travers une approche pharmacologique et une approche génétique. Nous rapportons 1) que la restauration de la fonction barrière de l'intestin atténue certains effets de la séparation maternelle et 2) qu'une hyperperméabilité intestinale chez des souris transgéniques non soumises à un stress produit des effets similaires à ceux de la séparation maternelle. Enfin, nous avons examiné les effets d'une adversité précoce multifactorielle sur le cerveau et l'intestin (perméabilité et microbiote) chez la descendance adulte mâle et femelle dans un modèle combinant infection prénatale et séparation maternelle. Nos résultats mettent en évidence un effet sexe très marqué sur les phénotypes comportements et intestinaux. D'autres études sont nécessaires pour identifier les mécanismes sous-tendant les effets de la perméabilité et la dysbiose intestinale sur la vulnérabilité émotionnelle associée au stress précoce.

Mots clés : modèles animaux de désordres psychiatriques ; comportement ; adversité précoce ; perméabilité intestinale ; microbiote intestinal ; axe corticotrope

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Title : **Role of the gut-brain axis in early stress-induced emotional vulnerability**

Abstract : Early-life adversity is a main risk factor for psychiatric disorders at adulthood; however the mechanisms underlying the programming effect of stress during development are still unknown. In rodents, chronic maternal separation has long lasting effects in adult offspring, including hyper-anxiety and hyper-responsiveness to a novel stress, along with gastrointestinal dysfunctions. Moreover, recent studies report gut barrier hyper-permeability in rat pups submitted to maternal separation, an effect that could potentially lead to dysbiosis and altered gut-brain communication. Therefore, the aim of my PhD was to unravel the role of the gut-brain axis in the neurobehavioral effects of early-life stress. We recently reported that some neural, behavioral and endocrine alterations associated with maternal separation in rats could be prevented by maternal exposure to a high-fat diet. We first addressed the effects of maternal high-fat diet on brain and gut during development in the maternal separation model. We show that maternal high-fat diet prevents the stress-induced decrease in spine density and altered dendritic morphology in the medial prefrontal cortex. Moreover, maternal high-fat diet also attenuates the exacerbated intestinal permeability associated with maternal separation. To explore a potential causal impact of gut leakiness on brain functions, we then examined the impact of pharmacological and genetic manipulations of intestinal permeability on brain and behavior. We report 1) that restoration of gut barrier function attenuates some of the behavioral alterations associated with maternal separation and 2) that chronic gut leakiness in naive adult transgenic mice recapitulates the effects of maternal separation. Finally, we examined the effects of multifactorial early-life adversity on behavior, gut function and microbiota composition in males and females using a combination of prenatal inflammation and maternal separation in mice. At adulthood, offspring exposed to early adversity displayed sex-specific behavioral (social behavior deficits in males and increased anxiety in females) and intestinal phenotypes. In conclusion, our work demonstrates an impact of gut dysfunctions, in particular gut leakiness, on the emergence of emotional alterations. Further studies are needed to unravel the role of the gut dysbiosis in the expression of the behavioral phenotypes associated with early-life adversity.

Keywords : animal models of psychiatric disorders; behavior; early-life adversity; gut permeability; gut microbiota; HPA axis

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Résumé des objectifs et principaux résultats

Contexte et objectifs de la thèse

Depuis une dizaine d'années, de plus en plus de travaux mettent en évidence un rôle clé du microbiote intestinal dans la communication entre intestin et cerveau (Mayer et al., 2014a). En particulier, de nombreuses données chez l'animal, et plus récemment chez l'homme, montrent le rôle de manipulations du microbiote dans la régulation des comportements de type anxieux et dépressifs et la sensibilité au stress (Dinan et al., 2013; Sarkar et al., 2016). De plus, la plupart des maladies psychiatriques présentent une importante co-morbidité avec des désordres gastrointestinaux (Folks, 2004; Buie et al., 2010), suggérant l'existence de bases physiopathologiques communes. De façon remarquable, la prévalence des désordres psychiatriques comme la dépression et les troubles anxieux, comme celle des désordres gastrointestinaux, est différente chez les femmes et chez les hommes (environ 2 fois plus élevée chez les femmes que les hommes) (Mayer et al., 1999; Fombonne, 2003; Altemus, 2006; Werling and Geschwind, 2013). Une littérature abondante démontre que l'adversité dans l'enfance (traumas, abus, négligence) augmente la vulnérabilité aux troubles anxieux et dépressifs à l'âge adulte (Chapman et al., 2004; Rutter, 2005), mais également au syndrome de l'intestin irritable (Chitkara et al., 2008; Bradford et al., 2012), un désordre fonctionnel de l'intestin associé à des douleurs abdominales chroniques (Öhman and Simrén, 2010). Chez le rongeur, la séparation maternelle chronique est un modèle de stress précoce qui induit chez la descendance adulte une hyper-anxiété associée à une hypersensibilité au stress (Ladd et al., 2000), ainsi que des atteintes digestives (O'Mahony et al., 2011) telles qu'une dysbiose (déséquilibre de la composition du microbiote intestinal) (De Palma et al., 2015). Comme chez l'homme, les altérations rapportées dans ce modèle sont sensiblement différentes entre mâles et femelles, aussi bien au niveau comportemental qu'intestinal (Slotten et al., 2006; Kokras and Dalla, 2014; Prusator and Greenwood-Van Meerveld, 2016). Des études récentes ont montré que le stress néonatal augmente la perméabilité de la barrière intestinale pendant le développement (Moussaoui et al., 2014), un effet participant potentiellement à la dysbiose et à la perturbation de la communication intestin-cerveau (Borre et al., 2014; Kelly et al., 2015). En effet, il a été montré que le microbiote interagit avec l'épithélium intestinal et module sa fonction barrière (Zakostelska et al., 2011; Jakobsson et al., 2015; Reunanen et al., 2015). En revanche, le rôle intrinsèque de la perméabilité intestinale sur la composition du microbiote est mal connu, et son implication potentielle dans la communication intestin-cerveau reste à explorer. **Cette thèse vise à comprendre le rôle de la perméabilité intestinale et du microbiote intestinal dans la mise en place d'une vulnérabilité neuropsychiatrique après stress précoce, en tenant compte des potentielles différences mâles-femelles.**

Résultats obtenus

La première partie de cette thèse est centrée sur l'étude du rôle de la perméabilité intestinale dans la vulnérabilité émotionnelle dans des modèles précliniques, à travers 3 approches complémentaires (nutritionnelle, pharmacologique et génétique) permettant d'inhiber ou exacerber la perméabilité intestinale. Notre hypothèse était que l'hyper-perméabilité intestinale induite par le stress précoce affecte le développement cérébral et contribue à la vulnérabilité neuropsychiatrique à long terme.

Nos travaux ont montré que chez le rat mâle, l'exposition à un régime hyperlipidique pendant la période périnatale chez des animaux soumis au stress de séparation maternelle atténue certaines altérations observées à l'âge adulte, en particulier les altérations émotionnelles et cognitives, l'hyper-activité de l'axe corticotrope, mais également l'hypersensibilité viscérale (Rincel et al., 2016, voir **ANNEXE 1**). De plus, chez les ratons exposés au stress précoce, le régime hyperlipidique restaure lors du développement les niveaux d'expression de gènes tels que le Bdnf, 5HTr1A et Rest4 dans le cortex préfrontal. Ces résultats nous ont conduit à proposer l'hypothèse selon laquelle le régime hyperlipidique pourrait protéger le cerveau et l'intestin en développement des effets du stress précoce (**Article 1 : Rincel et al., 2017**). Dans cette première étude, nous avons montré que le régime hyperlipidique périnatal atténue le déficit d'épines dendritiques dans les neurones pyramidaux du cortex préfrontal médian des ratons soumis au stress et par ailleurs ce même régime supprime l'hyper-perméabilité intestinale induite par la séparation maternelle à l'âge de 10 jours. Le régime hyperlipidique semble avoir un effet protecteur chez des animaux soumis au stress précoce et ceci tant sur le plan de la maturation neuronale que sur le plan de la barrière intestinale. Cependant, nos données ne permettent pas de déterminer si les effets protecteurs de ce régime sur le cerveau et les comportements mettent en jeu l'atténuation de l'hyper-perméabilité intestinale induite par la séparation maternelle.

La fonction barrière de l'intestin est régulée par l'ouverture/fermeture de jonctions serrées au niveau de l'épithélium intestinal, contrôlée par l'activité d'une enzyme, la MLCK (myosin light chain kinase) (Clayburgh et al., 2005). Afin d'explorer le lien causal entre hyperperméabilité intestinale développementale et vulnérabilité émotionnelle à l'âge adulte, nous avons utilisé un inhibiteur pharmacologique de la MLCK, le ML-7, pour restaurer la fonction de la barrière intestinale des ratons exposés au stress néonatal **(Article 2: Rincel et al., en préparation)**. Nos données montrent que l'inhibition de la MLCK restaure la perméabilité intestinale et protège de certaines altérations induites par le stress néonatal (anhédonie et hyper-réactivité de l'axe corticotrope au stress). Cet effet protecteur du ML-7

est accompagné, à l'âge adulte, d'une atténuation de la dysbiose intestinale engendrée par la séparation maternelle. L'analyse approfondie des communautés bactériennes dont l'abondance est altérée par le stress et normalisée par le ML-7 a permis d'identifier des populations d'intérêt pour d'éventuelles approches préventives ou thérapeutiques, y compris des Bifidobactéries qui sont déjà largement utilisées en tant que probiotiques. Par ailleurs, bien que la MLCK soit présente au niveau des jonctions serrées de la barrière hémato-encéphalique (BHE), nos mesures de l'expression des protéines des jonctions serrées par immunohistochimie et de la perméabilité de la BHE *in vivo* suggèrent que les effets du ML-7 chez les animaux exposés au stress précoce sont indépendants de modifications de la BHE.

Afin de confirmer la spécificité de notre effet, nous avons travaillé sur un modèle de souris transgéniques exprimant une MLCK constitutivement active (CA-MLCK) spécifiquement dans les cellules épithéliales de la barrière intestinale (Su et al., 2009a). Ces souris présentent une hyper-perméabilité intestinale et de façon très intéressante, un phénotype comportemental et endocrine similaire à celui induit par le stress précoce **(Article 3: Rincel et al., en préparation)**. En outre, l'étude des mâles et des femelles transgéniques a permis de mettre en évidence des différences importantes entre les deux sexes. En effet, les mâles CA-MLCK présentent une anhédonie semblable à celle observée chez le rat soumis à la séparation maternelle, tandis que les femelles CA-MLCK sont hyper-anxieuses par rapport aux souris WT. L'hyper-réactivité de l'axe corticotrope en réponse à un stress, classiquement rapportée chez les animaux exposés au stress précoce, a été retrouvée chez les deux sexes. Ces altérations sont accompagnées au niveau cérébral dans le cortex préfrontal médian, le noyau accumbens et l'hippocampe, par des différences d'expression de certains gènes associés au stress (5HTr1A, Crf, Tnfa, Fkpb5…), ainsi que des changements de connectivité entre différentes structures sensibles au stress et impliquées dans la régulation des comportements (hippocampe, noyau du tractus solitaire). Si les mécanismes par lesquels une augmentation de la perméabilité intestinale affecte le cerveau et les comportements restent à explorer, notre travail a permis de démontrer, *in vivo*, que la perméabilité intestinale, en plus du microbiote, joue un rôle crucial dans la communication intestin-cerveau.

Le deuxième volet de cette thèse visait à tester l'hypothèse selon laquelle les effets du stress précoce sur la composition du microbiote intestinal diffèrent en fonction du sexe, cette différence contribuant à l'émergence d'altérations comportementales sexe-dépendantes **(Article 4: Rincel et al., en préparation)**. Par ailleurs, nous avons pris en considération les données cliniques soulignant la multiplicité et les effets cumulatifs des facteurs environnementaux précoces dans l'étiologie des troubles neuropsychiatriques. Pour ce faire, nous avons utilisé un modèle d'adversité multifactorielle combinant une inflammation maternelle lors de la gestation et la séparation maternelle. Nous avons montré que les effets à long terme de cette adversité précoce multifactorielle sur les comportements émotionnels, les fonctions neurodigestives et le microbiote intestinal de la descendance diffèrent entre mâles et femelles. Plus particulièrement, les mâles soumis à l'adversité précoce multiple présentent une diminution du comportement social alors que les femelles sont plus anxieuses. En ce qui concerne le microbiote intestinal, il semble que les mâles soient plus sensibles au stress que les femelles, dans la mesure où plus de communautés bactériennes sont affectées chez les mâles. De façon intéressante, nous avons pu mettre en évidence des corrélations entre certains genres bactériens et les comportements chez les deux sexes. Par ailleurs, si la comparaison du microbiote de rat et de souris est délicate, nos résultats mettent en évidence une famille bactérienne affectée par le stress précoce dans ces deux espèces, les Lachnospiraceae. Ces bactéries sont présentes dans la couche du mucus intestinal, directement au contact de l'épithélium et des cellules du système immunitaire inné (Van den Abbeele et al., 2013). De ce fait, elles jouent potentiellement un rôle important dans l'homéostasie intestinale et leur perturbation pourrait contribuer aux altérations de la communication intestin-cerveau. Au niveau cérébral, nous avons observé une interaction sexe x adversité précoce significative pour l'expression de 8 gènes dans le cortex préfrontal médian, indiquant des effets opposés du stress précoce en fonction du sexe. Parmi ces gènes, l'expression de Klf2 (Krüppel-like factor 2) est augmentée spécifiquement chez les femelles exposées au stress précoce, or un article récemment publié dans Nature suggère que ce facteur pourrait être régulé par le microbiote intestinal (Tang et al., 2017a). D'autres études seront nécessaires pour déterminer si les différences de dysbiose intestinale entre mâles et femelles contribuent aux différences comportementales entre les deux sexes suite à l'adversité précoce multifactorielle.

Conclusion

En conclusion, les résultats obtenus dans le cadre de cette thèse confortent l'hypothèse selon laquelle l'adversité précoce contribue à l'émergence d'une vulnérabilité neuropsychiatrique et affecte à long terme la sphère gastrointestinale. Par ailleurs, nos travaux suggèrent un rôle causal de la perméabilité intestinale dans la mise en place de certaines altérations à long-terme dans un contexte de stress précoce. D'autres études sont nécessaires afin de déterminer le rôle causal éventuel de la dysbiose intestinale dans les effets observés, en particulier dans le cadre des effets différentiels de l'adversité précoce en fonction du sexe. Plus généralement, notre travail souligne l'importance d'étudier les mâles et les femelles, mais également les effets d'adversités précoces multiples dans les modèles précliniques en psychiatrie. Les futures études devront explorer comment l'intestin pourrait constituer une cible pour les traitements en psychiatrie, mais également comment les altérations intestinales pourraient contribuer à l'émergence d'une vulnérabilité neuropsychiatrique ou à la résistance aux traitements.

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FOREWORD

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The concept of brain-gut communication is not recent. For instance, each of us has experienced the effects of stress on their bowel. However, the contribution of gut microbes to brain function has been ignored for centuries until a revolution has begun. Since the last 10 years, advancements in sequencing technologies brought the concept of gut-brain axis in the spotlight of neuroscience research. The discovery that the number of gut microorganisms and their combined genetic information far exceed that of their host led to the concept of superorganism (also holobiont or hologenome) (Zilber-Rosenberg and Rosenberg, 2008) and raised important questions as regards the role of this "forgotten organ" in the host's physiology and health.

In particular, there is accumulating evidence that gut-brain communication is altered in a number of neuropsychiatric and neurodegenerative disorders. For instance, gastrointestinal (GI) symptoms are commonly found in major depressive disorder (Gros et al., 2009), autism spectrum disorder (ASD) (Buie et al., 2010), but also Parkinson and Alzheimer's diseases (Rao and Gershon, 2016). The possible implication of gut alterations in these disorders is currently being investigated. Specifically, the role of gut microbiota in the neurobehavioral phenotypes associated with these pathologies has been explored in animal models (Hsiao et al., 2013; Kelly et al., 2016; Sampson et al., 2016). Above all, converging data suggest a pivotal role of gut microbiota in the etiology of psychiatric symptoms in stress-related psychiatric disorders such as anxiety and depression (Rogers et al., 2016). Anxiety and depression are highly co-morbid with each other, as well as with cardiovascular, metabolic and more interestingly with regard to the gut-brain axis, GI disorders. Of these GI disorders, the irritable bowel syndrome (IBS) has shown the strongest and most frequent associations with anxiety and depression (Fond et al., 2014). The etiology of psychiatric disorders is not fully understood. However, exposure to stressful events during childhood has been repeatedly associated with increased vulnerability to both psychiatric and GI disorders such as IBS (Chitkara et al., 2008; Nemeroff, 2016). Among the leading causes of disability in the world in 2015, depression (3^{rd}) and anxiety disorders (9^{th}) represent considerable socioeconomic pressures on the individual and overwhelming costs to global health economies (GBD 2015 Disease and Injury Incidence and Prevalence Collaborators, 2016). A consistent gender effect in the prevalence of these psychiatric conditions has been reported with higher rates of mood and anxiety disorders in women than men (Steel et al., 2014). Existing therapeutic approaches target the central nervous system (CNS) and broadly affect its functioning, leading to numerous side effects. Most importantly, a large proportion of the patients are resistant to

pharmacological treatments (Millan et al., 2015). A better understanding of the role of the gut-brain axis in psychiatric vulnerability could open novel avenues for gut-directed therapeutic or preventive strategies. Especially, whether gut dysfunctions are a causal factor of psychiatric disorders remains to be determined. In this context, this PhD work aimed at identifying the mechanisms underlying the relationship between the gut and the brain in animal models of early-life adversity.

INTRODUCTION

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In this introduction, we first describe the different components of the gut-brain axis and potential mechanisms of bi-directional communication. Second, we review the evidence for gut manipulations affecting brain and behavior in both animal models and humans. Finally, we address the consequences of early-life adversity on the brain and intestinal tract with a particular focus on the rodent maternal separation model.

CHAPTER I - The gut-brain axis

The gut (also called bowel or lower GI tract) is the main organ involved in the uptake of nutrients and water. At the same time, it constitutes an essential barrier against harmful substances and pathogens from the external environment. The gut comprises the small (duodenum, jejunum and ileum) and the large (colon and rectum) intestines, separated by the caecum. It is a highly specialized organ involved in digestive function and especially in nutrient absorption, thanks to its outsized epithelial surface area. The gut is also provided with coordinated muscular segments (longitudinal and circular) that act to mix and move the luminal content. In addition to the numerous enzymes secreted in the lumen to breakdown the nutrients, epithelial cells secrete mucus to lubricate the gut walls and thus favor absorption but also protect the mucosa against gastric acid. In the large intestine, remaining molecules are further digested by microbial fermentation. In this chapter, we will describe the main components of the gut and the possible routes of communications linking them to the brain.

1.1. Anatomy and function of the main gut components

1.1.1. *Gut microbiota*

The gut microbiota can be viewed as an organ by itself and it is recognized to play an important role in gut digestive, metabolic and immunological functions in the healthy individual (see Sekirov et al., 2010 and Jandhyala et al., 2015 for reviews). It is defined as the ensemble of microorganisms residing in the gut, including bacteria, fungi and viruses. In the gut-brain axis literature, most is known as regards bacteria and their related molecules (Kelly et al., 2015), whereas the nature and function of fungi and viruses remain to be deeper documented. For this reason, we will focus on bacteria in the following.

The microbiota of a given subject weighs 1.5-2 kg and contains around 1000 different bacterial species (1/3 are common to most people and the other 2/3 are specific to each individual). In both humans and rodents, the most represented bacterial phyla in the gut are Firmicutes (such as *Lactobacillus, Clostridium* and *Enterococcus*) and Bacteroidetes (such as

Bacteroides) (Eckburg et al., 2005). Other phyla with lower relative abundance can be found, such as Actinobacteria (*Bifidobacteria*), Proteobacteria (*Escherichia coli*), Fusobacteria, Verrucomicrobia and Cyanobacteria. Notably, there are differences in the composition of the microbiota depending upon its specific location along the GI tract, but also with regard to the proximity to the mucus layer **[Figure 1]** (Hollister et al., 2014; Donaldson et al., 2016). An important consideration is also that the microbiota differs according to gender (Markle et al., 2013; Dominianni et al., 2015; Jašarević et al., 2016; Fransen et al., 2017) **[Figure 2]**.

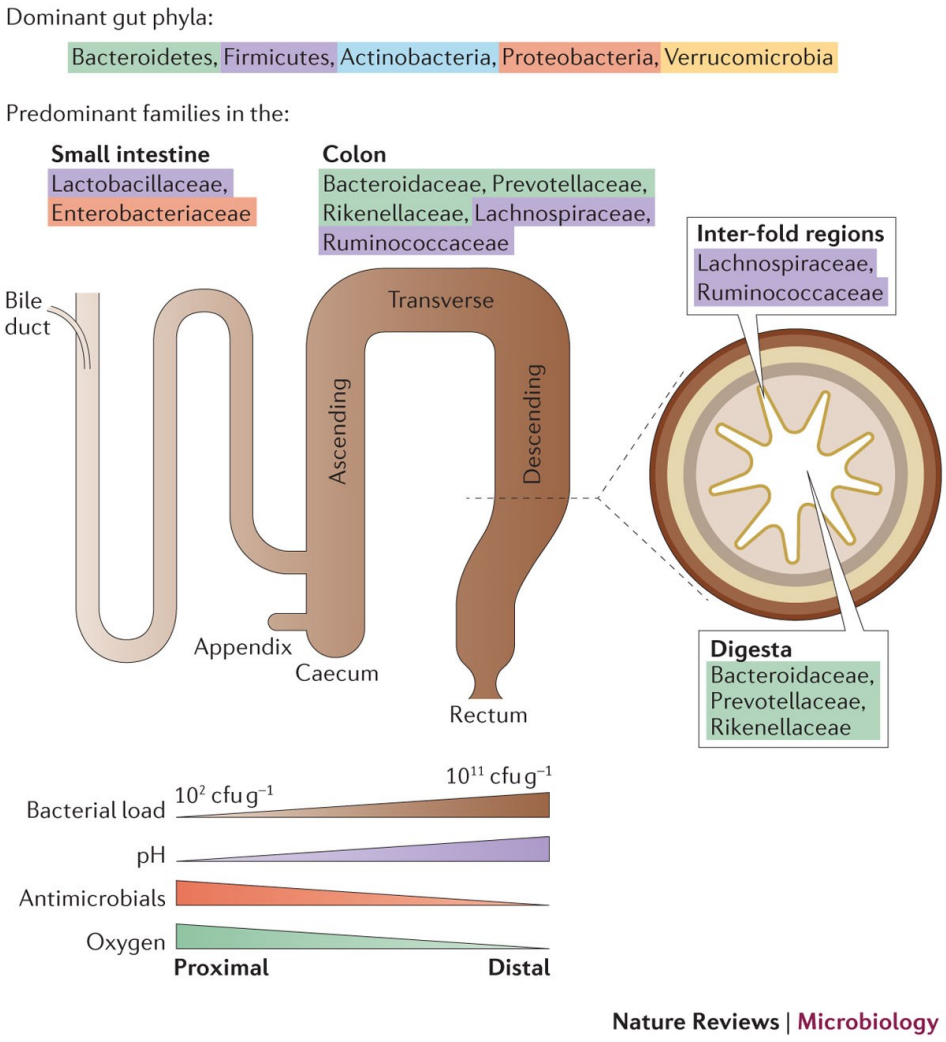

Figure 1 | Microbial habitats in the human lower gastrointestinal tract. Figure from Donaldson et al., 2016. The dominant bacterial phyla in the gut are Bacteroidetes, Firmicutes, Actinobacteria, Proteobacteria and Verrucomicrobia. The dominant bacterial families of the small intestine and colon reflect physiological differences along the length of the gut. For example, a gradient of oxygen, antimicrobial peptides (including bile acids, secreted by the bile duct) and pH limits the bacterial density in the small intestinal community, whereas the colon carries high bacterial loads. In the small intestine, the families Lactobacillaceae and Enterobacteriaceae dominate, whereas the colon is characterized by the presence of species from the families Bacteroidaceae, Prevotellaceae, Rikenellaceae, Lachnospiraceae and Ruminococcaceae (colors correspond with the relevant phyla). A cross-section of the colon shows the digesta, which is dominated by Bacteroidaceae, Prevotellaceae and Rikenellaceae, and the inter-fold regions of the lumen, which are dominated by Lachnospiraceae and Ruminococcaceae. cfu, colony-forming units.

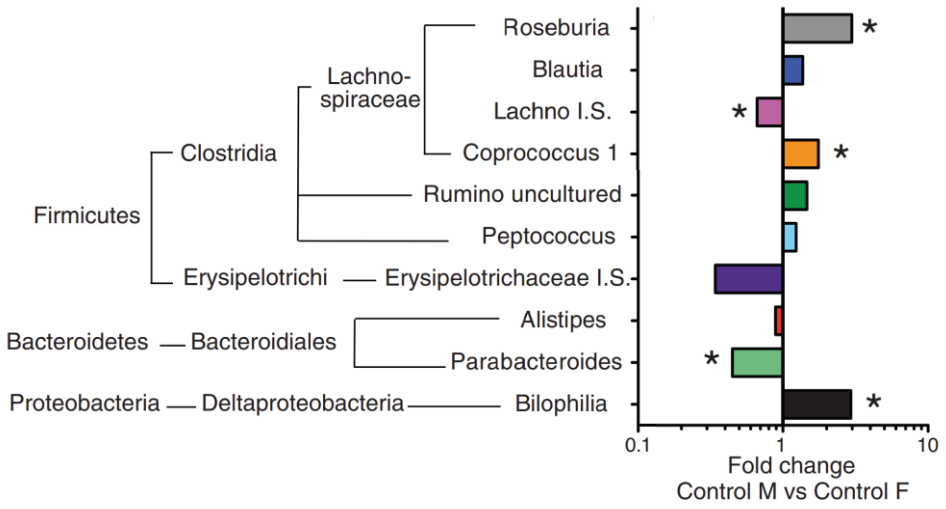

Figure 2 | Sex-differences in gut microbiota composition of adult mice. **Figure from Markle et al., 2013.** Genera with significantly different abundance revealed by 16S rRNA sequencing in 14 week-old males (M) and females (F).

The gut microbiota is acquired at birth and its initial composition is highly sensitive to the delivery mode (Dominguez-Bello et al., 2010). Initial colonization is crucial for healthy immune development. Several factors influence the trajectory of microbiota development including gestational age, antibiotic use, breastfeeding and exposure to family members and pets (Penders et al., 2006). In the first weeks, the gut microbiota is characterized by a low diversity and stability. By age three, however, the microbiota composition resembles that of an adult-like profile (Voreades et al., 2014). Although the common dogma states that the intrauterine environment and fetus are sterile until delivery, some evidence demonstrates bacterial presence in the intrauterine environment (Collado et al., 2016), suggesting that these bacteria may influence the microbiota of the infant even before birth (Gomez de Agüero et al., 2016)**.**

1.1.2. Gut epithelial barrier

The gut mucosa constitutes a physical barrier between the gut lumen and the host's inside (see König et al., 2016 for review). This barrier is mainly composed of the mucus layer, the epithelial layer, and the underlying lamina propria. The epithelium is organized as a monolayer mainly composed of enterocytes, but also contains a variety of other cell-types including enterochromaffin cells (enteroendocrine cells), goblet cells, paneth cells and immune cells **[Figure 3]**. Enterochromaffin cells release gut hormones such as cholecystokinin (CCK), glucagon-like peptide (GLP), peptide tyrosine tyrosine (PYY), but also neurotransmitters such as serotonin (Diwakarla et al., 2017). Goblet cells are involved in the production of mucin, a main component of the mucus layer which can be considered as a first bulwark of the barrier (Deplancke and Gaskins, 2001).

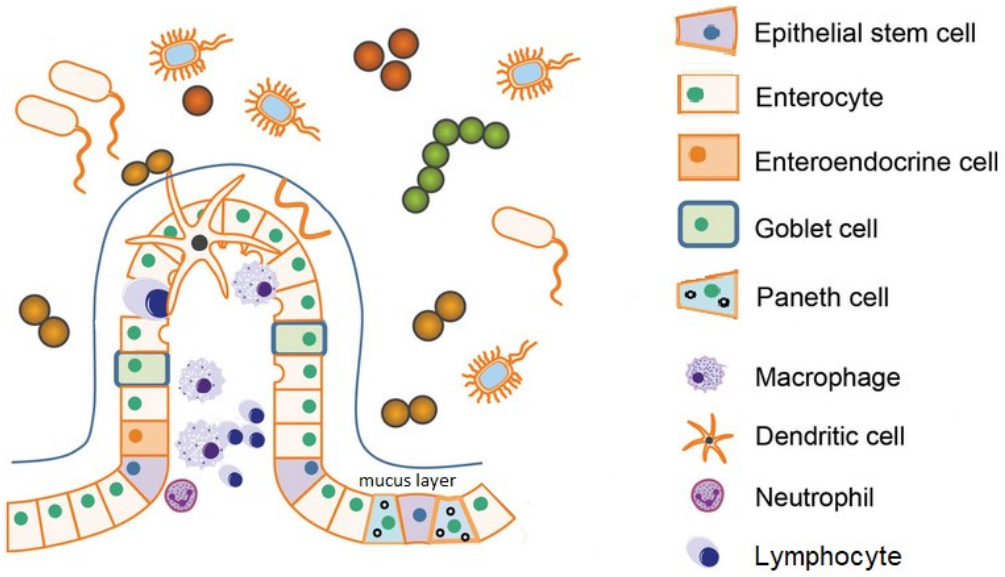

Figure 3 | The intestinal epithelial barrier. Adapted from Meng et al., 2015. Epithelial stem cells located in the crypt differentiate into four cell types: the absorptive enterocytes, enteroendocrine cells, goblet cells, and paneth cells. These cells are in close contact with immune cell populations including macrophages, dendritic cells, neutrophils, T lymphocytes, and B lymphocytes.

The main function of the intestinal barrier is to regulate the absorption of nutrients, electrolytes and water from the lumen into the circulation, while preventing the entry of pathogenic microorganisms and toxic luminal substances (König et al., 2016). This is achieved thanks to a dynamic regulation of the permeability between cells (paracellular permeability) and through cells (transcellular permeability) (Ménard et al., 2010) **[Figure 4]**. The mucus layer is also particularly important for the containment of microorganisms, as it contains high concentrations of secretory immunoglobulin (Ig) A and antimicrobial peptides.

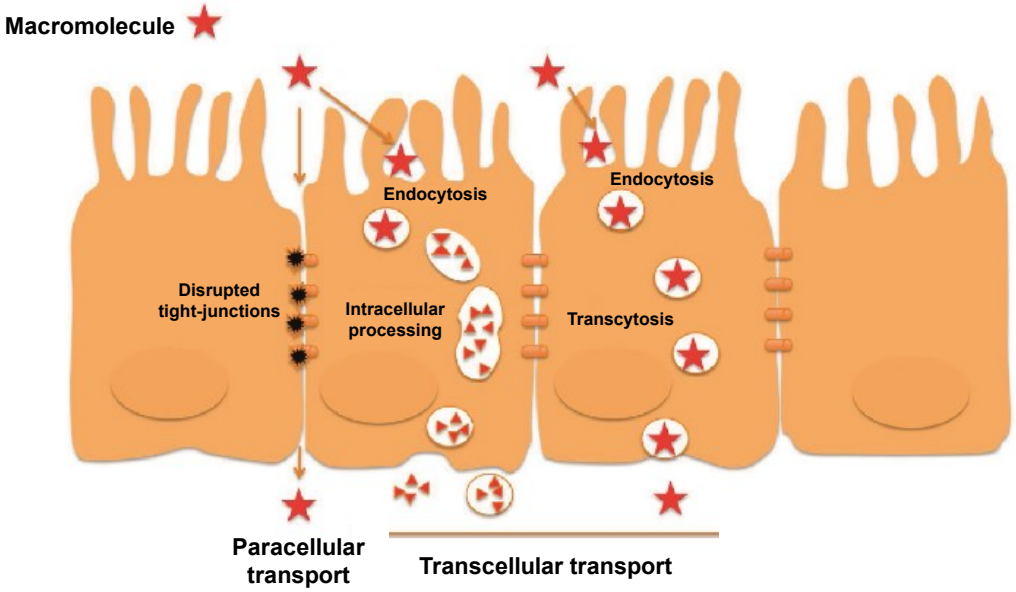

Figure 4 | Representation of the different selective permeability routes through the gut epithelium. Adapted from Heyman et al., 2012 and van Bilsen et al., 2017. Paracellular permeability between cells is

regulated by tight-junctions and allows the passage of small hydrophilic molecules *via* passive diffusion. In contrast, transcellular permeability involves active transport through channels, transporters or endocytosis. There are several ways to measure gut permeability in humans and animals (see Camilleri et al., 2012 for review). *In vivo* methods consist in quantifying the amount of a selected probe in the plasma or urine following oral administration. These methods are non invasive and can be done several times in the same individual. Probes of different molecular weight and physicochemical properties are used to evaluate para and transcellular intestinal permeability. In humans, intestinal permeability is classically measured using orally administered, non-degradable sugars or other molecules such as [51Cr]EDTA or polyethylene glycol (König et al., 2016). In animals, fluorescent probes such as FITC-dextran (a dextran labeled with fluorescein isothiocyanate) are widely used for paracellular permeability assessment, although plasma autofluorescence may interfere with the measure. Macromolecular transcytosis can be measured using horseradish peroxidase flux. However, changes in renal clearance or liver catabolism can affect these measurements and constitute a potential bias (Mattioli et al., 2011). Renal clearance of probes can be evaluated by injecting the probes directly into the penile vein and collecting urine for the ensuing 24 hours (Meddings and Gibbons, 1998). *Ex vivo* measurements using Ussing chambers (see Clarke, 2009 for review) circumvent renal clearance and offer the opportunity to control the electrochemical potential difference across the intestinal epithelium. The Ussing chamber provides a valuable method for the measurement of electrolyte and macromolecule active transport.

Paracellular permeability involves tight-junctions between epithelial cells **[Figure 5]**. Clayburgh and colleagues have shown that T cell activation causes intestinal epithelial barrier dysfunction characterized by increased paracellular protein flux, changes in tight junction protein distribution, and increased myosin light chain (MLC) phosphorylation (Clayburgh et al., 2005) **[Figure 5]**.

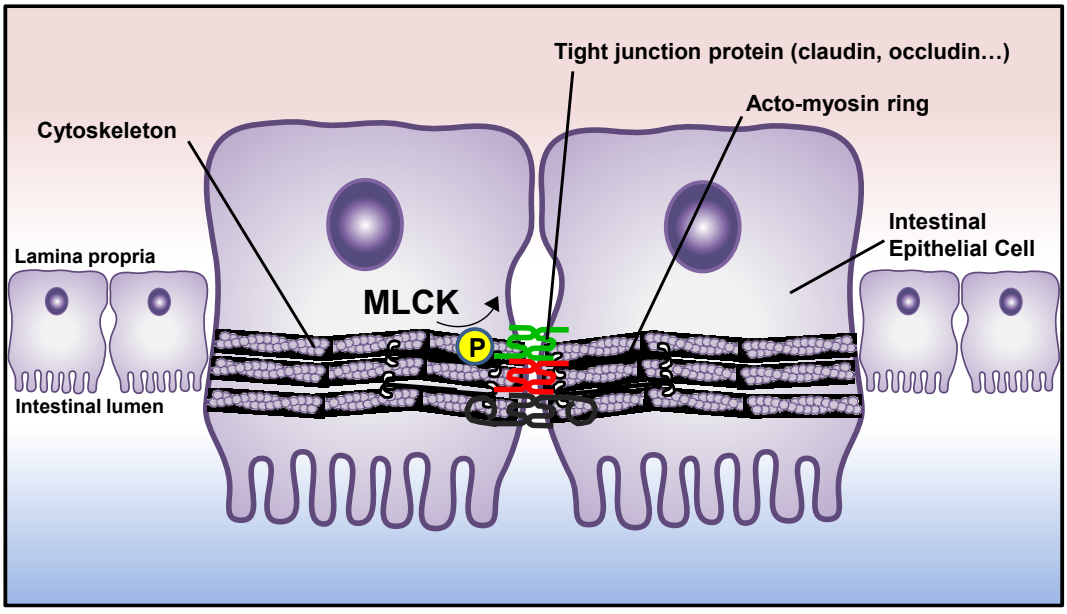

Figure 5 | The MLCK regulates tight-junction-dependent paracellular permeability. Tight junctions are complex protein structures that consist of transmembrane proteins such as claudin and occludin (Dörfel and Huber, 2012). Upon activation, the myosin II regulatory light chain kinase (MLCK) phosphorylates the myosin, thereby inducing a cytoskeleton remodeling and the opening of the junction (Zolotarevsky et al., 2002; Clayburgh et al., 2005; Shen et al., 2006).

In vitro and *in vivo* experiments revealed that this barrier dysfunction involves cytoskeleton-mediated epithelial tight junction opening, upon activation of the myosin II regulatory light chain kinase (MLCK) (Zolotarevsky et al., 2002; Clayburgh et al., 2005; Shen et al., 2006). Indeed, both genetic knockout and pharmacological MLCK inhibition effectively prevented T-cell-dependent loss of barrier function. The authors further suggested that the inhibition of MLCK may represent a novel non-immunomodulatory therapeutic approach. Moreover, increased MLCK activity was recently reported in IBS patients, suggesting that this mechanism is relevant to human disease (Wu et al., 2017).

The structure of the intestinal barrier is formed by the end of the first trimester (Montgomery et al., 1999). Maturation and functional development of the intestinal barrier continue in the post-natal period and are influenced by both feeding mode and diet (Cummins and Thompson, 2002). During early-life, gut permeability is relatively high as compared with the adult (about 10-fold) (Moussaoui et al., 2014).

1.1.3. Gut enteric nervous system

The enteric nervous system (ENS) is a complex neuronal network which extends from the esophagus to the anal sphincter (see Furness, 2012 for reviews). It is composed of a considerable number of neurons (over 80–100 million in rodents and 400–600 million in humans, that is, the same number as in the spinal cord), enteric glia (up to 4–7 times more numerous than neurons) and a network of nerve fibers communicating and projecting to effector tissues. It is the most complex division of the peripheral nervous system and is also coined as the "second brain" (Gershon, 1999). Indeed, while the sympathetic and parasympathetic autonomic nervous systems (ANS) provide extrinsic innervation to the GI tract and can modulate ENS activity, the ENS is capable of completely autonomous function without input from the brain or spinal cord. Key component of the gut, it coordinates the processes of digestion including mixing and propagation of GI luminal contents (motility), supply of digestive enzymes, absorption, fluid exchanges, storage and excretion), and is also involved in epithelial barrier function, nociception and immune responses. ENS neurons are organized into two main concentric ganglionated plexi **[Figure 6]**, the myenteric plexus (Auerbach) located between the longitudinal and circular muscle layers, and the submucosal plexus (Meissner) sandwiched between the submucosal matrix and external circular smooth muscle layer. The enteric glia populations are distributed within the plexi and the mucosa (Sharkey, 2015). Functionally, ENS neurons can be identified as motor neurons, intrinsic sensory (primary afferent) neurons and interneurons (Furness, 2012). They interact with each

other as well as with the other cell types including immune cells, enterocytes and neuroendocrine cells to control almost all gut functions. ENS neurons synthesize and use over 30 neuromediators that are similar to those present in the CNS (Furness, 2012). Cholinergic and nitrergic neurotransmissions are among the most abundant in the ENS and play key roles in the secretomotor, mucosal barrier and immune functions. Migration, differentiation and organization of enteric neurons mainly take place during early in-utero life. Microbial colonization and immune system development continue to shape the ENS during post-natal life (Kabouridis and Pachnis, 2015).

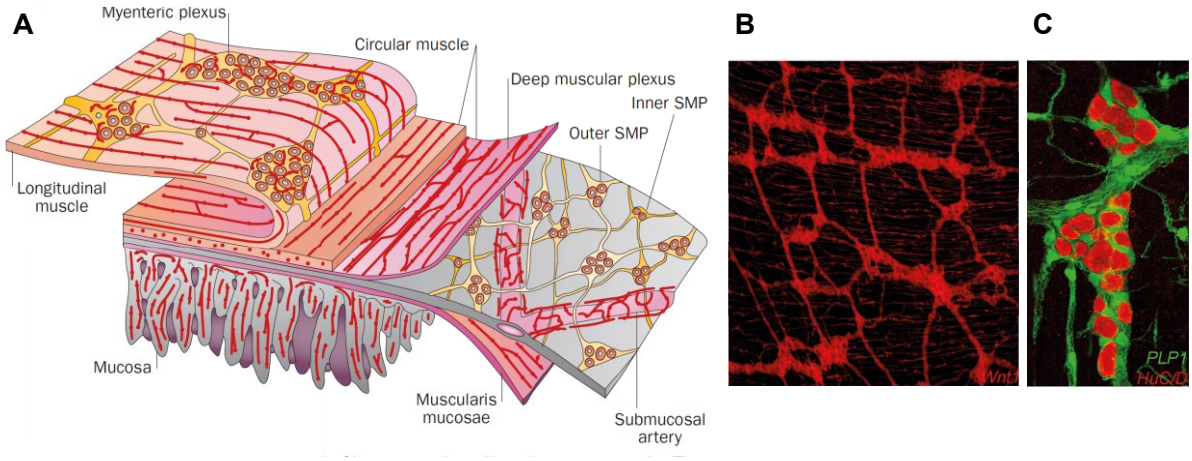

Figure 6 | Organization of the enteric nervous system. Adapted from Furness, 2012 and Nagy and Goldstein, 2017. (A) The enteric nervous system has ganglionated plexi, the myenteric plexus between the longitudinal and circular layers of the external musculature and the submucosal plexus that has outer and inner components. Nerve fiber bundles connect the ganglia and also form plexi that innervate the longitudinal muscle, circular muscle, muscularis mucosae, intrinsic arteries and the mucosa. SMP, submucosal plexus. Photomicrographs illustrating enteric neurons from the myenteric plexus (labeled in red) (B) and enteric ganglia with glia labeled in green and neurons in red (C).

1.1.4. Gut immune system

Intestinal homeostasis depends on complex interactions between the microbiota, the gut epithelium and the host immune system (see Macpherson and Harris, 2004, Cerf-Bensussan and Gaboriau-Routhiau, 2010 and Eberl, 2010; 2016 for reviews). Indeed, gut microorganisms stimulate immune cells at the mucosal surface **[Figure 3]** and the latter regulate microbial density via the release of IgA by B cells and the stimulation of antimicrobial peptides production by epithelial cells. Moreover, the immune system stimulates mucus secretion by goblet cells through the expression of interleukin (IL)-13. Other cytokines have an important role in the containment of microbiota, such as IL-17 or IL-22, that induce the recruitment of neutrophils and the production of antibacterial peptides including S100 protein (Eberl, 2010). Cytokines are small polypeptides produced by

numerous cell types including monocytes, macrophages and mast cells (Callard et al., 1999). They can act locally or travel to distal tissues as endocrine mediators. Importantly, only very low concentrations are needed for their biological effects via the binding to specific membrane receptors. Within the gut, cytokines are secreted by antigen-presenting cells and T cells and play a pivotal role in directing appropriate immune responses with respect to the nature of the encountered microorganisms (Eberl, 2016). They are capable of both promoting or dampening inflammatory environments, with a given cytokine generally recognized as intrinsically pro- or anti-inflammatory (O'Shea et al., 2002). However, this dichotomy is currently challenged as there is a dynamic equilibrium between pro- and anti-inflammatory immune responses (Eberl, 2010). The main cytokines are classified as interferons (IFN), interleukins, chemokines and the tumor necrosis factor (TNF)-α. In physiological conditions, dendritic cells and macrophages produce a set of specific cytokines depending upon the microbial environment (more specifically, whether gut microbes are intra- or extracellular threats) (Eberl, 2016). According to these cytokines, naive T helper (Th) cells adjust their expression of key transcription factors (T-bet, GATA-3 or RORγt) and thereby acquire a polarized phenotype (Th1, Th2 or Th17 subtypes, respectively) (Klatzmann and Abbas, 2015). In turn, these Th cells produce specific cytokines, with Th1 being the primary producer of IFNɣ, Th2 being associated with IL-4, IL-13, IL-10 and transforming growth factor (TGF) β, and Th17 with IL-17 and IL-22. Th17 cells are typically induced by the normal gut microbiota (healthy condition). In the context of strong inflammation and leaky gut, both Th17 and Th1 are likely recruited. On the other hand, type 2 polarization can occur in response to large organisms such as helminths – which are not supposed to be present in a healthy gut –, or to tissue damage. Innate immune cells permanently sense microbial antigens *via* a range of highly specific receptors (pattern recognition receptors) (Eberl, 2010). Toll-like receptors (TLRs) recognize conserved molecular motifs on microorganisms, such as, for example, lipopolysaccharide (LPS) from gram-negative bacteria (TLR4). In addition, peptidoglycan (a major component of bacterial cell wall) is recognized by nucleotide oligomerization domain (NOD) receptors 1 and 2 in the lamina propria. Physiological activation of these receptors is essential for colonic homeostasis (Bouskra et al., 2008; Wheeler et al., 2014). However, inappropriate activation of their signaling pathways may lead to deleterious inflammation and tissue injury.

Gut microbiota is critically involved in the shaping of immune development during early postnatal life. Indeed, in absence of microbiota, lymphoid tissues remain immature and the number of lymphocytes in the gut is dramatically reduced (Round and Mazmanian, 2009).

Even more striking is the need of microbial presence to induce the development of isolated lymphoid follicles (Bouskra et al., 2008), which constitute B cell reservoirs crucial for IgA production (Eberl and Lochner, 2009). Interestingly, the effects of gut microbes on immune maturation depend upon the nature of the bacterial communities. For instance, segmented filamentous bacteria, which have been shown to predominantly reside within the mucus layer in close proximity to the epithelium, are potent activators of gut Th cells including Th17 cells and likely play a unique role in the postnatal maturation of gut immune functions (Gaboriau-Routhiau et al., 2009; Ivanov et al., 2009; Cerf-Bensussan and Gaboriau-Routhiau, 2010). Furthermore, as for gut microbiota, there are sex differences in gut immune function and sensitivity in both humans and animals (Fransen et al., 2017).

1.2. Mechanisms of bidirectional gut-brain communication

The bidirectional interplay between gut and brain is illustrated in population survey studies revealing a strong correlation between anxiety, depression, and functional GI disorders. Furthermore, psychological distress can predict later onset of a functional GI disorder and the converse (Koloski et al., 2012). The mechanisms underlying this communication are beginning to be unraveled **[Figure 7]** (see Grenham et al., 2011; Mayer, 2011 and Cryan and Dinan, 2012 for reviews). From anatomical perspectives, the most obvious route of gut-brain communication is the ANS (neural route). ANS innervation connects the ENS and the CNS mainly via the vagal and splanchnic nerves (Furness, 2012; Bonaz and Bernstein, 2013). In addition, there exist a wide array of molecules arising from both gut and brain that travel in the bloodstream (humoral pathway) and may be involved in gut-brain axis bidirectional communication.

1.2.1. Top-down communication

Descending signals from the brain have been shown to modulate the motor, sensory, secretory, and immune functions of the gut in physiological conditions. This top-down communication is particularly evident to each of us in the manifestation of stress effects. Indeed, acute intense stress can rapidly trigger bloating, abdominal pain or discomfort and even diarrhea. In the following, we will describe the central and peripheral stress responses and discuss their impact on the gut.

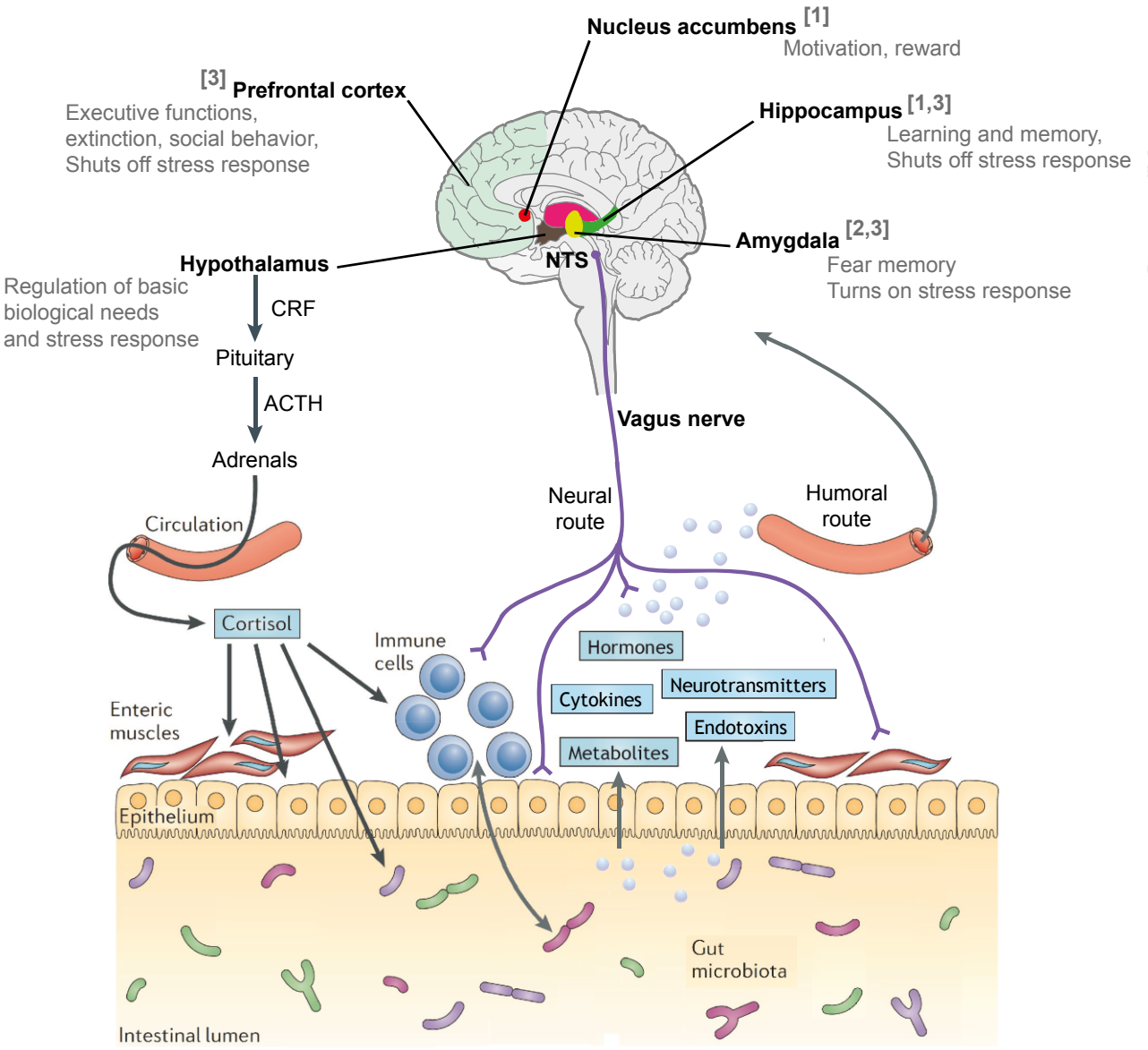

Figure 7 | The gut-brain axis. Adapted from Cryan and Dinan, 2012. Multiple potential direct and indirect pathways exist through which the gut and the brain communicate bidirectionally. They include endocrine (cortisol), immune (cytokines) and neural (vagus and enteric nervous system) pathways. The brain recruits these mechanisms to influence gut physiology, for example under conditions of stress. The hypothalamus–pituitary–adrenal axis regulates cortisol secretion, and cortisol can affect immune cells (including cytokine secretion) both locally in the gut and systemically. Cortisol can also alter gut permeability and barrier function, and change gut microbiota composition. Conversely, the gut can influence the brain *via* an array of molecules including hormones, cytokines, neurotransmitters and metabolites, but also endotoxins such as lipopolysaccharide. These gut-derived molecules may stimulate primary afferent nerve fibers of the vagus nerve (neural pathway), which relay information to brain areas through activation of the nucleus of the tractus solitarius (NTS). Gut-derived molecules may also travel in the bloodstream (humoral pathway) and access the brain through leaky regions of the blood-brain barrier such as the choroid plexus and circumventricular organs. [1] Brain areas associated with major depression (Maletic et al., 2007) or [2] anxiety disorders (Charney and Deutch, 1996). [3] Brain areas active in response to colorectal distension in IBS (Irritable bowel syndrome) patients (see paragraph 1.2.1.2.) (Guleria et al., 2017). ACTH, adrenocorticotropic hormone; CRF, corticotropin-releasing factor, NTS, nucleus tractus solitarius.

1.2.1.1. The stress system

When the brain detects a threat, a coordinated physiological response involving autonomic, neuroendocrine, metabolic and immune components is activated (see Herman et al., 2003, 2016 and Ulrich-Lai and Herman, 2009for reviews) **[Figure 8]**.

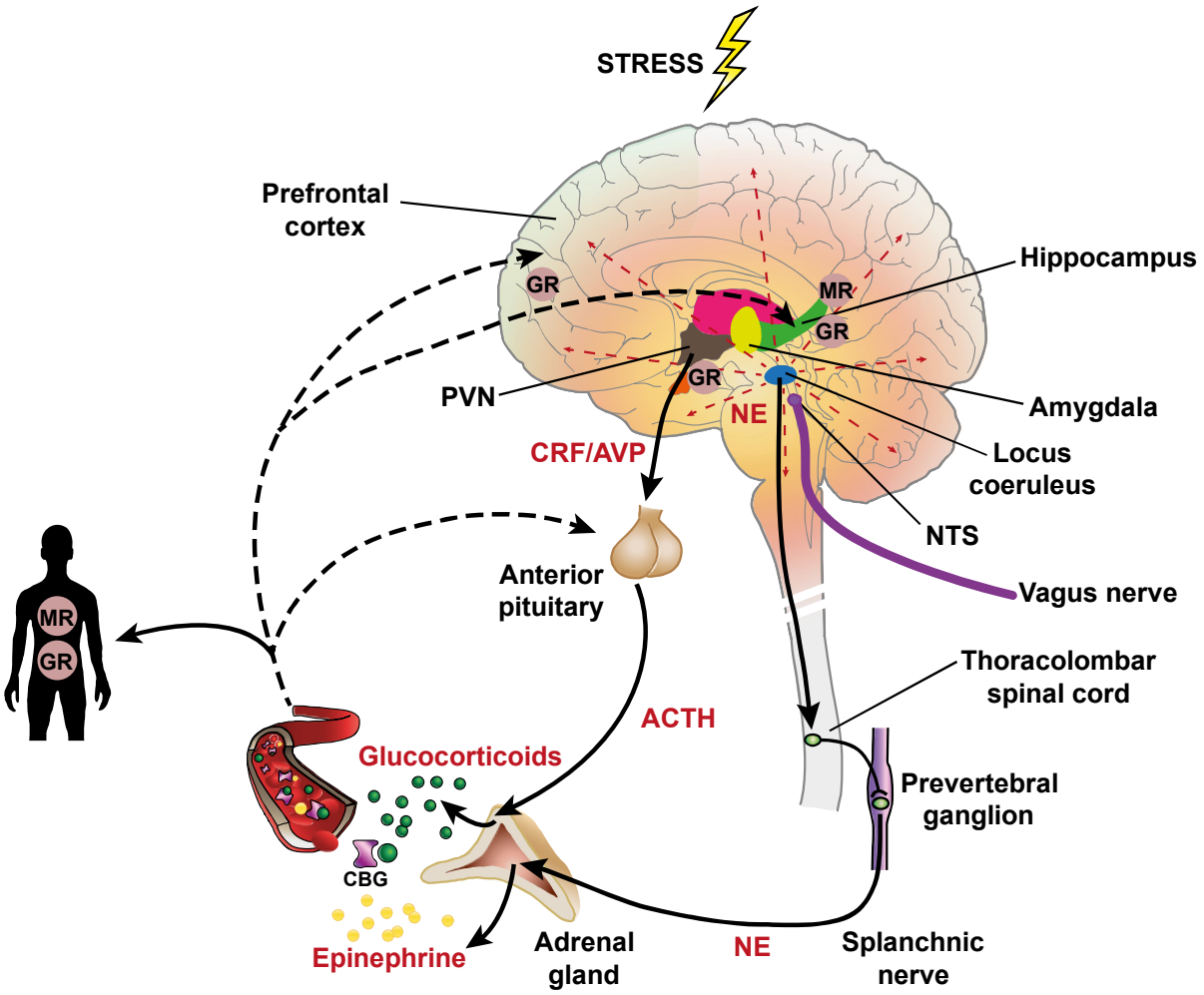

Figure 8 | The different components of the stress response. Adapted from Moisan and Le Moal, 2012 and Raabe and Spengler, 2013. ACTH, Adenocorticotropic hormone; AVP, Arginine-vasopressin; CBG, Corticosteroid binding globulin; CRF, Corticotropin releasing factor; GR, Glucocorticoid receptors; MR, Mineralocorticoid receptors; NE, Norepinephrine; PVN, Paraventricular nucleus of the hypothalamus; NTS, Nucleus tractus solitarius.

Psychogenic stress (such as fear, novelty) and systemic stress (such as blood loss, respiratory distress, immune stress and visceral pain) are relayed via limbic forebrain structures (prefrontal cortex (PFC), hippocampus, amygdala) and brainstem nuclei (nucleus tractus solitarius (NTS), parabrachial nucleus…), respectively, to the major stress-integrative brain centers located in the hypothalamus and in the brainstem. They include the parvocellular neurons of the paraventricular nucleus of the hypothalamus (PVN) and the locus coeruleus noradrenergic cell groups of the pons and medulla. The locus coeruleus noradrenergic system releases norepinephrine from a dense network of neurons throughout the brain, resulting in

enhanced arousal and vigilance; it also activates the ANS through descending projections to the preganglionic sympathetic nervous system. The autonomic sympathetic and parasympathetic systems provide the most immediate response to stress, and provoke rapid alterations in physiological state through neural innervation of end organs. The locus coeruleus activates the sympathetic nervous system by sending signals through the autonomic nerves to the medulla part of the adrenal glands which secretes epinephrine into the bloodstream. Epinephrine brings on a number of physiological changes (increasing blood flow to muscles, output of the heart, pupil dilation, and glucose and fat release) supporting the 'fight or flight' survival response.

A second key system in the stress response is the neuroendocrine hypothalamus-pituitary-adrenal (HPA) axis. Neurons in the medial parvocellular region of the PVN release corticotropin-releasing factor (CRF) and arginine-vasopressin (AVP). This triggers the subsequent secretion of adrenocorticotropic hormone (ACTH) from the anterior part of the pituitary gland (adenohypophysis), leading to the production of glucocorticoids (GC) by the adrenal cortex (cortisol in humans, corticosterone in rodents). As lipids, GC in the blood need to be transported: 95% of circulating GC are bound to either corticosterone binding globulin (CBG) (80%) or albumin (15%). CBG maintains a circulating GC pool and ensures GC delivery to target tissues. GC levels are also regulated by 11β-hydroxysteroid dehydrogenase (11β-HSD) 1 and 2, that interconvert active (cortisol or corticosterone) and inactive GC (cortisone or 11-dehydrocorticosterone). GC exert a wide range of effects both in the periphery and in the CNS via their glucocorticoid (type 2, low affinity) and mineralocorticoid (type 1, high affinity) receptors (GR and MR), expressed in many body tissues, including brain, bone, skin, liver and gut among many others. Within the brain, MR are specifically expressed in limbic areas, whereas GR are ubiquitous. At the cellular level, MR and GR are located in the cytosol, at the cell membrane and on mitochondrial membranes. Notably, MR and GR belong to the superfamily of nuclear receptors. When inactive, they reside in the cytoplasmic compartment, but when bound to their ligands, they translocate to the nucleus where they bind DNA sequences known as glucocorticoid/mineralocorticoid-response elements (GRE/MRE) to modulate the transcriptional activity of a plethora of target genes (Datson et al., 2008; Surjit et al., 2011). They can also interact with other transcription factors, such as nuclear factor-κB (NFκB), cyclic AMP response element-binding (CREB), activator protein-1 (AP-1) and several signal transducers and activators of transcription (STATs) (Datson et al., 2008). Many neurotransmitter systems are under GC control, including the serotonin receptor 1A (5HTr1A), the adenosine receptor A1, the vasopressin

receptor V1a and oxytocin receptors as well as monoamine oxidase (MAO) A (monoamines degradation enzyme). GC also regulate the expression of neurotrophic factors and their receptors and genes involved in neuronal shaping. They exert an effect on both exocytosis and endocytosis by affecting transcription of genes involved in vesicle recycling. Other GC-regulated genes are associated with apoptosis, cell cycle progression, intracellular signaling and circadian rhythms. In peripheral tissues, GC target genes regulate immune response and a variety of metabolic processes, including lipogenesis, insulin secretion, glucose clearance, lipid accumulation and nutrient absorption. Regulation of transcription of genes involved in gluconeogenesis and glycolysis not only occurs in key energy-consuming tissues such as the liver and muscles, but also in the brain. Finally, GR and MR at the cell membrane and on mitochondria can rapidly alter neuronal function upon ligand binding, independent of translocation to the nucleus, by modulating processes such as excitatory amino acid release. Moreover, GC also play a major role in the regulation of their own secretion. Negative feedback mechanisms on PVN neurons through GC action at different levels of the HPA axis as well as on PFC and hippocampus lead to the inhibition of GC secretion (Dallman et al., 1994). These processes are activated to counteract the physiological activation and reinstate the internal equilibrium of the organism (Chrousos and Kino, 2009), through a process called allostasis or "stability through changes" (McEwen, 1998). However, if the stressor persists and becomes chronic, the body enters a resistance phase and tries to adapt to the strains and demands of the environment by engaging coping mechanisms. When the severity and/or chronicity of the stressors exceeds certain limits, the adaptive system becomes defective, leading to a state of allostatic load (McEwen, 1998). This state, reflecting the "cost" of the adaptation, is harmful to the organism and lies at the origin of a variety of stress-related diseases that develop in the context of a vulnerable background (Chrousos and Kino, 2009). The pathogenesis of stress-induced disorders affects the whole body, including the viscera, of which the GI tract is a sensitive target (Chrousos and Kino, 2009; Stengel and Taché, 2010).

1.2.1.2. Effects of stress on the intestinal tract

Exposure to stress and GC profoundly affects GI function and immunity. As previously mentioned, acute stress response involves both sympathetic and parasympathetic autonomic systems. While the sympathetic nerves are activated (including splanchnic and pelvic nerves), the parasympathetic component (including the vagus nerve) is blunted, resulting in inhibition of digestive functions (see Bonaz and Bernstein, 2013 for review). This nervous stress response is mediated by acetylcholine, norepinephrine and adrenaline and leads to immediate increase in gut motility and thus accelerated intestinal transit. Moreover, chronic stress

decreases vagal efferent outflow (Taché and Bonaz, 2007) and this effect has been shown to favor intestinal inflammation (Straub et al., 2006). Intriguingly, vagal stimulation is used in humans to attenuate systemic or intestinal inflammation, suggesting that the vagal nerve could exert anti-inflammatory actions. Indeed, it has been shown that vagal stimulation decreases the production of TNF by macrophages through cholinergic neurotransmission (Borovikova et al., 2000). However, vagal stimulation likely affects both efferent and afferent fibers, and the underlying mechanisms are unclear.

Another consistent effect of stress exposure is the induction of gut barrier leakiness as well as visceral pain hypersensitivity (Barreau et al., 2004a, 2004b; Ait-Belgnaoui et al., 2005, 2009, 2014, 2012; Schwetz et al., 2005; Gareau et al., 2006; Tjong et al., 2011; Øines et al., 2012; Chen et al., 2013; Da Silva et al., 2014; Moussaoui et al., 2014, 2016a, 2016b). It is worth mentioning that if the stress-induced gut leakiness was first showed in animals, a recent human study reported similar effects of acute stress in healthy subjects (Vanuytsel et al., 2014). In addition to its effects on gut permeability and visceral pain, chronic stress affects gut microbiota composition, induces upregulation of TLRs expression in the colonic mucosa, affects the levels of intestinal secretory IgA and increases circulating cytokines levels (Bonaz and Bernstein, 2013). Moreover, animals submitted to chronic stress are more susceptible to experimental colitis. Importantly, acute water avoidance and restraint stress, but also early-life stress such as maternal separation, are among the most widely used paradigms to model IBS in preclinical studies (Moloney et al., 2015a). Remarkably, stress-induced intestinal hyperpermeability appears to be GC-dependent, as it is evoked by the synthetic GC dexamethasone and prevented by administration of a GR antagonist (RU486) (Moussaoui et al., 2014). Likewise, GR antagonism also prevents stress-induced visceral hyperalgesia (Myers and Greenwood-Van Meerveld, 2012; Prusator and Greenwood-Van Meerveld, 2017). On the other hand, the CRF peptide also plays a role in the effects of stress on the gut (Taché and Perdue, 2004). For instance, it has been shown that CRF administration directly into the amygdala increases visceral pain (Su et al., 2009a), while amygdalar CRF antagonism reverses visceral hypersensitivity (Johnson et al., 2012). Similarly, peripheral administration of a CRF receptor antagonist blocks the stress-induced visceral hyper-sensitivity and gut leakiness in rats (Schwetz et al., 2005; Million et al., 2006; Barreau et al., 2007a). Importantly, stress also induces CRF local expression in the gut and CRF receptors are widely expressed in the colonic mucosa, in particular in immune cells including macrophages, lymphocytes and mast cells (Taché and Perdue, 2004).

1.2.2. Bottom-up communication

There is increasing evidence that the gut also signals to the brain and can have causal effects on behavior, especially stress-related behaviors. In particular, numerous studies demonstrate a key role of the gut microbiota as a central node in gut-brain communication. An outstanding question is how can microbes located in the gut, separated by tight barriers from the host's inside, exert such effects? As for top-down communication, several lines of evidence show that bottom-up communication involve the neural pathway (vagus nerve) and humoral pathway (circulating factors) **[Figure 7]**.

1.2.2.1. Potential role of the vagus nerve in gut to brain signaling

The vagus nerve is a mixed nerve containing both efferent and afferent fibers. However, 90% of these fibers are afferent fibers, suggesting that the vagus nerve is particularly important for gut to brain signaling. So far, it has been shown that the vagus nerve conveys information regarding intestinal mucosa distortion, luminal osmolarity, the quality and quantity of luminal nutrients and the presence of bacterial products (Bonaz and Bernstein, 2013). Vagal afferents terminate in the dorsal vagal complex located in the brainstem (including the NTS). The NTS is further connected with limbic structures *via* the PVN (Rinaman et al., 2011; Herman, 2017). Importantly, vagal terminals are in close contact with mucosal mast cells and express cytokines receptors, suggesting that local immune activation in the gut could impact the brain through electrical inputs. Indeed, it has been shown that proinflammatory cytokines such as IL-1β, IL-6 and TNFα can stimulate vagal afferents and lead to activation of CRF neurons in the PVN (Dantzer et al., 2000). On the other hand, brain imaging studies in humans have shown that rectal distension, induced by inflating a balloon cathether inserted into the rectum, altered brain activation patterns in healthy volunteers (Baciu et al., 1999). Moreover, differential brain responses to visceral noxious, but also, non-noxious stimuli, which is characteristic of visceral hypersensitivity, were reported in IBS patients compared with controls (Andresen et al., 2005; Labus et al., 2008; Elsenbruch et al., 2010; Tillisch et al., 2011; Larsson et al., 2012). In these imaging studies, the changes in brain activity and connectivity were observed within a few seconds following visceral stimulation (Liu et al., 2016; Guleria et al., 2017), suggesting an underlying nervous communication. In the last 15 years, a role of the vagus nerve in mediating gut-brain axis effects on anxiety and emotional behavior has been documented in several animal studies using vagotomy or inactivation of the NTS. For instance, anxiolytic and antidepressant-like effects of probiotics are lost in vagotomized mice (Bravo et al., 2011). In addition, it has been shown that LPS exposure in mice decreases social interaction, but this effect is lost in animals that undergo vagotomy or

NTS pharmacological inactivation before LPS injection (Konsman et al., 2000; Marvel et al., 2004). Similarly, hyperanxiety produced by chemically-induced GI inflammation with dextran sodium sulfate (DSS), a widely used agent for the induction of colitis, could be reversed by a probiotic treatment in a vagal-dependent manner (Bercik et al., 2011a). However, another study reported no effect of vagotomy on hyperanxiety induced by infection with the non invasive parasite *Trichuris* muris (Bercik et al., 2010). On the other hand, vagal stimulation has been found to exert anxiolytic and antidepressant-like effects in rodents (Furmaga et al., 2011; Shah et al., 2016) and is currently used as a therapy in resistant epileptic and depressive patients (Kosel and Schlaepfer, 2002). However, as previously mentioned, vagal stimulation or vagotomy likely involves both bottom-up and top-down communication between the gut and the brain. Moreover, vagus nerve innervation is not limited to the GI tract (as a matter of fact, it only innervates the upper part of it, i.e. the stomach and proximal small intestine) but rather extends to the heart, lungs, pancreas and endocrine glands including thyroid and adrenals. Although their contribution remains underexplored, the splanchnic and pelvic autonomic nerves – which do innervate the lower intestine including the colon – could also play a role in the gut-brain axis.

1.2.2.2. Potential role of gut-derived molecules in gut to brain signaling

Another important route of gut-brain communication is the bloodstream. Circulating factors of different nature and origin can eventually reach the brain to regulate its function and behavioral responses. These gut-derived molecules include gut hormones, neurotransmitters, metabolites (arising from both the host and gut microbes), endotoxins or other microbial antigens, and inflammatory cytokines. Importantly, the potential role of these molecules in gut-brain communication must be cautiously addressed with regard to their intrinsic ability to cross or not the blood-brain barrier (BBB) (Engelhardt and Ransohoff, 2012). Nevertheless, it is worth mentioning that if the BBB prevents the passage of gut-derived molecules in healthy adult individuals, these molecules may cross the barrier in young subjects or under pathological context. Moreover, the gut microbiota and its associated products have been shown to influence BBB function (Braniste et al., 2014). On the other hand, the circumventricular organs, located outside the BBB, are sensitive to the vascular content and can modulate the activity of neighboring neurons.

As previously mentioned, the GI tract releases numerous hormones such as GLP-1, ghrelin, PYY, CCK, vasoactive intestinal peptide (VIP), gastric inhibitory polypeptide (GIP), gastrin and secretin, that are known to be involved in digestive processes as well as metabolic,

cardiovascular and immune processes. Interestingly, numerous studies have demonstrated that these molecules also modulate brain functions and behavior including food intake, emotion and cognition (Holzer, 2016). For instance, they have been implicated in visceral pain (CCK) (Wang et al., 2015), but also neuroprotection, neurogenesis, fear and anxiety, depression and memory (Lee and Soltesz, 2011; Bowers et al., 2012; Jiao et al., 2017). PYY and GLP-1 administration to rodents have significant effects on neurotransmitters and behavior, including learning and memory (Stadlbauer et al., 2015), but also anxiety and depressive-like behavior (Morales-Medina et al., 2012; Anderberg et al., 2016). The mechanism by which gut peptides affect brain functions is still a matter of debate. Some of them such as ghrelin or GLP-1 cross the BBB, or such as CCK may be released by vagal afferents fibers in the NTS (Sartor and Verberne, 2008), but most of them are also secreted directly within the brain as neuropeptides. Therefore, it is often difficult to determine whether the effects of gut hormones on the brain are mediated by peripheral (neuroactives peptides) or central (neuropeptides) sources of these peptides.

In addition, gut bacteria modulate various host metabolic reactions, resulting in the production of a vast repertoire of metabolites that have been identified as potential key players in gut-brain communication, such as bile acids, tryptophan (an amino acid essential for the synthesis of serotonin by the host), acetylcholine, dopamine, norepinephrine and short-chain fatty acids (SCFAs) (see Lyte, 2014; El Aidy et al., 2015 and Rooks and Garrett, 2016 for reviews). In particular, tryptophan and neurotransmitter metabolism are known for their neuroactive properties. Whether they can reach the CNS – and in sufficiently high concentrations – is currently unclear. However, they may modulate neural signaling within the ENS, or act directly on primary afferent axons of the ANS. Moreover, recent studies demonstrated that SCFAs such as butyrate can impact brain and behavior *via* different actions including epigenetic-related mechanisms (Bourassa et al., 2016; Stilling et al., 2016). For instance, SCFAs modulate the maturation and function of microglia, which are the resident macrophages of the CNS (Erny et al., 2015). Microbial antigens (e.g. LPS, peptidoglycans) can also cross the intestinal epithelium and enter the bloodstream. A recent study showed that bacterial peptidoglycan can be translocated into the brain and sensed by specific receptors, which were found to be highly expressed in the developing brain during postnatal development in both males and females (Arentsen et al., 2017). They further showed that knocking-out one of these receptors resulted in sex-dependent changes in social behavior. Alternatively, microbial antigens also stimulate local cytokine production by mucosal immune cells. Cytokines and even activated immune cells can in turn travel to the brain or

modulate ENS and vagal activity (Capuron and Miller, 2011). In the CNS, circulating cytokines can have direct effects or trigger *de novo* production of cytokines by glial cells, including in brain areas involved in the regulation of stress response, emotion and cognition (Capuron and Miller, 2011). Cytokines have been shown to modulate HPA axis activity, the metabolism and function of several neurotransmitters, neuronal plasticity and behavior. As an example, during infection the synthesis of brain cytokines acts to coordinate the behavioral changes reffered to as 'sickness behavior' (anxiety, fatigue…)(Dantzer, 2001). This sickness behavior is a transient adaptive response that contributes to the elimination of the pathogen and the resolution of inflammation. However, several pathological conditions such as depression are associated with a chronic low grade inflammation, which has been proposed to induce behavioral alterations (Raison et al., 2006; Maes, 2008; Nusslock and Miller, 2016).

During the last decade, a growing number of studies have explored this gut-brain communication through the evaluation of the impact of gut microbiota manipulations on brain function and behaviors. We will review these studies in the next chapter.

CHAPTER II - Impact of gut-directed interventions on brain and behaviors

The gut microbiota is highly sensitive to the environment and alterations of its composition (dysbiosis) have been described in conditions ranging from IBS and obesity to depression and autism (Zhao, 2013; Collins, 2014; Wang and Kasper, 2014; Mangiola et al., 2016). Of the many environmental factors, stress emerges as a consistent detrimental factor for the gut microbiota. However, the mechanisms underlying the ability of stress to modulate microbiota composition remain to be unraveled. Moreover, it is unclear whether dysbiosis is a causative factor in the etiology of the above-mentioned pathologies. Interestingly, studies using different, but complementary, gut microbiota-directed interventions (Germ-free (GF) rodents, antibiotics, probiotics, GI infection and fecal microbiota transplantation) have demonstrated that gut bacteria can signal to the brain through a variety of mechanisms and influence processes such as neurotransmission, neurogenesis, neuroinflammation, neuroendocrine stress response, and modulate behavior (see Cryan and Dinan, 2012; Burokas et al., 2015; Sampson and Mazmanian, 2015 and Sharon et al., 2016 for reviews). In particular, certain gut bacteria can have a benefical effect on emotional behaviors and, as such, have been proposed for potential therapeutic interventions in psychiatry (concept of psychobiotics) (Dinan et al., 2013; Sherwin et al., 2016). In this chapter, we will briefly mention some of the findings on cognitive function and memory and rather more extensively review the impact of gut-directed

interventions on emotionality and stress response. Furthermore, we will mention a number of studies demonstrating a key role of the gut in brain development.

The basis of this microbiota-gut-brain research stems from rodent studies. Psychiatric diseases and psychiatric symptoms cannot be directly assessed in animals. Available tools to evaluate emotionality are mostly limited to tests with a poor ethological validity but rather good predictive validity, which means that these tests are sensitive to pharmacological treatments used in psychiatry (anxiolytics, antidepressants). A summary of the different tests used to assess anxiety and depressive-like behaviors in rats and mice is presented in **Figure 9** (also see Cryan and Holmes, 2005; Nestler and Hyman, 2010 and York et al., 2012 for reviews).

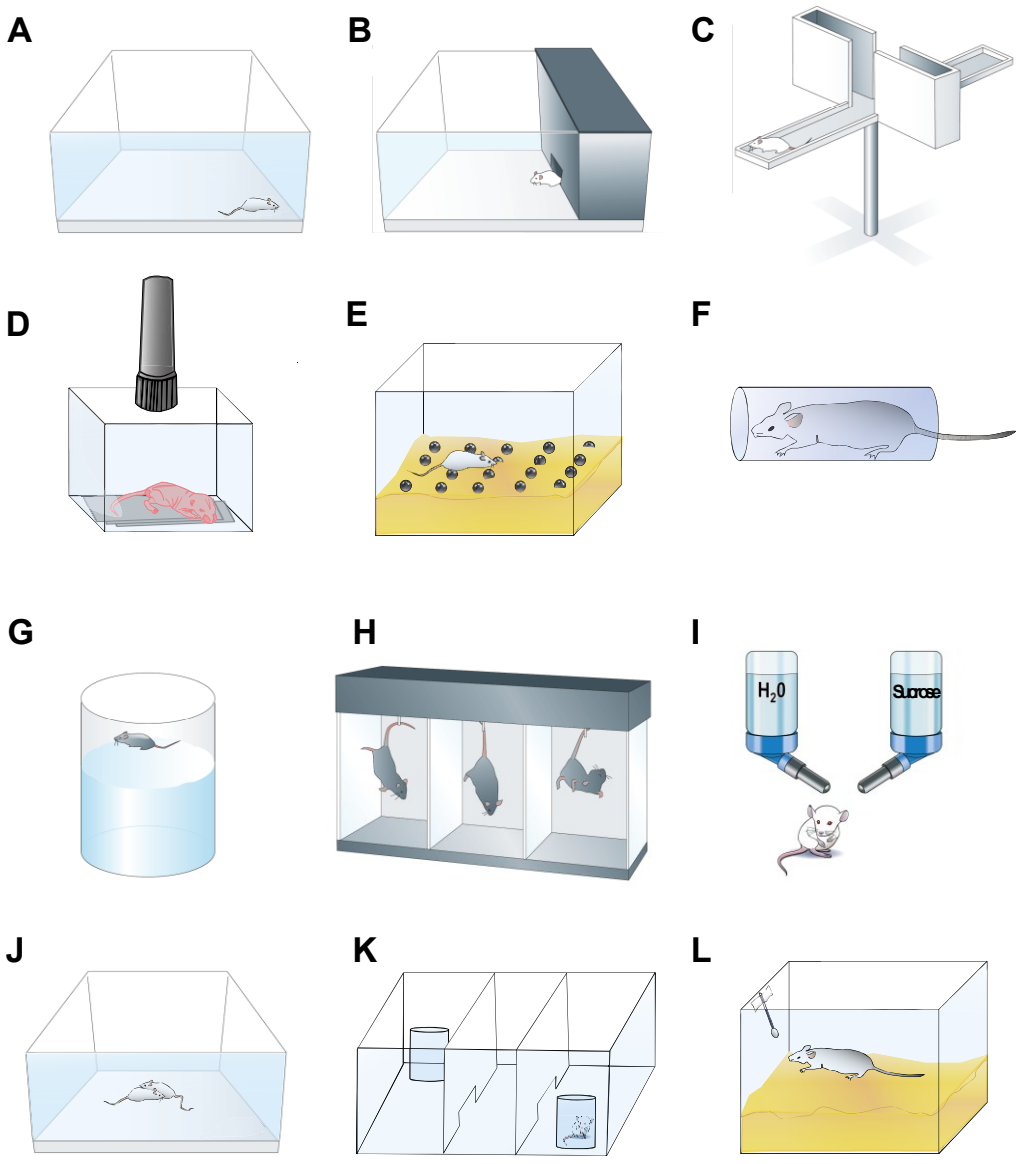

Figure 9 | Behavioral testing for emotionality in rodents. Adapted from Cryan and Holmes, 2005 and Talukdar et al., 2016. The most widely used tests to measure anxiety-like behavior in rodents are the open-field (A), the light/dark box (B), and the elevated plus maze (C) tests. These tests are based on rodents' natural fear of open spaces and preference for dark and confined places. Thus, an animal that spends less time exploring the

open areas and rather sticks around the shady or closed spots is considered anxious. Indeed, anxiolytic treatments increase the time exploring the open arms of the elevated plus maze, the center of the open-field or the light compartment of the light/dark box (Lister, 1990). During development, it has been shown that the emission of ultrasonic vocalizations (USV) (D) can be used as an index of anxiety in pups separated from their dams for a short duration (<10 minutes). Indeed, anxiolytics blunt USV response (see (Iijima and Chaki, 2005) for review). Moreover, other complementary tests for anxiety such as, for instance, the marble burying test (E), can be used in mice (Deacon, 2006). This test consists in introducing marbles in the homecage (or after habituation to a novel cage lined with bedding). Mice will spontaneously bury the 'intruder' marbles and this behavior is sensitive to anxiolytics. An increase in the number of buried marbles is associated with increased anxiety. In addition to the behavioral assessment of anxiety, many studies examine the HPA (hypothalamus-pituitary-adrenal) axis response to stress using, for instance, restraint stress (F). The animal is stuck in a plastic cylinder for a variable duration (around 30 min) and blood samples are collected at several timepoints before, during and after this stress for ACTH/corticosterone determination. Likewise, the most used tests for depressive-like behavior are the forced-swimming (G) and the tail suspension (H) tests (FST and TST). The FST was developed by Porsolt (Porsolt et al., 1977) and consists in placing the animal in a cylinder filled with warm water for 2 successive sessions. During the second session, the time spent immobile is a reflection of the despair state. Similarly, the TST (used in mice only) is based on immobility of the mice when suspended by the tail. These tests are also pharmacologically validated as antidepressant treatments decrease immobility and promote struggling. In addition, depressive-like behaviors can also be evaluated through the assessment of anhedonia-like status, such as in the sucrose preference test (I) (Willner et al., 1987). This test is based on the natural preference of rodents for a sucrose solution *versus* tap water, presented in a free-choice situation. A decreased preference for sucrose is representative of an anhedonic state. More ethological tests have been developed to evaluate other types of anhedonic behaviors that involve social or sexual dimensions. For instance, since rodents are social animals, a simple measurement is the time spent in social interaction with a conspecific in a neutral environment (J). On the other hand, the three-chamber test (K) is used to quantify the preference of an animal for a social target *versus* an empty compartment (or an objet/inanimate conspecfic) (Kaidanovich-Beilin et al., 2011). This measure of sociability can be automated and thus is often preferred. Decreased time spent in social interaction or decreased preference for the social target can be interpreted as social anhedonia. A more specific test available in males is the female urine sniffing test (FUST) (Malkesman et al., 2010) (L). In this test, the time spent sniffing estrus female urine *versus* water (presented using a cotton Q-tip) is significantly greater in control animals displaying normal sexual reward seeking behavior. The loss of interest for female urine in the FUST is restored by antidepressants and therefore can be considered as representative of a depressive-like state.

2.1. Germ-free animals

The study of GF animals served as a proof of concept for the role of gut microbiota in the regulation of brain function and behavior. A large number of studies have explored GF-associated phenotypes both in the gut and the brain. Here, we will non exhaustively summarize these phenotypes **[Figure 10]** (see Luczynski et al., 2016a for review).

GF animals (most often mice) are generated by aseptic caesarean section and adopted by a GF foster mother in a sterile environment (isolator), and grow up in isolators throughout life and across generations (Gustafsson, 1959a; Smith et al., 2007). It is worth mentioning that GF status is completely artificial and is not specific to the gut microbiota. Nevertheless, the absence of microbial colonization is deleterious for the digestive function and GF animals consume more food than control animals to maintain the same body weight (Wostmann et al., 1983). Indeed, gut microbes synthesize essential nutrients otherwise unavailable for the host's metabolism (Sekirov et al., 2010). To survive, GF rodents are fed a diet enriched in several

vitamins including vitamin B and K (Gustafsson, 1959b; Sumi et al., 1977). Importantly, GF models imply that the animals are GF during the whole developmental period, not only in adulthood. This has a number of implications regarding GF phenotypes. Indeed, as seen earlier in this introduction, the gut microbiota is necessary for normal maturation of the mucosal immune system (Macpherson and Harris, 2004). Moreover, GF animals display markedly impaired ENS development, with overall decreased nerve density in the small intestine (Collins et al., 2014). These alterations are associated with altered intestinal motility (Husebye et al., 1994, 2001). A recent study also reported increased visceral pain sensitivity in GF mice compared specific pathogen-free controls (SPF; mice guaranteed to not harbor certain pathogens (Luczynski et al., 2017).

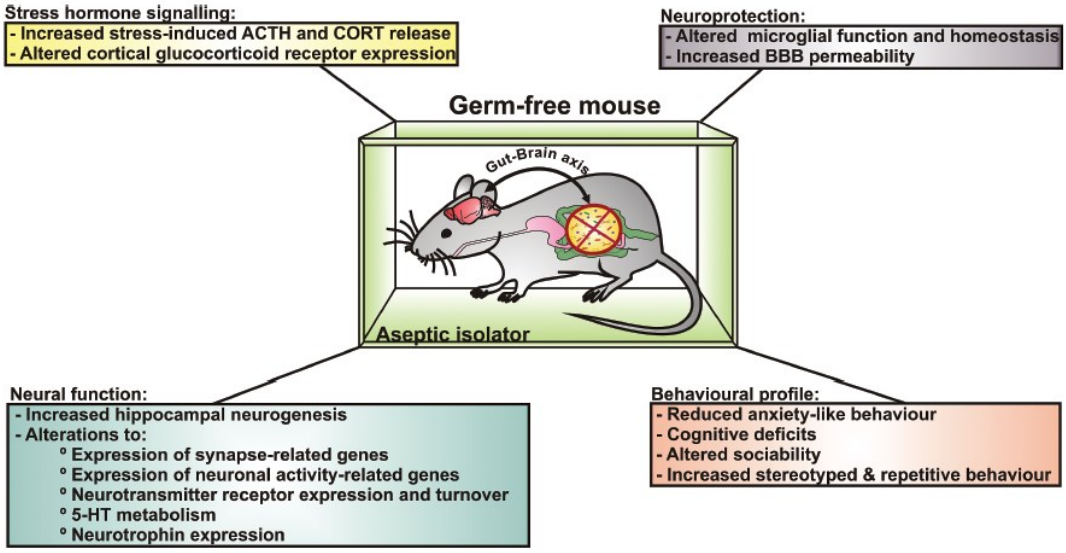

Figure 10 | Germ-free (GF) mice as a tool to study the microbiota-gut-brain axis. Figure from Luczynski et al., 2016a. GF mice are raised in isolation in a GF unit without any exposure to microorganisms. Gut-brain signaling is altered due to the lack of bacterial exposure throughout the lifetime. The microbiota is required for normal stress responsivity, anxiety-like behaviors, sociability, and cognition. Furthermore, the microbiota protects CNS homeostasis by regulating immune function and blood brain barrier (BBB) integrity. 5-HT, 5 hydroxytryptamine (serotonin).

Studies in GF rodents have revealed that the microbiota influences HPA axis responsiveness to stressors and associated emotional behaviors. Indeed, a study by De Palma and colleagues showed that early-life stress fails to induce long-term endocrine and behavioral alterations in GF mice compared with SPF controls (De Palma et al., 2015). On the other hand, adult GF animals show exaggerated release of corticosterone in response to stress compared with SPF controls (Sudo et al., 2004; Crumeyrolle-Arias et al., 2014). This HPA axis dysregulation is accompanied by alterations of stress-related behaviors such as anxiety (Heijtz et al., 2011; Neufeld et al., 2011a, 2011b). Interestingly, GF mice show reduced basal anxiety whereas GF rats are hyperanxious, suggesting that growing up GF has a species-specific directional effect

on anxiety levels (Crumeyrolle-Arias et al., 2014). Social behavior impairments have also been reported in GF mice (Desbonnet et al., 2014; Arentsen et al., 2015). Interestingly, the effects of GF status on sociability seem to be more important in males than in females. In addition to the observed deficits in sociability, male GF mice engage more in stereotyped and repetitive self-grooming behaviors compared with controls (Desbonnet et al., 2014). These alterations in sociability, locomotor activity, and repetitive, stereotyped behaviors in GF mice are comparable with the behavioral phenotype observed in rodent models of autism (Silverman et al., 2010). Intriguingly, patients with ASD have an altered composition of the gut microbiota and commonly suffer from GI complications such as constipation and increased intestinal permeability (Kohane et al., 2012; Mulle et al., 2013; Mayer et al., 2014a; Rosenfeld, 2015). GF animals also show cognitive disabilities including learning and memory deficits (see Luczynski et al., 2016a for review).

Although numerous findings in GF mice clearly demonstrate that the gut microbiota influences stress responsivity, sociability, and cognition, the molecular mechanisms underlying these effects remain largely unknown. Nevertheless, there is growing evidence that the GF status produces a variety of neurobiological alterations including both functional and structural alterations in the CNS. Some studies reported that the absence of microbiota affects serotonergic neurotransmission in the hippocampus specifically in males. Alterations in other neurotransmitter systems, including the noradrenergic, serotonergic, dopaminergic and glutamatergic systems, have been documented (Heijtz et al., 2011; Neufeld et al., 2011b; Clarke et al., 2013; Matsumoto et al., 2013). The study by Matsumoto and colleagues showed that the cerebral metabolome is different in GF *versus* ex-GF (conventionalized) mice (Matsumoto et al., 2013). On the other hand, brain derived neurotrophic factor (Bdnf) expression is altered in GF mice, with either increased or decreased expression depending on the studies and the brain areas (Sudo et al., 2004; Gareau et al., 2011; Heijtz et al., 2011; Neufeld et al., 2011b; Clarke et al., 2013). Altered levels of BDNF in the CNS are well known to be associated with affective disorders such as depression in humans (Autry and Monteggia, 2012). BDNF promotes the growth and development of new neurons and the survival of existing neurons (Park and Poo, 2012). Consistently, it was recently shown that GF mice exhibit alterations of neuronal morphology in the hippocampus, amygdala (Luczynski et al., 2016b) and medial PFC (Luczynski et al., 2017). In addition, a study revealed hypermyelination of prefrontal neurons in GF mice (Hoban et al., 2016a). Adult neurogenesis is a key process involved in memory processes, stress responsivity, and antidepressant treatment efficacy (Snyder et al., 2011; Marín-Burgin and Schinder, 2012;

Miller and Hen, 2015). Increased hippocampal neurogenesis is typically associated with improved memory, whereas its decrease is associated with memory impairments and depressive-like behavior (Deng et al., 2010; Eisch and Petrik, 2012). Surprisingly, the survival of newly-born neurons in the subgranuar zone of the dorsal hippocampus is increased in GF mice (Ogbonnaya et al., 2015). In contrast, the morphology of dorsal dentate granule cells of GF mice is less complex compared with SPF (Luczynski et al., 2016b).

Another important finding with respect to the gut-brain axis is that the BBB is underdeveloped in GF mice. Indeed, increased BBB permeability as well as tight junctions abnormalities have been reported in juvenile and adult GF animals (Braniste et al., 2014). These changes in BBB function likely underlie some of the reported alterations of brain function and behavior in GF animals. Finally, although the gut microbiota has long been known to impact the peripheral immune system, little was known until recently regarding its modulation of immune development and function in the CNS. GF mice have greater numbers of microglia throughout the entire brain compared with controls (Erny et al., 2015; Castillo-Ruiz et al., 2017). In addition, these microglia show abnormal morphology and an immature-like transcriptome (Matcovitch-Natan et al., 2016). Microglia are the resident macrophages of the CNS and act as the first line of immune protection for the brain and spinal cord (Prinz and Priller, 2014). Microglia are also involved in shaping neural circuits in the developing brain (Schafer and Stevens, 2013). Thus, alterations in microglia number and activity may also contribute to the reported physiological and behavioral alterations in GF animals.

Interestingly, many of the above GF phenotypes are normalized by colonization, although the effects largely depend upon the age of colonization and the animal species and strain (Clarke et al., 2013; Braniste et al., 2014; Desbonnet et al., 2014; Luczynski et al., 2017). As seen above, GF animals show altered brain development, suggesting that microbial colonization during a critical window in early-life could lastingly impact brain function and behavior. Supporting this, Sudo et al. reported the first evidence that colonization during early development, but not at a later age, could attenuate the increased HPA axis response to stress in GF mice (Sudo et al., 2004). In line with this study, Heijtz et al. further showed that some behavioral alterations (i.e. locomotor activity) in GF mice could be reversed by colonization early in life, whereas colonization at adulthood had no effect (Heijtz et al., 2011).

2.2. Antibiotics

GF models have, however, several limitations such as the absence of bacteria in the whole body and the difficulty to conduct behavioral tests in incubators or to reproduce housing

conditions in control groups (Luczynski et al., 2016a). The use of antibiotics allows to transiently reduce the abundance of certain gut bacterial communities at different developmental time points to examine the impact of gut microbiota on brain development and behavior.

Numerous studies have shown deleterious effects of antibiotics on brain and behavior. For instance, treatment with a mix of antibiotics increased depressive-like behaviors (Hoban et al., 2016b; Guida et al., 2017). This effect was associated with altered BDNF signaling and neuronal firing in the hippocampus and increased number of activated astrocytes and microglia in the hippocampus and cortex (Guida et al., 2017). Moreover, antibiotic depletion of gut microbiota during adolescence lastingly altered the expression of key neuromodulators in the hippocampus and hypothalamus and reduced anxiety, but induced cognitive deficits (Desbonnet et al., 2015). In line with the latter observation, several studies showed that different antibiotic treatments impaired spatial (Fröhlich et al., 2016; Hoban et al., 2016b) and non-spatial memory (Pyndt Jørgensen et al., 2015; Möhle et al., 2016), likely *via* decreased hippocampal neurogenesis proliferation and survival (Möhle et al., 2016). Furthermore, penicillin exposure early in life induces long-term hyperanxiety in males and females, social deficits in males and increased IL-6 and IL-10 expression in the frontal cortex together with altered tight junction protein expression in the hippocampus of both sexes (Leclercq et al., 2017). This suggests that the early perinatal period constitutes a critical window of sensitivity for the effects of dysbiosis on emotional behaviors. However, vancomycin treatment in early-life did not alter anxiety-like behavior in another study using male rats (O'Mahony et al., 2014). Overall, these studies indicate that antibiotics can bidirectionally modify behavior and emotionality and that the effects differ, notably, according to the antibiotic used.

On the other hand, beneficial effects of antibiotics have been found in different models producing behavioral alterations. In a post-infection model, ampicillin treatment attenuated the increased anxiety and showed antidepressant-like effects in control animals (Lotan et al., 2014). Likewise, a beneficial effect of antibiotics on social behavior was found in several studies. Indeed, social avoidance in non-obese diabetic mice was not observed in mice with antibiotic depleted microbiota (Gacias et al., 2016). Interestingly, 8 week-vancomycin treatment in autistic children transiently improved both GI symptoms and ASD symptoms, (Sandler et al., 2000). Similarly, in an animal model of autism (maternal immune activation), vancomycin pre-treatment in dams prevented the deleterious effects of prenatal infection in adult male offspring, including structural cortical abnormalities, social deficits and hyperanxiety (Kim et al., 2017). In another autism paradigm (prenatal valproate exposure),

oral treatment with minocycline was found to ameliorate autistic behaviors, including decreased sociability and hyperanxiety, and blood brain barrier leakiness in rats (Kumar and Sharma, 2016). Besides, other studies reported preventive antipsychotic-like effects of minocycline in different neurodevelopmental and adult animal models of schizophrenia (maternal immune activation and peripubertal stress, Mattei et al., 2014; Giovanoli et al., 2016; hippocampal inflammation, Zhu et al., 2014a, 2014b). Minocycline also prevented spatial memory deficits associated with neuroinflammation in stressed (McKim et al., 2016) or aged (Kohman et al., 2013) animals. However, in all these minocycline studies, although it was orally administered, the antibiotic was used for its anti-inflammatory or neuroprotective properties, without mention of the gut microbiota. Indeed, it is not clear whether these properties involve effects on the gut microbiota, as intraperitoneal or even central administration of minocycline also affect brain and behavior. This consideration also applies to other antibiotics including vancomycin. Therefore, conclusions on the role of gut microbiota in emotional behavior can only be drawned if appropriate controls are carried out, or when fecal transplantation or probiotic treatment following microbiota depletion reverse its effects. For instance, Bercik and coworkers showed that oral administration, but not i.p. injection, of antibiotics, increased exploratory behavior and hippocampal BDNF expression in mice (Bercik et al., 2011b). Moreover, the authors reported that orally delivered antibiotics had no effects on these parameters in GF mice, further demonstrating the involvement of gut microbiota in the effects of antibiotics.

2.3. Fecal transplantation

The important role of gut microbiota in the regulation of behavior was further confirmed by demonstrating the successful adoptive transfer of host behavioral phenotypes between mice of different strains and with different behavioral profiles (see Collins et al., 2013 for review). In addition to providing much information as regards the intrinsic role of the gut microbiota in the brain-gut axis, GF animals are also a useful tool for these fecal transplantation studies. Another possibility is to treat conventional animals with broad spectrum antibiotics before transplantation (Lundberg et al., 2016). In animals, fecal transplantation can be achieved by oral gavage of fresh fecal 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 fecal transplantation in animals showed a critical role of gut microbiota in host metabolism and energy balance (Turnbaugh et al., 2008; Wang et al., 2016). Since then, accumulating data have demonstrated that fecal

transplantation can affect brain and behavior in rodents. For instance, the cognitive impairment associated with diet-induced obesity (high-fat diet, HFD) was transferred to recipient mice fed a control diet (Bruce-Keller et al., 2015). Another study showed that social deficits in offspring from HFD-fed dams could be reversed by co-housing with offspring from dams fed a regular diet (Buffington et al., 2016), an effect accompanied by restored synaptic plasticity in the brain following social interaction. Similarly, social deficits in non obese diabetic mice could be transferred to control microbiota-depleted C57/Bl6 mice via fecal transplantation (Gacias et al., 2016). This study also reported changes in gene expression in the PFC of the recipient mice. Moreover, several studies indicate that anxiety and depressive-like behaviors can be modulated by fecal transplantation. A study using high and low anxiety mouse strains (NIH Swiss and Balb/C, respectively) demonstrated that microbiota transfer between strains also transferred their anxiety levels (Bercik et al., 2011b). Recent elegant studies have used gut microbiota from human patients and healthy volunteers for fecal transplantation in animals. Zheng and colleagues showed that fecal transplantation with the microbiota of depressed subjects induced significant depressive-like behaviors in GF mice, as compared with colonization with the microbiota of healthy control individuals (Zheng et al., 2016). Similar findings were also reported in the rat using antibiotic-driven microbiota depletion before transplantation (Kelly et al., 2016). Together these studies strongly suggest that the gut microbiota play a causal role in the development of features of depression and may represent a novel target in the treatment and prevention of this disorder.

On the other hand, colonization with the microbiota of IBS patients with diarrhea *versus* healthy controls recapitulated several features of IBS in GF mice, including faster GI transit, intestinal barrier dysfunction, innate immune activation, but also anxiety-like behavior (De Palma et al., 2017). Intriguingly, antibiotic-induced decrease in hippocampal neuronal progenitor survival as well as deficient non-spatial memory was not improved by fecal transfer with normal SPF microbiota, but fully restored by oral administration of a probiotic mixture containing eight bacterial strains of the *Bifidobacterium* and *Lactobacillus* genera (Möhle et al., 2016).

Overall, the potential clinical value of fecal transplantation for the treatment of disorders of the gut-brain axis is promising (Borody and Khoruts, 2011; Brandt and Aroniadis, 2013) and currently represents an active area of research. To date, the only indication for fecal transplantation in humans is the treatment of severe infections with *Clostridium difficile*, resulting in high success rates (Khoruts, 2014). Interestingly, a recent clinical trial evaluated the beneficial impact of fecal transplantation in a small cohort of 18 ASD-diagnosed children

(Kang et al., 2017). This study reports attenuated GI symptoms (e.g. constipation, diarrhea, abdominal pain) as well as behavioral ASD symptoms, both persisting up to 8 weeks after treatment, and associated with stable increases in the abundance of several bacterial taxa, including *Bifidobacterium, Prevotella,* and *Desulfovibrio*.

2.4. 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 (Eberl, 2010). The use of probiotics in animal studies has provided further evidence that the gut microbiota possess some level of antidepressant or anxiolytic activity, leading to the concept of psychobiotics (see Dinan et al., 2013 and Sarkar et al., 2016 for reviews). Furthermore, probiotics interventions are generally restricted to one or few bacterial species, thereby allowing the association between a given bug and a particular behavioral effect. The most used are members of the *Bifidobacteria* and *Lactobacillus* genera. In the last decade, beneficial effects of these probiotics on behavioral outcomes have been demonstrated in several rodent models, especially in a context of stress.

Pretreatment with a probiotic formulation (*Lactobacillus helveticus* and *Bifidobacterium longum*) has been shown to prevent the increase in plasma corticosterone and neuronal activation in the PVN of adult male C57Bl6 mice submitted to acute stress (water avoidance) (Ait-Belgnaoui et al., 2014). In addition, gut permeability and tight junction proteins levels were also restored by the probiotic. Similar results were reported with the probiotic *Lactobacillus casei Shirota* (Takada et al., 2016). Moreover, the authors demonstrated a dose-dependent modulation of vagal afferent activity by intragastric administration of *L casei*, providing potential insight into the underlying mechanisms. Another study conducted in female rats reported beneficial effects of *Lactobacillus* probiotics (*L farciminis*) on gut barrier function and HPA axis activity after acute stress (Ait-Belgnaoui et al., 2012). Interestingly, prevention of gut barrier leakiness using a pharmacological agent (MLCK inhibitor, ML-7) led to similar effects on HPA axis markers, suggesting that the effects of the probiotic on the latter are mediated by the promotion of gut barrier function (Ait-Belgnaoui et al., 2012). However, no behavioral outcome was tested in this study. In a later study, Emge and colleagues showed that a mixture of *Lactobacillus rhamnosus* and *Lactobacillus helveticus* could prevent the increased anxiety in a model of intestinal inflammation induced by DSS (Emge et al., 2016). Using the DSS model, it has been reported that intestinal inflammation leads to immune activation in the hippocampus and reduces cell proliferation in this area (Zonis et al., 2015). Taken together, these works suggest that probiotics can modulate

anxiety-like behavior *via* the buffering of HPA axis reactivity and possibly of neuroinflammatory processes. *Lactobacillus helveticus* was also shown to protect against the deleterious effects of chronic stress in adult rats (Liang et al., 2015). Indeed, chronic restraint stress led to decreased sucrose preference, but this effect on depressive-like behavior was comparably ameliorated by the probiotic or the antidepressant citalopram. Moreover, whereas the antidepressant did not affect stress-induced anxiety or increased corticosterone levels, *L. helveticus* also normalized these features in stressed rats. The probiotic group also showed increases in the anti-inflammatory cytokine IL-10, and in hippocampal Bdnf mRNA as well as norepinephrine and serotonin levels. Likewise, *Lactobacillus casei* has been shown to prevent antibiotic-induced depressive-like behaviors, altered hippocampal Bdnf and increased glial activation in the hippocampus and cortex (Guida et al., 2017). One study examined the effects of *Lactobacillus rhamnosus* in adult male BALB/c mice, a mouse strain showing innately high anxiety levels compared with other strains (Bravo et al., 2011). Both anxiety and depressive-like behaviors (in the elevated plus maze and forced swim tests, respectively) were ameliorated by the probiotic. These changes were accompanied by a blunted corticosterone response to stress and changes in mRNA expression of the GABA (gamma amino butyric acid) receptor GABAB1b in the PFC, hippocampus and amygdala. Furthermore, the behavioral and molecular changes were lost in vagotomized mice, suggesting that the modulation of synaptic transmission and HPA axis could mediate the effects of probiotics on anxiety and depressive-like behaviors *via* the vagus nerve. Another study showed that probiotic strains of the *Bifidobacteria* family *(B longum* and *B breve*) can also reduce anxiety and depressive-like behaviors in male BALB/c mice (Savignac et al., 2014). Moreover, *B longum* has been reported to normalize the increased anxiety in mouse models of GI inflammation induced by infection with the parasite *Trichuris muris* (Bercik et al., 2010) or by oral exposure to DSS (Bercik et al., 2011a). Intringuingly in the parasite study, the authors also tested the probiotic *L rhamnosus,* but the treatment with this bacterium was not able to prevent the elevated anxiety levels. This finding contrasts with the study by Bravo et al. in BalB/C mice and suggests that the effects of probiotics on anxiety depend on the mechanisms underlying the alterations of this behavior (Bravo et al., 2011). Likewise, treatment with the probiotic *Mycobacterium vaccae* in male BALB/c mice enhances learning performances in the spatial Hebb-Williams maze (Matthews and Jenks, 2013). Interestingly, the improved performance in probiotic treated mice was associated with a reduction of anxiety-related behaviors such as immobilization and grooming during the test.

Beneficial effects of probiotics have also been reported in paradigms involving early-life adverse events. For instance, the probiotic *Bifidobacterium infantis* administered at adulthood was reported to exert antidepressant-like effects in animals exposed to early-life stress (maternal separation) (Desbonnet et al., 2010). Indeed, stressed animals spent more time immobile in the forced swimming test compared with controls, but this effect was corrected by both antidepressant (citalopram) and the probiotic treatment. In addition, the increased peripheral levels of the proinflammatory cytokine IL-6 as well as the increased Crf mRNA levels in the amygdala in stressed animals were also normalized by both interventions. However, in a previous study, the authors found no behavioral change in adult naive rats treated with the same probiotic, although the anti-inflammatory effect on IL-6 was present (Desbonnet et al., 2008). Besides, in an elegant study, Hsiao and colleagues have demonstrated that oral gavage of *Bacteroides fragilis* is sufficient to correct gut permeability defects, impaired sociability and increased anxiety in a mouse model of prenatal infection (Hsiao et al., 2013). Interestingly, in this study, *B fragilis,* which is not known as a classic probiotic, was identified as one of the most altered bacterium by comparing the gut microbiota profiles in the prenatal infection and control groups. In addition to the beneficial effects on gut barrier and behavior, *B fragilis* treatment normalized the increase in 4-ethylphenylsulfate in the serum of prenatally infected mice. The authors further demonstrated that administration of this metabolite in naive mice was sufficient to induce hyperanxiety, but not social deficits. This study suggests that novel probiotic-like bacterial species can be identified using animal models. However, to date, the beneficial effects of this bacterium on behavior have not been tested in another study, and the presence of this bacterium in the serum is thought to cause systemic inflammation (Lukiw, 2016).

In addition to the beneficial effects of probiotics in adult animals, there is non-negligible literature showing 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 early-stressed (maternally separated) juvenile rats (post-natal day (PND) 20), in addition to mitigating the associated increased gut permeability (Gareau et al., 2007a). Similar findings have been reported in a mouse model of maternal separation where mice received the probiotic *Bifidobacterium pseudocatenulatum* during the perinatal period (Moya-Pérez et al., 2017). 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. Using another early adversity (i.e. perinatal

antibiotic exposure), Leclercq et al. reported that *Lactobacillus rhamnosus* supplementation in lactating dams prevented some of the deleterious effects of antibiotics on anxiety and social behavior, cytokine expression in the frontal cortex and BBB integrity in a sex-dependent manner (Leclercq et al., 2017).

Although the initial studies have been conducted in animal models, there is now a growing number of human trials providing evidence for psychotropic-like effects of probiotics on mood and anxiety. However, most of the trials were performed in healthy subjects (Benton et al., 2007; Messaoudi et al., 2011; Tillisch et al., 2013; Steenbergen et al., 2015). Interestingly, it has been recently demonstrated in double-blinded, placebo-controlled studies that *Lactobacillus casei Shirota* reduces stress-induced increase in salivary cortisol in healthy students (Takada et al., 2016) and decreases anxiety symptoms in subjects suffering from chronic fatigue syndrome (Rao et al., 2009). Probiotic treatment during two months significantly decreased anxiety symptoms compared with the placebo. A recent study reported that pregnant women supplemented with *Lactobacillus rhamnosus* until 6 months postpartum had significantly lower depression and anxiety scores in the postpartum period (Slykerman et al., 2017). Furthermore, a study reported that treatment with the probiotic *B longum* reduced depression but not anxiety scores in IBS patients compared with the placebo group. Using functional brain imaging, the authors also reported decreased brain activity in responses to negative emotional stimuli in probiotic-treated versus placebo-treated IBS subjects (Pinto-Sanchez et al., 2017). 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 (Vlainić et al., 2016). To date, only sparse evidence for antidepressant effects of probiotics has been reported in major depressive disorder (Akkasheh et al., 2016; Bambling et al., 2017).

2.5. Prebiotics

Prebiotics are nutrients that can be fermented by gut microbes in the gut and thus favor the growth of certain microbial communities (Gibson and Roberfroid, 1995). In comparison with probiotics, a much smaller number of studies have examined the effects of prebiotics on behavior (see Kao et al., 2016 for review). These include investigations of galacto-oligosaccharides (GOS) and fructo-oligosaccharides (FOS), which are a source of nutrition for *Bifidobacteria* and *Lactobacilli*. For instance, the effects of GOS have been tested in response to acute inflammation in adult male CD1 mice (Savignac et al., 2016). The prebiotic treatment did not affect the early hypolocomotion induced by LPS injection (sickness behavior), however, it did prevent the increased anxiety observed 24h after injection, as well

as the increased IL-1β levels in the frontal cortex. Another recent study in C57BL/6J male mice reported that the combination of both prebiotics had anxiolytic and antidepressant effects both in basal and chronic stress situations (Burokas et al., 2017). These effects were consistent across several behavioral tests and accompanied by attenuated HPA axis hyper-reactivity to stress as well as changes in mRNA expression of stress-related genes in the hypothalamus and hippocampus. GOS of FOS alone showed some levels of protective effects but to a much lower extent, suggesting that combining multiple prebiotics, including other oligosaccharides, may increase their beneficial potential. Human milk oligosaccharides (HMO) have been reported to impact brain development and cognitive functions (Wang and Brand-Miller, 2003; Wang et al., 2003). Mice supplemented with HMO in their diet (2 weeks) were protected against stress-induced hyperanxiety (Tarr et al., 2015). Apart from these effects on emotional behaviors, other studies have reported improved learning and memory performance in male rats and mice supplemented with different oligosaccharides including HMO (Vázquez et al., 2015; Jia et al., 2016; Oliveros et al., 2016; Yen et al., 2017).

2.6. Gut permeability

Strikingly, we have seen in this chapter that almost all the manipulations affecting the gut used to explore the role of the gut-brain axis in the regulation of behavior are focused on the gut microbiota. This is probably due to the fact that the initial findings in this research area come from GF animal studies. However, several studies highlight a potentially synergistic role of gut permeability in the effects of microbiota manipulations. Notably, gut microbes have been shown to influence gut barrier function and accordingly, gut dysbiosis is often concomitant with gut leakiness (Zakostelska et al., 2011; Hsiao et al., 2013; Jakobsson et al., 2015; Reunanen et al., 2015). For instance, Hsiao et al. have demonstrated that the long-term behavioral (autistic-like) alterations induced by prenatal inflammation were reversed by oral gavage of *Bacteroides fragilis*, an effect that was accompanied by a partial restoration of gut microbiota composition and a full restoration of gut barrier function (Hsiao et al., 2013). Likewise, in the study by Ait-Belgnaoui et al., acute stress led to increased corticosterone levels along with gut leakiness, and the beneficial effects of probiotics on corticosterone levels were accompanied by a restoration of gut barrier function (Ait-Belgnaoui et al., 2012). Furthermore, this study was the first to address the role of gut permeability *per se* in the stress-induced HPA axis alterations. Indeed, the authors demonstrated that treatment with ML-7, an inhibitor of the MLCK, which is responsible for tight junction permeability (Shen et al., 2006), was able to normalize HPA axis response. However, ML-7 inhibitory action is not specific to the gut MLCK and has been shown to also modulate BBB permeability (Kuhlmann

et al., 2007; Luh et al., 2010). Nonetheless, these results suggest that gut permeability could play a role in the regulation of behavior and stress responsivity. As previously mentioned, De Palma and colleagues have demonstrated that early-life stress (i.e. maternal separation) fails to induce hyperanxiety and depressive-like behaviors in GF mice compared with SPF controls (De Palma et al., 2015). Interestingly, colonization with the gut microbiota of a conventional SPF control mouse unmasked the effects of early-life stress in GF mice. However, colonization with the microbiota of an early-stressed animal did not transfer the stress-associated behavioral phenotype in naive GF mice, suggesting that gut bacteria are necessary but not sufficient to mediate the behavioral effects of early-life stress. Although the authors did not measure it, the increased permeability generally associated with early-life stress (Barreau et al., 2004a, 2004b; Gareau et al., 2006; Øines et al., 2012; Moussaoui et al., 2014, 2016a, 2016b) could be responsible for its deleterious effects upon colonization. Thus, it is conceivable that animals showing gut dysbiosis without concomitant gut leakiness display unspoiled behavior.

In conclusion, we have seen that stress affects both the brain and the gut and that gut microbiota plays a key role in the effects of stress on emotional behaviors. However, it is still not clear whether gut dysbiosis, leakiness or inflammation precede each-other and whether they are the cause or the consequence of psychiatric symptoms. In this respect, there is a need for other specific gut-directed interventions to better understand the effects of stress on the gut-brain axis. Tools for the specific manipulation of gut permeability, in particular, would provide new insight in the field, even though it is likely that they would affect the gut microbiota at some point. Moreover, despite widespread sex differences in both gut physiology and neuropsychiatric vulnerability, most of the studies ignore the issue of sex.

Finally, this chapter called attention to the early-life period as particularly sensitive for gut-brain communication alterations. As seen in chapter I, the gut mucosal barrier remains immature until mid-infancy [Figure 11]. Moreover, it is greatly influenced by gut microbiota colonization and diversification during the lactating period. The interaction between epithelial cells and the mucosal immune system during the same period is crucial for future health including mental health. Indeed, the brain also undergoes important maturation during early post-natal life (Gutman and Nemeroff, 2002; Andersen and Teicher, 2008) [Figure 11]. In the following chapter, we will discuss the impact of stress during this particular developmental window.

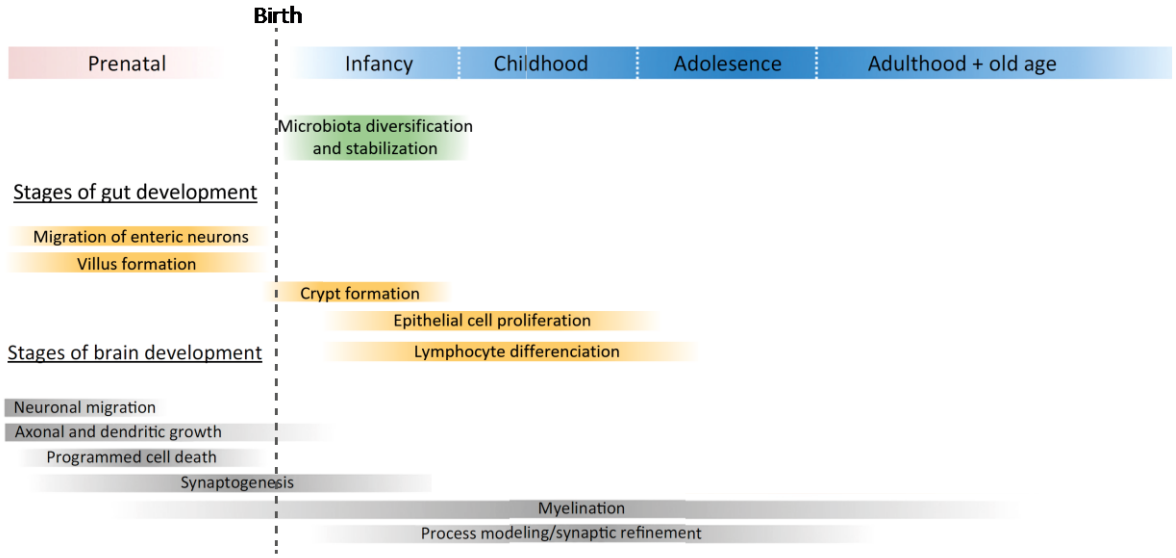

Figure 11 | Temporal sequences of gut and brain development. Adapted from Sasselli et al., 2012; Borre et al., 2014 and Jain and Walker, 2015.

CHAPTER III – Early-life stress, neuropsychiatric vulnerability and intestinal dysfunctions

3.1. Early-life adversity in humans

The "Developmental Origins of Health and Disease" (DOHaD) concept states that "environmental factors acting during the phase of developmental plasticity interact with genotypic variation to change the capacity of the organism to cope with its environment in later life" (Gluckman and Hanson, 2004). Although the main focus of the DOHaD originally was on nutritional factors (Barker, 1998), this concept was extended to a broader range of environmental factors such as stress during different phases of development (Heindel et al., 2015). This concept suggests that the early-life environment is determinant for the individual's future phenotype and health (Gluckman and Hanson, 2004; Boersma et al., 2014). Exposure to trauma or chronic stress, especially during critical periods such as infancy, may increase the allostatic load and can vulnerabilize to a plethora of health problems across lifespan, including neuropsychiatric disorders, bowel disease, metabolic and cardiac disorders (Danese and McEwen, 2012).

Childhood adversity is among the factors that affect the most brain function and mental health at adult age (see Bick and Nelson, 2016 for review). Indeed, a vast literature has shown that childhood adversity is a major risk factor for the development and persistence of neuropsychiatric disorders such as anxiety and depression at adulthood (Famularo et al., 1992; Kendler et al., 2004; Mullen et al., 1996; Stein et al., 1996; Young et al., 1997; Agid et al.,

1999; Heim and Nemeroff, 2001; Chapman et al., 2004; Grover et al., 2005; Phillips et al., 2005; Faravelli, 2012; Bruce et al., 2013; Reiser et al., 2014). An adverse childhood experience most often refers to parental maltreatment, neglect and sexual abuse. However, it has been shown that several other stressors such as war, pollution, illness or low socio-economic status may constitute risk factors for mental health, in particular anxiety and depression (Weich et al., 2009; McLaughlin et al., 2010), but also ASD, schizophrenia and addiction. It has been established that there is a dose-dependent relationship between the severity of childhood adversity and the occurrence of depressive episodes in adulthood (Chapman et al., 2004). Moreover, the occurrence or aggravation of these neuropsychiatric disorders is often associated with additional adversities at adulthood (Hammen et al., 1992, 2000; Norman and Malla, 1994), suggesting that childhood life adversity could increase long-term vulnerability to stress. Nevertheless, the effects of early-life adversity on neuropsychiatric vulnerability seem to differ according to gender (Carpenter et al., 2017).

Childhood adversity is associated with structural alterations in brain structures involved in the regulation of emotions and stress response. In particular, the PFC, amygdala and hippocampus have been consistently reported to be impacted by early adversity. More precisely, individuals having suffered from childhood adverse events generally exhibit reduced PFC (De Bellis et al., 2002; Frodl et al., 2010a, 2010b; van Harmelen et al., 2010) and hippocampus (Vythilingam et al., 2002; Woon and Hedges, 2008; Frodl et al., 2010a, 2010b; Rao et al., 2010) volume and an increased volume of the amygdala, despite some inconsistent findings (i.e. decreased volume) in the latter area (Mehta et al., 2009; Lupien et al., 2011). Functional brain imaging studies have also revealed that, relative to control subjects, previously maltreated individuals exhibit decreased activation in the basal ganglia (ventral striatum, globus pallidus) following money reward (Dillon et al., 2009; Mehta et al., 2010).

In addition to its long-term effects on the brain and neuropsychiatric vulnerability, numerous studies have shown that childhood adversity is a risk factor for GI disorders, especially IBS (Hislop, 1979; Klooker et al., 2009; Halland et al., 2014; see Chitkara et al., 2008 for review). Notably, emotional abuse seems to be the most potent predictor for IBS (Bradford et al., 2012). How does adversity “get under the skin” to influence the physiology of the developing child is still unclear. Alterations in neuroendocrine stress systems, metabolism and inflammatory processes, associated with allostasis overload, probably play a major role in the health problems associated with early-life stress (Barboza Solís et al., 2015; Nusslock and Miller, 2016).

Finally, a large literature also suggests that stress occurring before birth and even before conception has similar detrimental effects on brain and neuropsychiatric vulnerability (Glover, 2011; Entringer et al., 2015; Gröger et al., 2016), but no robust link between prenatal stress and functional GI disorders has been established. Although several evidences for long-term effects of early adversity on brain vulnerability exist in the human literature, most of the relationships have been demonstrated in animal models.

3.2. Animal models of early-life adversity: focus on rodent maternal separation

Pioneering work from Levine, Denenberg, Meaney and Plotsky in rodents has shown that the early environment shapes emotional behavior as well as stress responsivity in adult life (Levine, 1957; Denenberg et al., 1962; Meaney et al., 1991a; Plotsky and Meaney, 1993). Since then, a vast body of literature has documented these effects (Meaney, 2001; Chapillon et al., 2002; Weaver et al., 2002; Champagne et al., 2003; Cirulli et al., 2003; see Maccari et al., 2014 for review). In particular, there is compelling evidence that maternal care (including arched-back nursing and anogenital licking and grooming of the pups) is crucial for brain maturation and long-term stress sensitivity in the offspring (Maccari et al., 1995; Barbazanges et al., 1996; Champagne et al., 2003; Darnaudéry et al., 2004). Indeed, when compared with offspring from dams showing high levels of active maternal care, offspring from dams with low levels of maternal care display hyperanxiety accompanied by exacerbated HPA axis response to stress and decreased expression of GR in the hippocampus (see Hackman et al., 2010 for review). Moreover, cross fostering experiments showed that adoption of pups from high maternal care biological mothers by low maternal care dams induced similar alterations in offspring. Consistent with these demonstrations of the pivotal role of maternal care on brain vulnerability, disruption of the mother-infant relationship in rodents, best known as maternal separation (MS) or deprivation, induces a wide range of neurobehavioral and endocrine alterations both in juvenile and adult offspring. In the following, we will review the adverse consequences of MS, which is the most used model of early adversity in the gut-brain axis field. This model produces emotional and endocrine, but also GI alterations with good face validity.

3.2.1. Maternal separation and emotional vulnerability

MS models are based on dam-pups separations in rats, during the first weeks of life (1-3 weeks). The most common MS paradigm consists in daily 3h separations between postnatal days 2 and 14 (Lippmann et al., 2007). However, there are other models using different separation durations (3-8 h per day) or an acute 24h separation (Barna et al., 2003; Schmidt et

al., 2004; Roman et al., 2006; Viveros et al., 2009). MS results in different degrees of perceived stress in dams and pups according to the protocol used. Dams can be placed in a novel cage while their litter remains together in the homecage, or the whole litter can also be transferred into a novel environment. In addition, pups can be individually separated in a novel environment, resulting in an additional isolation stress. Finally, a key factor is the choice of the control group. The most widely used is the condition where dams and their pups remain undisturbed throughout the lactation period. In some studies, however, dams are daily separated from the litter for a short period (i.e 15 min) (Vallée et al., 1996, 1997, 1999; Pryce et al., 2003; Wilber et al., 2009). This condition, referred to as 'early handling', was designed to mimic the short separations observed in the wild when the dams leave the nest to find some food. Moreover, this daily separation allows similar handling between MS and control groups, with the only difference being separation duration. However, it has been shown that this procedure *per se* is beneficial and leads, for instance, to improved stress sensitivity and anxiety in the offspring, likely through an enhancement of active maternal care following separation (Meaney et al., 1991a, 1991b, 1991c; Vallée et al., 1996, 1997, 1999). The different models and their respective effects are reviewed in Pryce and Feldon, 2003; Korosi and Baram, 2010 and Vetulani, 2013. In any case, pups are deprived of maternal care during the separation. 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 24h MS (Suchecki et al., 1993; van Oers et al., 1998). Initially, MS was developed in rats, but mother-infant separation-based models have also been developed in other rodents (e.g. guinea pigs and mice) and in primates (rhesus macaques) (see Cirulli et al., 2009 for review). The largest literature still involves rats, with mice being more and more used; we will focus on these species in this introduction. It appears that mice are less sensitive to this early-life stress than rats (see Millstein and Holmes, 2007 for review). This might be attributable to species specificities in neurodevelopment and maternal care behavior patterns. Indeed, brain developmental trajectories in mice and rats differ significantly. Mice exhibit more rapid neurodevelopment and consequently earlier maturation of the HPA axis (Schmidt et al., 2003). Another possible reason is that mouse studies more often involve genetically inbred as well as transgenic strains (Millstein and Holmes, 2007). C57/Bl6 is the most widely used mouse strain and this strain shows particularly low levels of active maternal care as well as stress hyposensitivity compared with other strains and with rats, which could also explain why dam-pups separation has fewer impact in mouse studies.

Furthermore, as reviewed by Tractenberg et al., MS studies in mice show important inconsistencies in their separation procedures (Tractenberg et al., 2016). Accordingly, Mansuy developed a new mouse model combining MS and unpredictable chronic mild stress in dams during separation sessions (Weiss et al., 2011). With this model, they demonstrate that the effects of early adversity are transmitted to the next generation.

In the following, we will describe the effects of MS on behavior, endocrine stress response and neurobiological correlates.

3.2.1.1. Long-term psychoneuroendocrine alterations

Behavior

The long-term consequences of MS on emotional behavior have been extensively documented. Typically, MS leads to increased anxiety and depressive-like behaviors. Indeed, adult 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 **[Figure 9A-C]** compared with non separated controls (Caldji et al., 2000; Huot et al., 2001; Kalinichev et al., 2002; Daniels et al., 2004; Lee et al., 2007, 2014; Lambás-Señas et al., 2009; Troakes and Ingram, 2009; Maniam and Morris, 2010a; De Palma et al., 2015; Shu et al., 2015; Gracia-Rubio et al., 2016; Koe et al., 2016; Rincel et al., 2016, see **ANNEXE 1**; Shin et al., 2016; Moya-Pérez et al., 2017). Moreover, it has been shown that exposure to a novel stress at adulthood aggravates these anxiety-like behaviors in maternally separated rats (Marais et al., 2008; Eiland and McEwen, 2012). Numerous studies also report increased depressive-like behaviors in the forced swimming test or tail suspension test **[Figure 9G,H]**. Indeed, adult maternally separated rodents show greater immobility time in these tests compared with controls (MacQueen et al., 2003; Lee et al., 2007; Lambás-Señas et al., 2009; Desbonnet et al., 2010; Maniam and Morris, 2010a; Sung et al., 2010; Uchida et al., 2010; Hui et al., 2011; Réus et al., 2011, 2017; Bai et al., 2012; Amiri et al., 2016; Gracia-Rubio et al., 2016; Paternain et al., 2016; Sadeghi et al., 2016; Amini-Khoei et al., 2017). Anhedonic behaviors with respect to different rewards (sucrose, social target… **[Figure 9I-K]**) are also sensitive to antidepressants and can be used as more ethological assessment of depressive-like behaviors. MS has been associated with decreased sucrose preference in numerous studies (Maniam and Morris, 2010b; Hui et al., 2011; Bai et al., 2012; Øines et al., 2012; Kundakovic et al., 2013; Shu et al., 2015; Amiri et al., 2016; Sadeghi et al., 2016; Yang et al., 2016a; Amini-Khoei et al., 2017). In addition, social behavior was repeatedly found to be altered by MS. Specifically, social interaction with a conspecific has been shown to be decreased (Zimmerberg and Sageser, 2011; Kundakovic et al., 2013; Tsuda et al., 2014; Farrell et al., 2016; Rincel et al.,

2016, see **ANNEXE 1**). Moreover, male aggressivity towards a counterpart is either increased (Shin et al., 2016) or decreased (Tsuda et al., 2011;2014), indicative of social behavior impairment.

However, inconsistencies can be found in the MS literature, with a number of studies reporting no alterations of certain emotional behaviors (Shalev and Kafkafi, 2002; Zimmerberg and Kajunski, 2004; Rüedi-Bettschen et al., 2005; Marais et al., 2008; Farkas et al., 2009; Uchida et al., 2010; Hulshof et al., 2011; Mourlon et al., 2011; Eiland and McEwen, 2012; Klug and van den Buuse, 2012; Øines et al., 2012; Park et al., 2012; Zhang et al., 2012; Ferreira et al., 2013; Hill et al., 2014; Rincel et al., 2016, see **ANNEXE 1**; Sadeghi et al., 2016) and a some others reporting opposite effects (e.g. lower anxiety or increased sucrose preference) (Eklund and Arborelius, 2006; Slotten et al., 2006; Michaels and Holtzman, 2007; Maniam and Morris, 2010b; León Rodríguez and Dueñas, 2013; Tsuda et al., 2014; Chocyk et al., 2015; Aya-Ramos et al., 2017). These discrepancies could 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). The majority of these findings were obtained using males only.

The effects of MS are not limited to the above alterations of emotional behaviors. For instance, MS leads to exacerbated motivation for alcohol and drugs of abuse (Kosten et al., 2000, 2006a; Huot et al., 2001; Ploj et al., 2003a; Jaworski et al., 2005; Lynch et al., 2005; Roman and Nylander, 2005; Roman et al., 2005; Vazquez et al., 2005, 2006; Naudon et al., 2013; Gondré-Lewis et al., 2016; see Moffett et al., 2007 for review).

Finally, several studies have also shown deleterious effects of MS on cognition (see Kosten et al., 2012 for review). Briefly, these effects include impaired hippocampal-dependent spatial learning and memory (Son et al., 2006; Hui et al., 2011; Couto et al., 2012; Rincel et al., 2016, see **ANNEXE 1**; Wang et al., 2011, 2014a), altered non-spatial memory (Aisa et al., 2007; Benetti et al., 2009; Hulshof et al., 2011; Pinheiro et al., 2014; Wang et al., 2011, 2014a) and impairments in PFC-dependent tasks (working memory, extinction, cognitive flexibility) (Wang et al., 2011; Baudin et al., 2012; Lejeune et al., 2013; do Prado et al., 2015; Thomas et al., 2016; Yang et al., 2016b). In contrast, amygdala-dependent aversive memory (e.g. fear conditioning) can be enhanced by MS (Wilber et al., 2009; Diehl et al., 2014; Toda et al., 2014). As for emotional behavior, some studies did not replicate these findings

(Lehmann et al., 1999; Pryce et al., 2003; Kosten et al., 2006b; Stevenson et al., 2009; Wang et al., 2011; Hill et al., 2014; Zhu et al., 2017).

Endocrine response and neurobiological correlates

MS also exerts long-lasting effects on HPA axis function, leading in most of the studies to endocrine hyper-responsivity to a novel stress (Rosenfeld et al., 1992a; Plotsky and Meaney, 1993; Ladd et al., 1996, 2000; Patchev et al., 1997; Biagini et al., 1998; Lehmann et al., 2002; Slotten et al., 2006; Aisa et al., 2007; Cotella et al., 2013). However, a few studies did not replicate these findings (Daniels et al., 2004; Hulshof et al., 2011). These discrepancies likely cannot be explained by differences in MS protocol, age, sex, strain or type of stressor, as we and others reported contrasting results using Wistar male adult rats exposed to the same separation paradigm (i.e. 3h per day from PND2-14 with separation of the whole litter and undisturbed controls) and a similar stressor at adulthood (i.e. exposure to a novel environment) (Hulshof et al., 2011; Rincel et al., 2016, see **ANNEXE 1**). Suprisingly, Hulshof and coworkers reported no alteration of ACTH or corticosterone response to different other stressors including odor predator, footshock or restraint stress, suggesting that the nature and intensity of the stressor does not impact HPA axis reactivity. However, these animals were individually housed as adults, whereas they were housed in collective cages in our study (Rincel et al., 2016, see **ANNEXE 1**). Importantly, HPA axis activity varies with respect to the circadian rhythm (Nicolaides et al., 2014), thus, although this information is not provided in most cases, the time of blood sampling could possibly account for some of the observed discrepancies. Moreover, a recent study suggests that the effects of early adversity depend upon the gut microbiota profile of the animals (which differs across animal suppliers, i.e. Jackson laboratories and Taconic Biosciences) (Kim et al., 2017).

Within the CNS, this HPA axis hyper-reactivity is associated with an up-regulation of CRF expression in the PVN and amygdala but also with CRF concentration and increased CRF receptor density in the locus coeruleus and raphe nucleus (Plotsky and Meaney, 1993; Ladd et al., 1996, 2000; see Rivarola and Renard, 2014 for review), as well as altered oxytocin and vasopressin expression (either up- or down-regulated) in the PVN (see Veenema, 2012 for review). MS also decreases GR expression in the hippocampus and PFC (Rivarola and Suárez, 2009; Wilber et al., 2009), two main brain areas involved in HPA axis negative feedback. In addition, MS decreases the number of GABA receptors in noradrenergic neurons of the locus coeruleus, as well as the number of benzodiazepine receptors in the amygdala, the locus coeruleus and the PFC (Caldji et al., 2000). The GABA/benzodiazepines system plays a role in CRF synthesis inhibition in the central amygdala, allowing a buffering of the

noradrenergic response to stress. Interestingly, short separations (early handling) produce opposite effects on behavior and neuroendocrine response to stress relative to prolonged separations. It has been proposed that the latter observation could be due to changes in other neurotransmission systems in different brain areas. These alterations include impaired glutamatergic (Pickering et al., 2006; Katsouli et al., 2014), serotonergic (Daniels et al., 2004; O'Mahony et al., 2008; Kawakami et al., 2013; Bravo et al., 2014), dopaminergic (Matthews et al., 2001; Ploj et al., 2003a; Brake et al., 2004; Arborelius and Eklund, 2007; Kawakami et al., 2013; Li et al. 2013; Romano-López et al. 2016; Moya-Pérez et al., 2017) and opioidergic (Ploj et al., 2003a, 2003b) transmission. In the CNS, serotonin is involved in neuronal development (Daubert and Condron, 2010), emotionality and also pain modulation (Kim and Camilleri, 2000; Sommer, 2004). Among other effects, MS reduces the expression of the serotonin transporter in the raphe nucleus (Bravo et al., 2014). Interestingly, SSRI antidepressants are inhibitors of this transporter (selective serotonin reuptake inhibitors). For instance, administration of the SSRI paroxetine normalizes HPA axis function as well as emotional behavior in maternally separated adult rats, suggesting that serotonin plays a major role in the long-term consequences of MS (Huot et al., 2001). Moreover, adult MS offspring display neuroinflammatory marks such as increased TNFα, IL-1β and TLR4 expression in the hippocampus (Pinheiro et al., 2014; Sadeghi et al., 2016; Amini-Khoei et al., 2017) and PVN (Tang et al., 2017b).

As in humans, MS has been shown to induce both functional and structural changes in several brain regions including the PFC, hippocampus, amygdala and nucleus accumbens (Muhammad et al. 2012; Li et al. 2013; Danielewicz and Hess, 2014; Soztutar et al., 2016). 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 maternally separated rats (Bock et al., 2005; Gos et al., 2008; Gruss et al., 2008; Monroy et al., 2010; Muhammad and Kolb 2011; Baudin et al. 2012; Chocyk et al. 2013; Cao et al., 2014; Sousa et al., 2014; Farrell et al. 2016; Romano-López et al. 2016; Shin et al., 2016). In addition, it has been shown that MS leads to hypomyelination in the medial PFC (Yang et al., 2016b). By contrast, MS has been shown to induce dendritic hypertrophy in the amygdala (Koe et al., 2016). These effects are accompanied by changes in the expression of neurotrophins such as NGF (nerve growth factor) and BDNF, that are known to play critical roles in dendrite growth and spinogenesis (see Park and Poo, 2012 for review). In particular, numerous studies have reported decreased NGF or BDNF expression in the hippocampus (Lippmann et al., 2007; Marais et al., 2008; Aisa et al., 2009; de Lima et al.,

2011; Réus et al., 2011). In addition, MS leads to alterations of hippocampal neurogenesis (either decreased or increased) at adulthood (Mirescu et al., 2004; Hulshof et al., 2011; Hays et al., 2012; Suri et al., 2013). 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 Autry and Monteggia, 2012 for review).

3.2.1.2. 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 in several studies (see Korosi, 2009 for review). In the following, we will discuss the effects of MS during development and highlight some evidences of their potential involvement in the programming of long-term phenotypes.

Mother infant communication and maternal care

As previously mentioned, maternal care is thought to play an important role in brain maturation and later vulnerability to stress. It has been established that rodent pups vocalize in response to isolation (30-90 Hz ultrasounds) (Branchi et al., 2001; Hofer et al., 2002) and MS has been shown to increase the number of these vocalizations compared with undisturbed pups in several mouse strains (Feifel et al., 2017). Because these isolation calls elicit retrieval behavior in the mother, they are thought to serve mother-pup communication and stimulate maternal care towards their pups (D'Amato et al., 2005; Brunelli et al., 2015). Moreover, this behavior is potentiated when pups are isolated for a second time immediately after dam contact (or return to the nest) (Hofer et al., 2002). Differences in maternal care have been shown to contribute to the differences in offspring's stress reactivity at adulthood (Maccari et al., 1995; Liu, 1997; Caldji et al., 1998; Francis et al., 1999; Francis and Meaney, 1999; Champagne et al., 2003, 2008; Cameron et al., 2005). 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 behavioral effects of acute 24h-MS can be prevented by pup tactile stimulation (van Oers et al. 1998). Nevertheless, the role of maternal care in the long-term effects of MS remains controversial. Several studies have reported increased overall (over 24h) maternal care in dams submitted to chronic MS (Macrí et al., 2004; Marmendal et al., 2004), but also to early handling (Macrí et al., 2004). As previously mentioned, these two separation protocols produce opposite effects on HPA axis reacitvity and emotionality in adult offspring.

MS also constitutes a potent stressor for the dams. Indeed, it has been reported that this psychological stress induces anxiety and depressive-like behaviors in dams (Boccia et al., 2007; Maniam and Morris, 2010c; Aguggia et al., 2013). 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. As an example, Huot and coworkers have shown that the 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 (Huot et al., 2004).

Endocrine, immune and neurobiological effects of MS in developing pups

The HPA axis is almost silenced during a short window of early post-natal development (i.e. from PND4 to 14) (Sapolsky and Meaney, 1986; Vázquez, 1998). This stress hypo-responsive period is characterized by extremely low basal corticosterone levels in the plasma as well as a limited amplitude of ACTH and corticosterone response following stress exposure. Nevertheless, this blunted HPA axis response is not absolute, since a potent stressor such as MS is able to induce its activation (Anisman et al., 1998; Vázquez, 1998; Gutman and Nemeroff, 2002). It has been proposed that stress and immune activation result in a cross-sensitization of both systems that possibility creates a self-perpetuating cycle contributing to the emergence of the alterations in animals subjected to early stress. MS has been shown to decrease the plasma levels of the anti-inflammatory cytokine IL-10 in adolescent rats (Grassi-Oliveira et al., 2016). In addition, altered circulating pro-inflammatory IL-1β, IL-6 and TNFα were observed in maternally separated pups and adolescent animals (Wieck et al., 2013; do Prado et al., 2015; Pinheiro et al., 2014; Roque et al., 2014, 2016; Réus et al., 2017). Furthermore, several pieces of evidence show that MS induces neuroinflammation in the PFC and hippocampus (Park et al., 2014; Réus et al., 2017). For instance, two-week-old MS pups display increased activated microglia in the PFC and hippocampus (Gracia-Rubio et al., 2016) and decreased number of astrocytes in the hippocampus (Musholt et al., 2009), along with increased IL-1β and TNFα expression in the same brain area compared with controls (Roque et al., 2016). Furthermore, the authors report increased hippocampal expression of TNFα in control pups submitted to a single separation on PND15, suggesting that long-lasting alterations in the hippocampus could be underlied by the altered TNFα expression during development.

Both altered HPA axis activity and neuroinflamamtion during development have been shown to be deleterious for the immature brain. In particular, processes of synaptogenesis, dendritic expansion, neurogenesis and apoptosis are highly dynamic during this period. MS disrupts the normal course of brain development and produces structural alterations including delayed

synaptic maturity (Andersen and Teicher, 2004) and increased neuronal and glial death (Zhang et al., 2002; Kuma et al., 2004; Mirescu et al., 2004). Altered expression of neurotrophins such as BDNF and NGF in separated pups could contribute to these effects (Cirulli et al., 2000; Kuma et al., 2004; Roceri et al., 2004). 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-day-old pups (Ohta et al., 2014). A recent study demonstrates that transient juvenile – but not adult – knockdown of Otx2 (orthodenticle homeobox 2) in the ventral tegmental area mimics early-life stress by increasing stress susceptibility, whereas its overexpression reverses the effects of early-life stress (Peña et al., 2017). Moreover, we and others have reported decreased mRNA expression of the transcription repressor Rest4 (RE-1 silencing transcription factor 4) in the PFC of pups submitted to MS (Uchida et al., 2010; Rincel et al., 2016, see **ANNEXE 1**). It has been shown that Rest4 targets include genes encoding Crf, Bdnf and 5HTr1A (Otto et al., 2007). Remarkably, Uchida and colleagues have demonstrated that Rest4 overexpression in the PFC specifically during development is sufficient to produce HPA axis hyper-responsivity to stress in adulthood, suggesting that this brain area is particularly relevant for the long-term effects of MS (Uchida et al., 2010). We recently demonstrated that exposure to a high-fat diet (HFD) during the perinatal period can prevent the long-term MS-associated neurobehavioral alterations including hyperanxiety, decreased social behavior and HPA hyperresponse to stress, possibly *via* a protective effect on gene expression in the PFC (Rincel et al., 2016, see **ANNEXE 1**). Indeed, perinatal HFD prevented the MS-induced alterations of Rest4, Bdnf and 5HTr1A expression in this brain area.

Epigenetic changes in MS offspring

Epigenetic regulation of DNA transcription can be achieved by several mechanisms including DNA methylation, histone modifications and interaction with non-coding RNAs such as microRNA (miRNA) (Goldberg et al., 2007). Epigenetic marks are dynamic and highly sensitive to environmental factors; furthermore they can last in time and even be transferred across generations (Bohacek and Mansuy, 2013). As such, they represent a potential mechanism that could underlie the long-term effects of early-life stress (Heim and Binder, 2012; Lutz and Turecki, 2014; Provençal and Binder, 2015; Silberman et al., 2016). Indeed, a number of studies have reported persistent epigenetic marks in the genome of animals submitted to MS (see Jawahar et al., 2015 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 (Meaney and Szyf, 2005; Murgatroyd et al., 2009; Roth et al., 2009; Roth and Sweatt, 2011; Wang et al., 2014a; Zhu et al., 2017). It has been shown that administration of a DNA methyl transferase (DNMT) inhibitor prevents the decreased prefrontal Bdnf mRNA expression induced by MS (Roth et al., 2009). Moreover, DNA methylation in the offspring has been shown to be associated with the level of maternal care (Weaver et al., 2004). Nonetheless, the group of Mansuy provided evidence for epigenetically-mediated transmission of behavioral traits induced by early-life stress across generations irrespective of crossfostering (Weiss et al., 2011).

Another major epigenetic process is histone modification, especially acetylation by histone acetyltransferases (HATs) or deacetylation by histone deacetylases (HDACs). Histone acetylation patterns as well as HAT and HDAC expression in the brain are also altered by MS (Pusalkar et al., 2016). For instance, Park and coworkers have shown that MS leads to decreased Bdnf and GR mRNA expression in the hippocampus, and that these effects were accompanied by decreased levels of histone acetylation at their respective promoters (Seo et al., 2016; Park et al., 2017). In addition, the authors reported increased hippocampal HDAC5 mRNA in MS animals compared with controls. Interestingly, chronic treatment with an antidepressant (escitalopram) attenuated all these effects, suggesting that the behavioral antidepressant effect could involve epigenetic changes. Furthermore, a recent study suggests that there is a cross-talk between histone acetylation and DNA methylation (Zhu et al., 2017). Indeed, the authors reported that treatment with a HDAC inhibitor reversed the MS-induced increased DNA methylation at the GR promoter region.

Finally, the possible role of brain miRNAs 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 (Uchida et al., 2010). 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 (Bai et al., 2012).

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

As seen earlier, MS is also used as a model of IBS (see Barreau et al., 2007b and O'Mahony et al., 2011 for reviews). In addition to its effects on stress vulnerability, it leads to several GI dysfunctions and increases the vulnerability to experimental colitis.

3.2.2.1. Effects of maternal separation on the enteric nervous system, visceral sensitivity and motility

MS induces dynamic structural and functional changes in the ENS (Barreau et al., 2008; Tominaga et al., 2016). For instance, MS has been reported to increase nerve density and synaptogenesis in juveniles, but these effects are no longer present at adulthood (Barreau et al., 2008). 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 ENS development in a sex-dependent manner, with females being more sensitive than males (Million and Larauche, 2016). It has been widely reported that adult maternally separated rats display visceral hyperalgesia during colorectal distension (Coutinho et al., 2002; Rosztóczy et al., 2003; Barreau et al., 2004b; Schwetz et al., 2005; O'Mahony et al., 2008; Gosselin et al., 2010; Tjong et al., 2011; Tsang et al., 2012; Hyland et al., 2015; Moloney et al., 2015b; Rincel et al., 2016, see **ANNEXE 1**; Tang et al., 2017b; Yi et al., 2017). This procedure consists in introducing a balloon in the rectum and measuring the visceromotor response (abdominal contractions or electromyogenic signal) in response to the balloon's inflation. Interestingly, this hyper-sensitivity to colorectal distension is larger in females than in males (Rosztóczy et al., 2003). Furthermore, the authors also showed that visceral hyperalgesia was greater when all pups were separated from the dam than when only half of littermates were removed, suggesting that the dam's perceived stress plays a role in the long-term effects of MS on visceral sensitivity. Indeed, in another study, it was demonstrated that MS-induced visceral hypersensitivity is transferred across generations and that this effect likely depends upon maternal care (Van den Wijngaard et al., 2013). In addition, MS produces increased intestinal motility in response to stress, as evidenced by reduced total transit time and increased number of fecal pellets (Schwetz et al., 2005; Hyland et al., 2015; Moloney et al., 2015b; Murakami et al., 2017).

3.2.2.2. Effects of maternal separation on gut microbiota composition

Gut microbiota composition can be determined by various approaches. The most common methods rely on 16S ribosomal RNA (rRNA) amplification or sequencing. 16S rRNA is ubiquitous in prokaryotes and has the advantage to contain both highly conserved domains and variable domains, which allow detection and identification of the taxa, respectively. 16S-high throughput sequencing is the most costly but provides much broader information, including α and β diversity (within- and between-community diversity, respectively). Although this method is widely used in the field of gut microbiota in general, it has been used in a limited number of studies investigating the effects of MS on microbiota composition. A

growing number of studies have reported altered gut microbiota composition both in juvenile and adult maternally separated animals. However, the use of different animals and 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 in rodents. The first study that has investigated the effects of MS on the gut microbiota showed overall reduced bacterial diversity in maternally separated animals versus controls (O'Mahony et al., 2009). This finding has been replicated in a more recent work (Zhou et al., 2016). However, another recent study reports no change in diversity (Moya-Pérez et al., 2017). Qualitatively, MS was shown to increase the Firmicutes to Bacteroidetes ratio at the phylum level in some studies (De Palma et al., 2015; Zhou et al., 2016; Li et al., 2017a; El Aidy et al., 2017), but again this finding is not consistent across studies as some report opposite (Pusceddu et al., 2015) or no effects (Zhou et al., 2016). A consistent finding, however, is that the effects of MS on microbiota composition vary both qualitatively and quantitatively with respect to age of investigation. Indeed, several studies comparing at least two time points show completely different patterns (García-Ródenas et al., 2006; Barouei et al., 2012; Zhou et al., 2016; Moya-Pérez et al., 2017). Overall, *Bacteroides* and Lachnospiraceae *(including Clostridium XIVa)* species seem to be consistently altered (either enriched or depleted) across several studies (De Palma et al., 2015; Zhou et al., 2016; Murakami et al., 2017). 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 behavioral effects of MS. Furthermore, considering the importance of sex differences in both stress effects and basal gut microbiota composition, future studies should be conducted in both males and females. To date, only one of the above studies investigated males anf females separately, but found no sex effect (El Aidy et al., 2017).

3.2.2.3. 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 (Estienne et al., 2010). 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 (Barreau et al., 2004a; O'Malley et al., 2010; Li et al., 2016, 2017a, 2047b). Moreover, MS animals show more colonic damage after DSS or TNBS-induced colitis than non stressed animals and as a result, they also lose more weight, indicating that they are more sensitive to experimental colitis (Varghese et al., 2006; Ghia et al., 2008; Veenema et al., 2008). There is mounting

evidence that MS produces long-term gut paracellular and transcellular hyper-permeability to ions and macromolecules (Söderholm et al., 2002; Barreau et al., 2004a, 2004b; García-Ródenas et al., 2006; Varghese et al., 2006; Gareau et al., 2007a, 2007b; Moussaoui et al., 2014; Li et al., 2016) **[Figure 4]**. In addition, exposure to a novel stress at adulthood potentiates gut hyperpermeablity in maternally separated rats (Söderholm et al., 2002; Øines et al., 2012). 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 (Barreau et al., 2004a; Gareau et al., 2006; Moussaoui et al., 2014).

Not surprisingly according to these observations, MS also produces several immune alterations in the colon. Indeed, MS animals show an infiltration of immune cells (i.e. polymorphonuclear neutrophils) (Barreau et al., 2004a; Ghia et al., 2008) and an increase in mucosal mast cell density (Barreau et al., 2004a, 2004b, 2008; Hyland et al., 2009). MS also increases the expression of numerous cytokines including IL-6, IL-1β, TNFα, IFNɣ, IL-4, IL-2, IL-22 and IL-10 in the colonic mucosa (Barreau et al., 2004a; Ghia et al., 2008; Barouei et al., 2015; Li et al., 2017a, 2017b; Moya-Pérez et al., 2017). It has been previously shown that MS increases IFNɣ and TNF secretion by mesenteric lymph node cells (Veenema et al., 2008). In addition, increased mRNA expression of TLR3, 4 and 5 has been reported in the colonic mucosa of MS adult rats (McKernan et al., 2009).

In conclusion, we have seen that MS induces long-term neurobehavioral alterations similar to that observed in human subjects with a history of early-life adversity. Moreover, MS leads to long-term GI dysfunctions resembling IBS symptoms in humans. Altogether, this suggests that MS is a good model to investigate the role of the gut-brain axis in long-term emotional vulnerability.

OBJECTIVES

OBJECTIVES

The etiology of psychiatric disorders is not fully understood, but there are strong evidences that early-life adversity is a major risk factor (Chapman et al., 2004; Rutter, 2005). Early-life adversity is generally defined as exposure to abuse, trauma or neglect during childhood. However, prenatal adverse experiences such as maternal stress or illness have also been reported to increase the vulnerability to neuropsychiatric disorders, including schizophrenia, autism and depression (Herbert, 2010; Brown, 2011; Entringer et al., 2015; Flinkkilä et al., 2016). Unfortunately, cumulating several of the above pre and postnatal events is frequent and likely leads to even higher emotional vulnerability (McEwen, 1998; Maynard et al., 2001; Nederhof and Schmidt, 2012). Importantly, anxiety and mood disorders are more frequent in women than men (Altemus, 2006), and the converse for ASD (Werling and Geschwind, 2013, suggesting that early-life adversity does not equally affect both genders (Carpenter et al., 2017). In addition, psychiatric disorders are comorbid with each other as well as with other conditions including cardiovascular and metabolic diseases (Luppino et al., 2010; Oladeji and Gureje, 2013), but also GI disorders (e.g. IBS) (Folks, 2004; Buie et al., 2010). Interestingly, early-life stress is also a risk factor for IBS (Chitkara et al., 2008; Bradford et al., 2012), the latter being more prevalent in women (Mayer et al., 1999). Retrospective epidemiological studies are poorly reliable tools to understand the complex effects of early-adversity, and prospective longitudinal studies are very costly and have to deal with numerous confounding factors. Animal models have succeeded in demonstrating the causal relationship between early-life adversity (especially stress) and emotional alterations (Sánchez et al., 2001; Cottrell and Seckl, 2009; Schmidt et al., 2011; Molet et al., 2014). However, few studies have taken into account the multiplicity of early stress sources and the potential resulting aggravation of the neurobehavioral alterations. A widely used model of early post-natal stress is maternal separation (MS) (Cirulli et al., 2003; Korosi, 2009). In rodents, chronic MS produces long lasting effects in adult offspring, including hyper-anxiety and hyper-responsiveness to a novel stress (Gutman and Nemeroff, 2002), but also GI dysfunctions (Barreau et al., 2007b; O'Mahony et al., 2011).

In the last decade, there has been huge interest in the field of gut-brain communication as regards mental disorders and especially the regulation of emotions (Mayer et al., 2014a). Exciting converging data from both animal and human studies strongly suggest that what happens in the gut can influence brain function and behavior. The gut microbiota has received particular attention thanks to the technological progress in DNA sequencing, showing altered relative abundance of some bacterial phyla, genera or species in a number of pathological

conditions (Sekirov et al., 2010; Rogers et al., 2016). Proof-of-concept studies in animal models using GF animals or probiotics demonstrate a causal effect of gut bacteria on brain and behavior (Mayer, 2011; Luczynski et al., 2016a; Sarkar et al., 2016; Sherwin et al., 2016), especially in early adversity models including MS studies (De Palma et al., 2015). Notably, gut microbiota composition is significantly different in men *versus* women, and the same finding was observed in animals (Markle et al., 2013; Dominianni et al., 2015; Jašarević et al., 2016; Fransen et al., 2017). This suggests that sex differences in emotional vulnerability could be due to differential microbiota-gut-brain interactions. A lot of expectancy lies on fecal transplantation as a potential novel, revolutionary medical tool for brain disorders (Borody and Khoruts, 2011; Lemon et al., 2012; Collins et al., 2013). However, to date, it has only proved efficient in severe forms of *Clostridium difficile* infections (Khoruts, 2014). On the other hand, pre- and probiotics treatments show mild, inconsistent effects. Overall, it remains unclear which bacterial species or communities are beneficial and by which mechanisms they can impact brain and behavior. Dietary interventions that more globally impact the GI tract could constitute powerful alternatives. Although it is known that gut microbes tightly interact with the gut epithelium and are important regulators of gut permeability (Jakobsson et al., 2015), few attention has been devoted to gut barrier function (Bischoff et al., 2014). Yet, is has been reported that gut permeability is increased in several psychiatric conditions (Julio-Pieper et al., 2014) and is highly sensitive to stress (Kelly et al., 2015; Pigrau et al., 2016). Moreover, gut leakiness has been recently reported in rat pups submitted to acute MS (Moussaoui et al., 2014). Loss of gut barrier function or leakiness is often concomitant with gut dysbiosis (Leclercq et al., 2014; Moussaoui et al., 2016a; Slyepchenko et al., 2016; Stevens et al., 2017) and can lead to bacterial translocation in the bloodstream and peripheral immune activation, that constitute potential mechanisms for altered gut to brain communication (Moriez et al., 2005; Maes and Leunis, 2008; Maes et al., 2008; Slyepchenko et al., 2016). However, the role of gut microbiota and gut permeability in the etiology of anxiety and mood disorders and the underlying mechanisms remain to be explored.

The general objective of this thesis was therefore to decipher the mechanisms underlying gut-brain axis communication in a context of early-life stress. To this end, we first aimed to explore the effects of a nutritional approach on brain and gut alterations induced by MS during development (objective 1). Our results suggested a role of gut permeability in the short and long-term neurobehavioral effects of MS. Thus, our second aim was to investigate the effects of gut permeability *per se* on brain and behavior but also on gut microbiota

composition (objective 2). Finally, we aimed to develop an animal model of multifactorial early-adversity that more closely reproduces what happens in humans (objective 3).

Considering the lack of studies involving both males and females and the gender differences reported in both humans and animals, we will endeavor to study the differential effects of early-life adversity on brain and gut in males and in females.

Objective 1 : What is the impact of perinatal high-fat diet exposure on early-stress-induced brain and gut alterations during development?

In rodents, MS induces enhanced anxiety-like behaviors and hyper-responsiveness to stress, along with increased gut permeability and visceral sensitivity in adulthood. We recently showed that the long-term effects of MS on anxiety, social behavior and stress endocrine response, but also visceral sensitivity, can be prevented by exposing the dams to high-fat diet during gestation and lactation (Rincel et al., 2016, see **ANNEXE 1**). In addition to this protective effect of perinatal high-fat diet in adult animals, we reported similar beneficial effects on the developing brain. Indeed, maternal high-fat diet 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. It has been proposed that abnormal density and organization of dendritic spines in the PFC may contribute to the behavioral alterations caused associated with chronic stress (Moench and Wellman, 2015). On the other hand, recent studies report increased gut permeability in stressed pups (Moussaoui et al., 2014), potentially impacting visceral sensitivity, gut microbes and immune processes, which could in turn affect brain development.

Here, we tested the hypothesis that maternal high-fat diet protects PFC neurons but also gut barrier function in pups submitted to MS. Our results are in line with this hypothesis, and suggest that altered gut permeability could contribute to the long-term effects of MS.

Objective 2 : Is there a causal role for gut leakiness in mediating the long-term effects of early-life stress on emotional vulnerability ?

Gut permeability is increased in several psychiatric conditions associated with vulnerability to early-life adverse events, including major depression and ASD (Maes et al., 2008; de Magistris et al., 2010). Moreover, gut leakiness is often concomitant with gut dysbiosis (Leclercq et al., 2014; Moussaoui et al., 2016a; Slyepchenko et al., 2016; Stevens et al., 2017). However, to date, most of the research on the gut-brain axis and early-life adversity

has been focusing on the gut microbiota, and the intrinsic role of gut permeability in the regulation of emotional behavior remains unexplored. Gut leakiness is associated with epithelial tight-junctions defects. A key regulator of tight junction permeability is the MLCK.

In this part, we used two complementary strategies to manipulate gut permeability by targeting this enzyme. First, we pharmacologically inhibited the MLCK during early-life to test whether we could restore normal behavior in maternally separated animals. Second, we used transgenic mice expressing a constitutively active form of the MLCK specifically in the gut (CA-MLCK mice) to test whether gut leakiness *per se* could lead to neurobehavioral alterations in naive animals. Our data strongly suggest that gut leakiness can impact emotional behavior and stress responsivity both in males and females.

Objective 3 : What is the impact of multifactorial early adversity on brain and gut in males and females ?

In the two first parts, we used the MS model to investigate the role of gut-brain communication in the effects of early-life adversity. It is worth mentioning that, as in humans, other early adverse events that can impact the prenatal and postnatal development have been associated with emotional and GI outcomes in adult animals. Indeed, stress exposure of the dams during gestation or before conception leads to anxiety and depressive-like behaviors in offspring (Schmidt et al., 2011; Maccari et al., 2014; Gröger et al., 2016). One study also reported GI defects including gut dysbiosis (Golubeva et al., 2015). On the other hand, models of early infection also produce emotional alterations, gut leakiness and gut dysbiosis (Enayati et al., 2012; Hsiao et al., 2013; Depino, 2015; Foley et al., 2015; Winston and Sarna, 2016; Kim et al., 2017). It has been proposed that accumulation of stressors across the life span can enhance neuropsychiatric vulnerability (Maynard et al., 2001; Nederhof and Schmidt, 2012). However, most of the animal models only deal with one stressor and one specific time window. Therefore, relevant models combining multiple early adversities could shed new light in the gut-brain axis field. In addition, sex differences are omnipresent in early adversity models, including MS (Zimmerberg and Kajunski, 2004; Slotten et al., 2006; Pohl et al., 2007; Weinstock, 2007; Chaloner and Greenwood-Van Meerveld, 2013; Clarke et al., 2013; Kundakovic et al., 2013; Foley et al., 2015; Gobinath et al., 2016; Prusator and Greenwood-Van Meerveld, 2016; Carpenter et al., 2017). However, most studies have focused on males and there is a lack of studies involving both sexes. On the other hand, gut microbiota profiling is often carried out in males and females pooled together (Hsiao et al.,

2013; De Palma et al., 2015) and the sex of the animals used is sometimes not even mentioned.

Here, we developed a new model of multifactorial early adversity combining maternal infection (LPS injection in late gestation) and MS in early post-natal life, in order to examine the consequences on behavior, gut function and microbiota composition in males and females. Preliminary results indicate marked sex differences in all above-mentioned outcomes.

RESULTS

ARTICLE 1

Maternal high-fat diet and early life stress differentially modulate spine density and dendritic morphology in the medial prefrontal cortex of juvenile and adult rats

ARTICLE 2

Early restoration of gut barrier function abrogates the long-term neurobehavioral effects of early-life stress in rats

In preparation

ARTICLE 2 in preparation:

Early restoration of gut barrier function abrogates the long-term neurobehavioral effects of early-life stress in rats

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ABSTRACT

Maternal separation (MS) in rats is associated with emotional, cognitive and endocrine alterations as well as long-lasting gastrointestinal dysfunctions. Recent studies report increased intestinal permeability in pups after MS. A major regulator of tight-junction permeability is the myosin light chain kinase (MLCK). The present study aims to determine whether the inhibition of MLCK-dependent gut leakiness during the neonatal period protects against the long-term effects of MS. Male Wistar rats were exposed to MS (3 h per day from postnatal day (PND)2 to PND14) or left undisturbed, and received daily injection of a MLCK inhibitor (ML-7, 5mg/kg; i.p.) or vehicle during the same period. Gut permeability as well as blood-brain barrier (BBB) function were evaluated in juvenile rats. At adulthood, emotional behaviors and corticosterone response to stress were analyzed. Finally, gut microbiota composition was analyzed in both juvenile and adult rats. We report that ML-7 restored normal gut barrier function in MS pups (PND14). BBB permeability was not affected by MS or the ML-7 treatment. Remarkably, ML-7 treatment during development prevents MS-induced sexual reward seeking impairment and altered corticosterone response to stress at adulthood. In contrast, ML-7 has no preventive effect on anxiety or sucrose preference. These effects of ML-7 were accompanied by normalization of the increased abundance of *Lachnospiraceae UCG-001 group*, *Clostridiales vadinBB60 group* and *Desulfovibrio* spp. and decreased abundance of *Bacteroidales S24-7*, *Enterorhabdus* and *Bifidobacterium* spp. in the feces of MS rats at adulthood. However, increased *Escherichia* spp. and *Acetitomaculum* spp abundance in MS rats was not prevented by ML-7. There were limited effects of MS (decreased *Enterorhabdus* spp.) and no effect of ML-7 on gut microbiota composition in juveniles. Altogether, our work suggests that gut barrier dysfunction during development plays a critical role in the long-term effects of early-life stress and provides new insight into the gut-brain communication in a context of stress.

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INTRODUCTION

Anxiety and mood disorders are highly comorbid with gastrointestinal disorders such as the irritable bowel syndrome (IBS) (Folks, 2004), suggesting that they may share common pathophysiological bases. Indeed, childhood adverse experience emerges as a common risk factor for both conditions (Chapman et al., 2004; Chitkara et al., 2008; Bradford et al., 2012). In rodents, chronic maternal separation (MS) is a widely used model to study the long-term impact of early-life stress on brain and behavior, but also on gut physiology and digestive functions (Barreau et al., 2007; O'Mahony et al., 2011). A large body of literature reports hyper-anxiety and exaggerated endocrine responsiveness to a novel stress in adult maternally separated animals (Gutman and Nemeroff, 2002). In addition, MS produces various gastrointestinal dysfunctions including visceral hyperalgesia, gut dysbiosis and impaired gut barrier function (Barreau et al., 2007). In the last decade, the gut microbiota has been implicated in a variety of behavioral processes, particularly emotional processes and stress vulnerability (Dinan and Cryan, 2012; Foster and McVeyNeufeld, 2013; Burokas et al., 2015; Luczynski et al., 2016). For instance, De Palma and colleagues have demonstrated that MS-induced anxiety and depressive-like behaviors are absent in germ-free (GF) mice, but can be unmasked upon colonization with the gut microbiota of a conventional control mouse (De Palma et al., 2015). However, colonization with the microbiota of a maternally separated mouse did not transfer the stress-associated behavioral phenotype in naive GF mice, suggesting that gut bacteria are necessary but not sufficient to mediate the behavioral effects of early-life stress. Although it is known that gut microbes tightly interact with the gut epithelium and are important regulators of gut permeability (Zakostelska et al., 2011; Jakobsson et al., 2015; Reunanen et al., 2015), few attention has been devoted to the potential role of gut barrier function in stress-induced emotional alterations. Yet, is has been reported that gut permeability is increased in several psychiatric conditions (Maes et al., 2008; de Magistris et al., 2010) and is highly sensitive to stress (Kelly et al., 2015; Pigrau et al., 2016). Loss of gut barrier function or leakiness is often concomitant with gut dysbiosis (Zakostelska et al., 2011; Hsiao et al., 2013; Jakobsson et al., 2015; Reunanen et al., 2015) and can lead to bacterial translocation in the bloodstream (Gareau et al., 2006; Moussaoui et al., 2014) and peripheral immune activation, that constitute potential mechanisms for altered gut to brain communication. Moreover, probiotic treatments have been shown to attenuate the behavioral and endocrine alterations induced by stress exposure, including stress during early-life. Interestingly, some studies reported that the beneficial effects of probiotics on behavior and hypothalamus-pituitary-adrenal (HPA) axis response to stress are accompanied by changes in gut microbiota composition, but also a restoration of gut barrier function (Gareau et al., 2007; Ait-Belgnaoui et al., 2012). Pharmacological inhibition of the myosin light chain kinase (MLCK) prevents stress-induced intestinal, tight-junction-

dependent leakiness in rat pups (Moussaoui et al., 2014) and HPA axis hyperactivity in adult rats (Ait-Belgnaoui et al., 2012). The latter findings suggest that increased gut permeability could contribute to the effects of early-life stress on brain vulnerability through alterations of gut-brain communication. The present study aims to determine whether the prevention of gut leakiness associated with MS during the neonatal period protects against the long-term behavioral and endocrine abnormalities in MS animals. Moreover, we also explored the effects of MS and ML-7 treatment on gut microbiota composition both in adult and juvenile rats.

METHODS

Animals

Male and female Wistar rats were used for breedings. All experiments were approved by the local Bioethical committees of our Universities (Bordeaux: N° 50120186-A ; Toulouse: ToxCom/0031) and by the régions Aquitaine (ID: A33-063-920) and Midi-Pyrénées Veterinary Services (National Animal Care Committee ID : 86) according to the European legislation (Directive 2010/63/EU, 22 September 2010). Animals were maintained in a 12-h light/12-h dark cycle (lights on at 0800 hours) in a temperature-controlled room (22 °C) with free access to food and water, unless otherwise mentioned.

Study design

3 cohorts were used in this study. **Figure 1** illustrates the experimental design with the different cohorts used.

Experiment 1 (cohort 1): determination of the dose of ML-7 for pharmacological inhibition of gut leakiness in MS pups. A first cohort was used to test the effects of chronic ML-7 injections in maternally separated male pups. Pups were separated from their dam for 3 hours daily on post-natal day (PND)2-14, or left undisturbed. Prior each separation session, MS pups received a ML-7 injection (10 µL i.p, Bio-Techne R&D Systems, Lille, France) or vehicle (saline). The ML-7 solution was prepared from ML-7 powder in NaCl 9‰ and sonicated 10s twice. Two ML-7 doses (1 mg/kg and 5 mg/kg) were tested based on previous work (Moussaoui et al., 2014). Intestinal permeability was assessed on PND14, PND21 and PND49. The dose of 5 mg/kg was chosen for the following experiments.

Experiment 2 (cohorts 2 and 3): impact of chronic ML-7 on short and long-term abnormalities associated with early-life stress. Dams were individually housed throughout gestation and lactation. At birth, litters were culled to 9 pups with balanced sex-ratio and randomly assigned to either MS or control groups. MS was carried out from PND2 to PND14 (between 9:00 ± 1hr and 12:00 ± 1hr) as previously described (Rincel et al., 2016).

Control litters remained undisturbed throughout the procedure. Within each litter (MS or control), 2 male pups received daily injections of ML-7 (5 mg/kg, i.p.) immediately before separation (or at the same time for controls), while 2 others were assigned to the vehicle group.

Molecular analyses

In vivo intestinal permeability. Intestinal permeability was measured at PND14, PND21 and PND49. Pups were gavaged with 250 µL of 1 or 5 mg/mL solution of Fluorescein-5-isothiocyanate (FITC)-labeled dextran 4kD (FD4, TdB consultancy AB, Uppsala, Sweden). After 4 hours, blood samples were taken from the facial vein (heparinized tubes). For the measure on PND14, oral gavage was performed immediately after the 180min of separation. Tubes were centrifuged for 10 minutes at 10,000 g and fluorescein concentration was determined against a standard curve on a microplate reader (Tecan Infinite M200, Lyon, France). Results are expressed as FD4 plasma concentration per gram of body weight.

In vivo BBB permeability. Gestant female Wistar (n=6) were purchased (Janvier Labs, Le Genest Saint Isle, France) at gestational day 16 and individually housed throughout gestation and lactation. MS and ML-7 injections were carried out between PND2 and PND14 as described in the experimental design. Evans blue (EB) extravasation was used as an index of BBB permeability (Kumar and Sharma, 2016). The following protocol was based on preliminary tests in naive animals (see **Supplementary methods** and **Supplementary Fig. 1** for EB dose-response). All groups (N=7 male pups/group) received 2% EB (Sigma Aldrich, St. Quentin Fallavier, France) injections (240 mg/kg, i.p.) on PND14 immediately after ML-7 or vehicle injection. EB was diluted in NaCl, vortexed and filtered 24h prior injection and stored at 4°C. ML-7/vehicle and EB injections were done at the opposite body side. 24h after EB injections (including the 3hr-MS on PND14), pups were deeply anesthetized with pentobarbital 150 µl and cardiac blood was collected before perfusion with 20ml NaCl. Brain tissues were split in left and right hemispheres (forebrain) and hindbrain (cerebellum + brainstem), weighed and stored at -20°C. 5 female pups (MS group) were killed as negative controls without EB injection (blank).

All samples remained protected from light throughout the procedure. Plasma samples were obtained following a series of centrifugations (10min at 1000g), diluted 10x in 50% trichloroacetate (TCA) and further centrifuged (10min at 10,000rpm). The supernatant was finally stored at -20°C. Brain tissues were homogenized in 1 ml 50% TCA (tissuelyser 3min 3Hz), centrifuged 30 min at 10000 rpm and the supernatant was stored at -20°C. EB fluorescence was measured in supernatants from plasma and brain tissues using a microplate reader (Tecan Infinite M200, excitation 620 nm / emission 680 nm). EB fluorescence was expressed relative to the blank group.

Plasma cytokines multiplex assay. Trunk blood was collected from PND14 pups after 180 min of MS and centrifuged at 4°C before plasma was stored at−20°C. Plasma interleukin (IL)-1b, IL-6, IL-10, interferon gamma (IFNg) and tumor necrosis factor alpha (TNFa) were measured by multiplex assay (RECYTMAG-65K MILLIPLEX MAP Rat Cytokine/Chemokine Magnetic Bead Panel, Millipore, Fontenay sous Bois, France) according to the manufacturer's instructions. Cytokines concentrations were determined using the Luminex xMap Technology (Bio-Rad, Marnes-la-Coquette, France). All samples were processed in duplicates. Intra- and inter-assay coefficients were below 5 and 15%, respectively and crossed reactions were insubstantial (0.01%). Minimum detectable concentrations were 2.8 pg/mL for IL-1b, 30.7 pg/mL for IL-6, 2.7 pg/mL for IL-10, 6.2 pg/mL for IFNg and 1.9 pg/mL for TNFa. Only IL-1b and IL-10 were detectable in our samples.

Corticosterone radioimmunoassay. Total plasma corticosterone was measured with an in-house radio immunoassay, by competition between cold corticosterone (B) and 3H-B (B*) for a specific anti-corticosterone antibody, as previously described (Rincel et al., 2016).The sensitivity of this assay is around 5 ng/ml. Intra- and inter-assay variations were <15%.

Immunohistochemistry

After deep anesthesia with sodium pentobarbital (100µL/10g BW), PND14 pups (n=7-8 per group) were transcardially perfused with 50mL of phosphate-buffered saline (PBS) followed by 50 mL of4% paraformaldehyde (PFA). The brains were removed, post-fixed in the same fixative for 12 hours and cryoprotected by immersion in 30% sucrose/PBS for 48 hours. Brains were finally frozen in isopentane and stored at -80°C.

Tight-junction proteins immunofluorescence. 20µm coronal sections containing the choroid plexus (anteroposteriority from bregma: -2.64nm/-3.72nm, according to Paxinos and Watson 2013) were obtained using a HM560 cryotome (MM, Francheville, France). Every 8th section was collected on the same slide so that the interval between sections within a given series was 160µm. Slides were incubated with the primary antibody (polyclonal rabbit anti-occludin, 1/2000, ab 31721Abcam, UK; polyclonal rabbit anti-zonulin-1 (zo-1), 1/50, 61-7300, Zymed, Thermo Fisher Scientific, USAor polyclonal rabbit anti-claudin-1, 1/300, ab 15098Abcam, UK) for 48h at 4°C followed by a 1h incubation with a goat anti-rabbit antibody coupled to Alexa 488 (1/1000, A11008, Molecular Probes, Thermo Fisher Scientific, USA) at 4°C and in contact with 4',6-diamidino-2-phenylindole (DAPI, 1 µM for 1 min, D3571 Molecular Probes, Thermo Fisher Scientific). Sections were coverslipped with Prolong Goldantifade (P36930, Molecular Probes, Thermo Fisher Scientific, USA) before analysis.

Quantification of immunoreactivities. Images were captured by a black and white Zeiss Camera (Axiocam 503) coupled with a Zeiss Axio-imager M2m microscope using the Zen.2 pro software. For occludin and zo-1 labeling in the choroid plexus, we obtained a Z-stack of

50 virtual optical slices of 0.250 µm each using the x63 objective. For claudin-1 labeling in the choroid plexus, we obtained a Z-stack of 10 virtual optical slices of 0.55 µm each using the x40 objective. The signal was quantified using Fiji (Schindelin et al., 2012) and expressed as Alexa 488 intensity/ Alexa area / DAPI area.

Behavioral assessment

All experiments were performed during the light phase (9h30-17h). For analyses involving manual quantifications, experimenters remained blind to the experimental groups.

Ultrasonic vocalizations (USVs). USVs were assessed in response to a 6min separation on PND6 (in the afternoon, 3 hours after the end of the MS episode). Pups were gently removed from the homecage and placed in a glass crystallizer bedded with thick cotton in an adjacent room. USVs emissions (range 0-70 kHz) were recorded using an ultrasound microphone coupled with the AvisoftSAS LabPro software (Avisoft Bioacoustics,Glienicke, Germany) and automatically quantified.

Sucrose preference (2 months). Rats were individually housed 3 days before the beginning of the experiment and underwent 3 days of habituation with two bottles of tap water. On the day of testing (afternoon), animals were presented one bottle filled with 1% sucrose solution and one bottle of water during 24h. Water and sucrose intakes were monitored before and after the 24h of test and sucrose preference was calculated as the percentage of sucrose intake over total fluid intake. Bottle side was randomized to control for any side bias.

Light-dark box (2.5 months). The apparatus was a two-compartment box with a dark compartment (31x31cm) wrapped with a cover and a light compartment (45x31cm) exposed to intense light (light intensity: 70 lux). Rats were placed in the dark compartment and exploration of the light box was recorded with a digital camera during 10 min. Time and number of entries in the light box were manually quantified using an ethological software (The observer, Noldus Information Technology, Wageningen, The Netherlands). An entry in the light box was scored as such when the rat placed all four limbs into the light compartment.

Female urine sniffing test (3 months). The test was performed as previously described (Malkesman et al., 2010). Females (3 month-old) from different litters were used for urine collection. To elicit estrus, a male was introduced in the females' homecage during 1 hr. Three days later, females were placed in a new cage without bedding for 3 hours and urine (approximately 3 ml per animal) was collected and stored at -20°C until use. Estral cycle phase was determined by vaginal cytology and only urine from estrus females (n=5) was used for the experiment. Under dim light (light intensity: 3 lux)**,** male rats were habituated to a dry Q-tip in the homecage for 60 min, and then presented with a Q-tip soaked with sterile water for 5 min. After 45 min without any Q-tip, rats were presented with a Q-tip soaked with

estrus female urine for another 5 min. Q-tips were soaked with 200 µl of sterile water or urine and taped to the cage wall. 5 min trials were videorecorded using a digital camera and Q-tip sniffing time was manually quantified using the Observer software (Noldus Information Technology).

HPA axis reactivity to stress (4.5 months). Rats were placed in transparent plexiglas restraint tubes for 30 min and put back in their homecages. Blood samples were collected from the tail vein at the beginning of the restraint stress (t0), 30 min (t30, end of restraint), 60 min (t60) and 120 min (t120) after the beginning of the stress. Blood samples were centrifuged (4000 rpm, 4°C) for 20 min and plasma was stored at -20°C until use.

16S rRNA sequencing and microbial community analysis.

Fecal content was collected at sacrifice and stored at -80°C until use. Genomic DNA from fecal homogenates was extracted using the ZR Fecal DNA Miniprep™ (Zymo Research) and DNA quantity was determined using TECAN Fluorometer (Qubit® dsDNA HS Assay Kit, molecular probes). The microbial 16S rRNA gene was amplified during the first PCR step with adapter fusion primers targeting the V3 to V4 regions (corresponding to a 460-bp region of *Escherichia coli* 16S rRNA gene, GenBank number J01695 with bacterial forward 343F (TACGGRAGGCAGCAG, Liu et al., 2007) and reverse 784R (TACCAGGGTATCTAATCCT, Andersson et al., 2008) primers. Pooled amplicon libraries were sequenced employing an Illumina MiSeq (2 x 250 bp) at the GeT-PlaGe platform in Toulouse (France). Sequence reads were quality controlled and first treated with the FROGS pipeline (Find Rapidly operational taxonomic units (OTU) with Galaxy Solution) (Escudie et al., 2015). Briefly, after merging the 250 bp reads, datasets were denoised and the software was further used for several quality filtering level of DNA sequences before and after clustering (Swarm (Mahé et al., 2014). Chimera (Vsearch), singletons and OTU representing low proportion of reads (0.00005, Bokulich et al., 2013) or found in less than 3 samples were removed. Taxonomic assignment at the lowest phylogenetic level (BLAST algorithm against the SILVA 123 database) and prevalence based filtering step allowed to obtain 643 OTUs (after correcting multi-affiliations and some misleading affiliations) among the 3,772,253 remaining reads. Between 25,718 and 54,362 valid sequences per sample were counted (an average of 48,393 sequences at PND 14 and 37,340 at PND 155). Richness and diversity indexes of bacterial community as well as clustering and ordinations were computed using Phyloseq package (v 1.19.1) in RStudio software (R Development Core Team, 2011; Mcmurdie and Holmes, 2012, 2013). Differences in overall bacterial communities composition were determined using two-way ANOVAs combined with Holm-Sidak's *post-hoc* tests, whereas microbiota differences in structure between groups were assessed using Adonis (permuted *p*-value was obtained by performing 9999 permutations). For further differential abundance

analysis, closely-related taxa were agglomerated at the genus rank, reducing the taxon list to 96 at PND14 and 94 at PND155. A negative binomial fit model for count data was run on all groups using the DESeq2 package (v 1.14.1) (Love et al., 2014; McMurdie and Holmes, 2014) and linear discriminant analysis (LDA) effect size was performed between two groups and plotted using LEfSe (Segata et al., 2011). Tests were corrected for multiple inferences using Benjamini-Hochberg method to control the False Discovery Rate (Hochberg and Benjamini, 1990). The complete method and statistical analysis are provided in the online supplementary methods.

Statistics

Data were analyzed using Statistica 6.0 (Statsoft) and Graphpad Prism 6. Normality was assessed by Shapiro–Wilk tests. Two-way ANOVAs followed by Fisher's LSD, Holm-Sidak's *post-hoc* tests or planned comparisons were used to test the effects of MS and ML-7 in juveniles and adults, unless otherwise mentioned. Three-way ANOVAs with repeated measures were used for the analysis of USVs and HPA axis responsiveness to stress. Student *t*-tests were used to test the effects of MS and ML-7 on intestinal permeability. Paired *t*-tests were used to analyze the preference for urine *versus* water in the FUST. Detailed statistics used for gut microbiota analysis are provided in the corresponding method section. Statistical significance was set at $p<0.05$, unless otherwise mentioned. Graphics were made using GraphPad Prism 6 and Adobe Illustrator CS5.1 was used for artwork. Data are expressed as means ± SEM or medians, unless otherwise stated (gut microbiota analyses).

RESULTS

Chronic MLCK inhibition from PND2 to PND14 prevents gut leakiness in MS pups specifically during early development and has no effect on BBB permeability

We first determined the ML-7 dose able to prevent gut leakiness in MS pups **(Fig. 2)**. In PND14 pups, chronic MS significantly increased gut permeability to FITC-Dextran (MS Veh *vs* Control, Student *t*-test, $t(14)=5.77$, $p<0.0001$) **(Fig. 2a)**. This effect was not modulated by 1 mg/kg ML-7 (MS ML-7 1mg/kg *vs* MS Veh, $t(14)<1$, n.s.); however, 5 mg/kg ML-7 fully restored gut permeability (MS ML-7 5mg/kg *vs* MS Veh, $t(14)=5.52$, $p<0.0001$) **(Fig. 2a)**. Consequently, we used this dose in all the following experiments. MS-induced intestinal hyper-permeability was still present on PND21 (MS Veh *vs* Control, $t(14)=5.11$, $p=0.000$) **(Fig. 2b)** and PND49 ($t(14)=5.55$, $p<0.0001$) **(Fig. 2c)**, but the ML-7 treatment from PND2-14 failed to prevent this long-lasting effect of early stress (MS ML-7 *vs* MS Veh, all $t(14)<1$, n.s.). These results suggest that chronic ML-7 treatment from PND2 to PND14 specifically

prevents the increase of intestinal permeability during the stress procedure, but this effect is not maintained after the end of the ML7 treatment.

Early-life stress has been shown to increase BBB permeability (Gómez-González and Escobar, 2009). As the intestinal barrier, the BBB is regulated by tight junction MLCKs and we cannot exclude that i.p. ML-7 treatment does not impact brain MLCK activity (Kuhlmann et al., 2007; Luh et al., 2010). Thus, we assessed markers of structural and functional BBB integrity in PND14 pups at the end of the MS procedure **(Fig. 3)**. Protein levels of occludin, claudin-1 and zo-1 in the choroid plexus were not modified by MS (early stress effect: all $F(1,16)<1.2$, n.s.)**(Fig. 3a-c)**. However, ML-7 significantly decreased occludin expression whatever the stress condition (treatment effect: $F(1,16)=4.81$, $p=0.0434$). We further explored BBB function by measuring Evans Blue (EB) extravasation in the brain after i.p. injection **(Fig. 3d-f)**. There was no significant difference in EB fluorescence between groups in the forebrain or hindbrain 24h after injection (all $F(1,25)<2$, n.s) **(Fig. 3d,e)**. Of note, EB fluorescence in plasma was similar in all groups and much higher than in brain tissues, indicating that there was no injection bias (all $F(1,24)<1$, n.s.)**(Fig. 3f)**. These results suggest that the BBB is unspoiled in our experimental conditions.

Effects of neonatal MLCK inhibition on MS-associated alterations in juvenile rats

We then investigated the effects of MS and ML-7 in juvenile rats (PND6-14). On PND6, rat pups were submitted to a short separation episode to assess ultrasonic vocalization (USV) emissions. USV response has been shown to serve mother-infant communication in rodents and can be used as an index of pups' anxiety (Branchi et al., 2001). There was no change in the number of calls across time (three-way ANOVA with repeated measures, time effect: $F(5,110)=1.98$, $p=0.0874$) **(Fig. 4a,b)**. Overall, MS significantly increased the number of USVs in response to the short separation (early stress effect: $F(1,22)=11.37$, $p=0.0027$). The ML-7 treatment did not significantly alter USV emission pattern ($F(1,22)<0.01$, n.s.). There was no effect of MS or ML-7 treatment on pups body weight at PND14 (two-way ANOVA, early stress effect, treatment effect, early stress x treatment effect, all $F(1,51)<0.5$, n.s.) **(Fig. 4c)**. However, as expected, there was an increase in plasma corticosterone in response to the 3hr separation, but this effect was not affected by the ML-7 treatment (early stress effect: $F(1,26)=28.93$, $p<0.001$) **(Fig. 4d)**. Both gut leakiness and MS are associated with peripheral immune activation (Ait-Belgnaoui et al., 2012; Wieck et al., 2013; Pinheiro et al., 2014; Roque et al., 2014; do Prado et al., 2015; Réus et al., 2017). We thus measured peripheral cytokine levels in the plasma. MS increased both IL-1b and IL-10 levels regardless the treatment (IL-1b, early stress effect: $F(1,36)=3.66$, $p=0.0639$; treatment effect, $F(1,36)=1.55$, n.s.; early stress x treatment effect: $F(1,36)=1.17$, n.s. and IL-10, early stress effect: $F(1,36)=5.41$, $p=0.0300$; treatment effect, $F(1,36)=0.53$, n.s.; early stress x treatment effect:

F(1,36)=0.48, n.s.) **(Fig. 4e,f)**. These results suggest that MS induces immune activation in the periphery. To assess whether MS leads to neuroinflammation, we also evaluated cytokine mRNA expression in the hippocampus and mPFC, two brain areas sensitive to stress and previously shown to exhibit altered cytokine expression in MS animals (Pinheiro et al., 2014; Roque et al., 2014; Réus et al., 2017) **(Supplementary Fig. 2a,b)**. Overall, brain cytokine mRNA levels were not altered by the perinatal manipulations (all $F(1,29)<3$, n.s. $F(1,29)<1.5$, n.s, except IL-1b in the hippocampus, early stress effect: $F(1,29)=3.51$, $p=0.0710$; treatment effect: $F(1,29)=6.88$, $p=0.01375$; early stress x treatment effect: $F(1,29)=3.67$, $p=0.0653$). IL-1b levels in the hippocampus were significantly higher in C ML-7 animals relative to all other groups (Fisher LSD's *post-hoc*, at least $p<0.05$). Altogether, these results suggest that MS is not associated with increased cytokines expression in the brain. Vagus nerve afferences to the nucleus tractus solitarius (NTS) constitute an important route of gut-brain communication (Bercik et al., 2011; Bravo et al. 2011). Therefore, were quantified neuronal activation in the NTS after the 3hr separation on PND14 **(Supplementary Fig. 2c)**. There was no effect of MS or ML-7 on the number of C-FOS immunoreactive cells (two-way ANOVA, all $F(1,10)<3$, n.s.).

Chronic MLCK inhibition during early-life prevents MS-induced impairment in sexual reward seeking and HPA axis hyper-responsiveness to stress at adulthood

To test the hypothesis that early restoration of gut barrier function would alleviate MS-associated long-term behavioral alterations, adult rats were tested for anxiety, anhedonia and social behaviors **(Fig. 5 and Supplementary Fig. 3)**. Regardless the treatment, MS significantly decreased the time spent in the light box, indicating that MS led to exacerbated anxiety (two-way ANOVA, early stress effect: $F(1,44)=5.35$, $p=0.0254$; treatment effect: $F(1,44)=0.93$, n.s.; early stress x treatment effect: $F(1,44)=0.13$, n.s.) **(Fig. 5a)**. In the sucrose preference test, there was no difference in water intake over the 24h (early stress effect, treatment effect and early stress x treatment effect, all $F(1,44)<1$, n.s.; data not shown). However, MS significantly reduced sucrose intake whatever the treatment (early stress effect: $F(1,44)=8.02$, $p=0.0070$; treatment effect: $F(1,44)=0.00$, n.s.; early stress x treatment effect: $F(1,44)=0.27$, n.s.) **(Fig. 5b)**. Furthermore, reward seeking behavior was assessed in the female urine sniffing test **(Fig. 5c)**, a more ethological paradigm. Control animals spent more time sniffing estrus female urine than water (paired *t*-test, $t(4)=2.82$, $p=0.0480$). This preference for female urine was not observed in MS Veh or C ML-7 groups (both $t(4)<1$, *n.s.*). However, it was restored in MS ML-7 rats ($t(3)=3.80$, $p=0.0320$). These results show that MS rats display anhedonia for both sucrose and female urine. Interestingly, ML-7 prevented MS-induced anhedonia for female urine but not sucrose. Of note, there was no significant impact of the perinatal manipulations on social interaction with a conspecific,

an aggressor or a juvenile (two-way ANOVAs, early stress effect, treatment effect and early stress x treatment effect, all $F(1,44)<2$, n.s.) **(Supplementary Fig. 3)**.

In order to assess HPA axis response to stress, rats underwent 30min restraint stress and plasma corticosterone was determined at 0, 30, 60 and 120 min **(Fig. 5d)**. Three-way ANOVA with repeated measures showed a significant effect of time ($F(1,132)=281.19$, $p<0.001$). The effects of MS varied according to the treatment, although the interaction did not reach statistical significance (early stress x treatment effect: $F(1,44)=2.67$, $p=0.1096$). Planned comparisons at 60 and 120 min revealed that MS led to sustained corticosterone levels after the end of stress relative to controls, suggesting impaired HPA axis negative feedback (MS Veh vs C Veh, t60: $p=0.1470$ and t120: $p=0.0107$). These effects were totally prevented by the ML-7 treatment (MS ML-7 vs MS Veh, t60: $p=0.0489$ and t120: $p=0.0004$) **(Fig. 5d)**. Consistently, the area under the curved (AUC) was differentially altered by MS depending upon the treatment (early stress x treatment effect: $F(1,44)=2.82$, $p=0.1004$, data not shown). The MS-induced increase in AUC was abrogated by the ML-7 treatment (Fisher LSD's *post-hoc,* MS Veh vs C Veh: $p=0.0493$ and MS ML-7 vs MS Veh: p=0.0182). We then tested whether the impaired HPA axis negative feedback was associated with altered expression of stress-related genes in the hippocampus, a key brain region involved in HPA axis regulation (Herman et al., 2003, 2016) **(Supplementary Fig. 4)**. Overall, there was no significant effect of MS or ML-7 on mRNA in the hippocampus of adult rats (two-way ANOVA, all $F(1,28)<2$, n.s., except for 11 beta hydroxysteroid dehydrogenase 1 (11bHSD1, treatment effect: $F(1,28)=4.14$, p=0.0514). The ML-7 treatment slightly downregulated 11bHSD1 expression whatever the stress condition **(Supplementary Fig. 4b)**.

Impact of MS and neonatal MLCK inhibition on gut microbiota composition

Several studies have reported altered microbiota composition in adult rats and mice with a history of MS (De Palma et al., 2015; Pusceddu et al., 2015; Zhou et al., 2016; El Aidy et al., 2017; Murakami et al., 2017) and some evidence exists that this dysbiosis is necessary for certain behavioral effects of MS (De Palma et al., 2015). Moreover, there is a cross-talk between gut barrier function and gut microbiota, suggesting that inhibition of gut leakiness could impact the microbiota. Therefore, at the end of the behavioral experiments (PND155), we conducted 16S rRNA illumina sequencing-based analysis of fecal microbiota in the same 4 experimental groups. Fecal microbiota composition was differentially affected according to the neonatal manipulations (**Fig. 6**). Specifically, the number of bacterial genera (α-diversity) was decreased by MS, but this effect was attenuated in rats treated with the ML-7 (early stress x treatment effect: $F(1,40)=5.23$, $p=0.0276$, Holm-Sidak's *post-hoc* tests, C Veh *vs* MS Veh $p=0,0174$; C Veh *vs* MS ML-7 $p=0.0617$) (**Fig. 6a**). Multidimensional scaling analysis based on Bray Curtis distances (β-diversity) showed partial separation of microbiota profiles

according to the neonatal manipulations, the MS Veh microbiota being the most distant from C Veh and MS ML-7 microbiotas (Fig.1b, MANOVA, early stress x treatment effect: $F(1,40)=3.72$, $p=0.0010$) (**Fig. 6b**). At the phylum level, ANOVA revealed an interaction between early stress and treatment for all the detected phyla except Tenericutes, that were unaffected by either manipulation (Firmicutes: $F(1,40)=5.72$, $p=0.0216$; Bacteroidetes: $F(1,40)=6.49$, $p=0.0148$; Proteobacteria: $F(1,40)=8.83$, $p=0.0050$; Actinobacteria: $F(1,40)=6.22$, $p=0.0169$; Deferribacteres: $F(1,40)=6.25$, $p=0.0166$; Tenericutes: $F(1,40)<1$, n.s.) (**Fig. 6c-h**). Proteobacteria were significantly enriched in MS Veh compared with controls (Holm-Sidak's *post-hoc* tests, C Veh *vs* MS Veh $p= 0.0064$). A trend towards increased Deferribacteres (only represented by the species *Mucispirillum schaedleri*) was also observed in MS Veh (C Veh *vs* MS Veh $p= 0.0601$). Strikingly, these MS-induced changes were not observed in rats treated with ML-7 (C Veh *vs* MS ML-7, all n.s.) (**Fig. 6e-g**). Over the 94 assigned bacterial taxa at the genus rank, 12 were significantly affected primarily by MS (**Fig. 7a**) (early stress effect: all $p$adj<0.05, see detailed statistics in **Supplementary Table 2**). Among these 12 genera altered by early-life stress, concomitant ML-7 administration normalized the relative abundance of 6 genera (**Fig. 7b**) (early stress x treatment effect: all $p$adj<0.05, see detailed statistics in **Supplementary Table 3**). Indeed, the increased abundance of *Lachnospiraceae UCG-001 group*, *Clostridiales vadinBB660* (both Firmicutes) and *Desulfovibrio* spp. (Proteobacteria), as well as the decreased abundance of *Bacteroidales S24-7* (Bacteroidetes), *Enterorhabdus* spp. and *Bifidobacterium* spp. (i.e. *animalis* and *pseudolongum*) (both Actinobacteria) found in MS Veh were not observed in MS ML-7 rats. Cladograms illustrating the changes in gut microbiota composition in MS Veh and MS ML-7 groups compared with controls are shown in **Figure 7c,d** (see LDA scores in **Supplementary Fig. 5**). Remarkably, **Figure 7d** underlines that altered abundance of *Escherichia* spp. and *Acetitomaculum* spp. in MS animals was not normalized by ML-7. Of note, there was no major impact of the ML-7 treatment in non-separated animals (**Supplementary Fig. 6).**

We then asked whether MS and ML7 similarly impacted these bacterial communities during development **(Supplementary Fig. 7 and 8)**. In PND14 pups, there was no significant impact of early-life manipulations on global composition of the fecal microbiota at phylum level, as evidenced by the lack of difference in α- and β-diversity or relative abundance of the detected phyla (two-way ANOVAs, all $F(1,39)<3,2$, n.s., **Supplementary Fig. 7**). Further analysis at the genus rank revealed a significant decrease in *Enterorhabdus spp.* in maternally separated pups, regardless their treatment group (early adversity effect: $p$adj=0.0470) **(Supplementary Fig. 8)**. Interestingly, this decreased abundance of *Enterorhabdus spp.* is observed in both juveniles and adult rats. However, normalization by the ML-7 treatment occurs in adults only.

DISCUSSION

There is mounting evidence that gut microbiota plays an important role in the regulation of stress-related behaviors and stress responsivity (Dinan and Cryan, 2012; Foster and McVeyNeufeld, 2013; Burokas et al., 2015; Luczynski et al., 2016). Numerous studies have shown that chronic stress exposure leads to gut dysbiosis concomitant with gut leakiness. Despite the close relationship between gut microbiota and gut epithelial barrier, the intrinsic role of gut barrier permeability in stress-induced emotional alterations has not been investigated. Previous work demonstrated that inhibition of the MLCK by ML-7 prevents stress-induced intestinal, tight-junction-dependent, increase in paracellular permeability in rat pups (Moussaoui et al., 2014) and HPA axis hyperactivity in adult rats (Ait-Belgnaoui et al., 2012). Here, we show in male rats with a history of chronic maternal separation that inhibition of gut leakiness during the neonatal period (PND2-PND14) prevents some of the long-term deleterious effects of maternal separation. These results suggest that altered gut permeability during this critical developmental period may contribute to HPA axis dysfunctions and impaired sensitivity to sexual reward later in life. Moreover, we also report a preventive effect of the attenuation of gut leakiness in early-stressed animals on gut dysbiosis at adulthood, suggesting that primary defects in barrier function can drive changes in gut microbial communities.

Previous studies have demonstrated that intestinal permeability is exacerbated in pups during maternal separation, this effect lasting at least 8 hours (Moussaoui et al 2014; Rincel et al., 2017). Interestingly, gut permeability seems to be also durably increased after the end of chronic MS, since adolescent or adult animals with an history of MS exhibit gut leakiness (Gareau et al., 2007a, 2007b). In line with these studies, we report increased gut permeability in PND21 and PND49 rats, but also in juveniles (PND14) submitted to chronic MS. Importantly, the neonatal ML-7 treatment prevents MS-induced gut leakiness specifically at PND14, but its effect is no longer present several days after treatment cessation (i.e. PND21 or PND49). Our results indicate that ML-7 from PND2-14 prevented the long-term MS-induced sexual reward seeking impairment and neuroendocrine hyper-response to stress. This suggests that gut leakiness may affect the maturation of the HPA axis and brain circuits involved in reward processes. In contrast, anxiety and sucrose preference in MS rats were not modified by the neonatal ML7 treatment. Since the beneficial effect of ML-7 treatment on intestinal permeability was not maintained after the end of the maternal separation period, we can not exclude that long-lasting hyper-permeability also contributes to the behavioral alterations reported in MS animals at adulthood (increased anxiety and decreased sucrose preference). Further experiments testing the effects of ML-7 treatment at adulthood are required to address this question.

Our results extend previous data showing that MLCK inhibition in adult animals suppresses acute restraint stress-induced hypercorticosteronemia and elevated corticotropin-releasing factor (Crf) expression in the paraventricular nucleus of the hypothalamus (PVN) (Ait-Belgnaoui et al., 2012). More importantly, our study provides the first evidence that neonatal intestinal hyper-permeability *per se* may causally affect behavior and HPA axis function. A study by De Palma and colleagues recently demonstrated that MS has no effect on emotional behaviors in germ-free mice devoid of gut microbiota, but leads to increased anxiety and depressive-like behaviors upon colonization with the gut microbiota of a conventional control mouse (De Palma et al., 2015). However, colonization with the microbiota of a maternally separated animal did not transfer the MS-associated behavioral phenotype in naive germ-free mice. These findings suggest that gut bacteria are necessary but not sufficient to mediate the behavioral effects of MS. A hypothesis could be that the altered microbiota in MS mice is associated with stress-induced gut leakiness. Thus, animals showing gut dysbiosis without gut barrier defect display unspoiled behavior. Together with these previous findings, our results suggest that both gut dysbiosis and gut leakiness are required for the long-term behavioral effects of MS.

It is worth mentioning that MLCK inhibition did not prevent other MS-induced behavioral alterations such as increased anxiety and anhedonia for sucrose. The lack of significant effect of ML-7 on reduced sucrose preference in MS animals was surprising because both decreased interest for sexual rewards and decreased sucrose preference are considered as measures of anhedonia. A recent study conducted in rats exposed to early stress demonstrated that anhedonia (assessed using sucrose preference and social play) is associated with aberrant interaction of reward (ventral tegmental area, nucleus accumbens) and anxiety (infra-limbic medial prefrontal cortex, amygdala) circuits (Bolton et al., 2017). However, brain circuits involved in these behaviors may differ with respect to the nature of the reward.

It has been shown that stress-induced HPA axis hyper-reactivity and hyperanxiety can be prevented by antibiotics or normalized by probiotic treatments (Gareau et al., 2007a; Ait-Belgnaoui et al., 2012, 2014; Desbonnet et al., 2015; Liang et al., 2015; Leclercq et al., 2017; Moya-Pérez et al., 2017). Here, we report a protective effect of chronic ML-7 treatment in early-life on MS-associated gut dysbiosis both at the phylum and genus levels. Specifically, increased abundance of *Lachnospiraceae UCG-001 group, Clostridiales vadinBB660* and *Desulfovibrio* spp., but also decreased abundance of *Bacteroidales S24-7, Enterorhabdus* and *Bifidobacterium* spp*.* are normalized in animals treated with ML-7. Whether these effects participate in the prevention of sexual reward seeking impairment and deficit in HPA axis negative feedback remains to be determined. Increased *Desulfovibrio spp*. and decreased *Bifidobacterium spp*., have been reported in IBS patients and have been suggested as a

signature of disrupted intestinal barrier homeostasis (Crouzet et al., 2013). Moreover, *Bifidobacteria* strains are among the most used probiotics and have been reported to prevent the hypercorticosteronemia in response to acute stress in mice (Ait-Belgnaoui et al., 2014) and to reduce anxiety and depressive-like behaviors in different mouse models (Bercik et al., 2010, 2011; Savignac et al., 2014). For instance, *Bifidobacterium infantis* administered at adulthood was reported to exert antidepressant-like effects in early-life stressed animals (Desbonnet et al., 2010). Here, the normalization of *Bifidobacteria* at the genus level is associated with restored HPA axis function and sexual reward motivation but not anxiety or sucrose preference. Our results suggest that the behavioral phenotype likely results from a unique combination of microbial alterations rather than altered abundance of a single bacterium.

A limitation in our study is that the ML-7 treatment is not specific of intestinal MLCK. The MLCK is expressed in all epithelia including lungs and kidney, but also in endothelial cells of the blood-brain barrier (Beard et al., 2014). Nevertheless, neither histological nor *in vivo* measures of BBB permeability revealed a major impact of ML-7 on the BBB. The mechanisms underlying its protective effect on neuroendocrine stress response and sexual reward seeking remain to be further explored. Surprinsingly, we did not observe any beneficial effect of the ML-7 in juvenile MS rats, suggesting that ML-7 during development may impact the maturation of some physiological systems. In contrast to the numerous gut microbiota alterations found in adult offspring, we did not observe major effects of MS in PND14 juveniles. Some studies have reported differentially abundant taxa in maternally separated animals at PND21, including increased *Bacteroidales spp.,* suggesting that gut dysbiosis may appear before adulthood in our animals (El Aidy et al., 2017; Moya-Pérez et al., 2017). Whether the dysbiosis is established early or not, gut leakiness during the perinatal period could lead to immune activation and/or abnormal leakage of gut-derived molecules such as bacterial antigens, gut hormones, neurotransmitters or other metabolites. Although there was no obvious sign of neuroinflammation in PND14 pups, the elevated circulating cytokine levels reported here suggest that MS pups undergo peripheral immune activation that could impact the brain at a later age.

In conclusion, our work demonstrates for the first time that gut leakiness during the neonatal period may contribute to long-term HPA axis altered response to stress and behavioral impairments associated with early stress. Our study also suggests that the development of pharmacological or nutritional strategies to modulate intestinal permeability and/or gut microbiota may be relevant in combination with standard treatments, in particular in neurosychiatric conditions associated with early-life adversity.

FIGURE 1

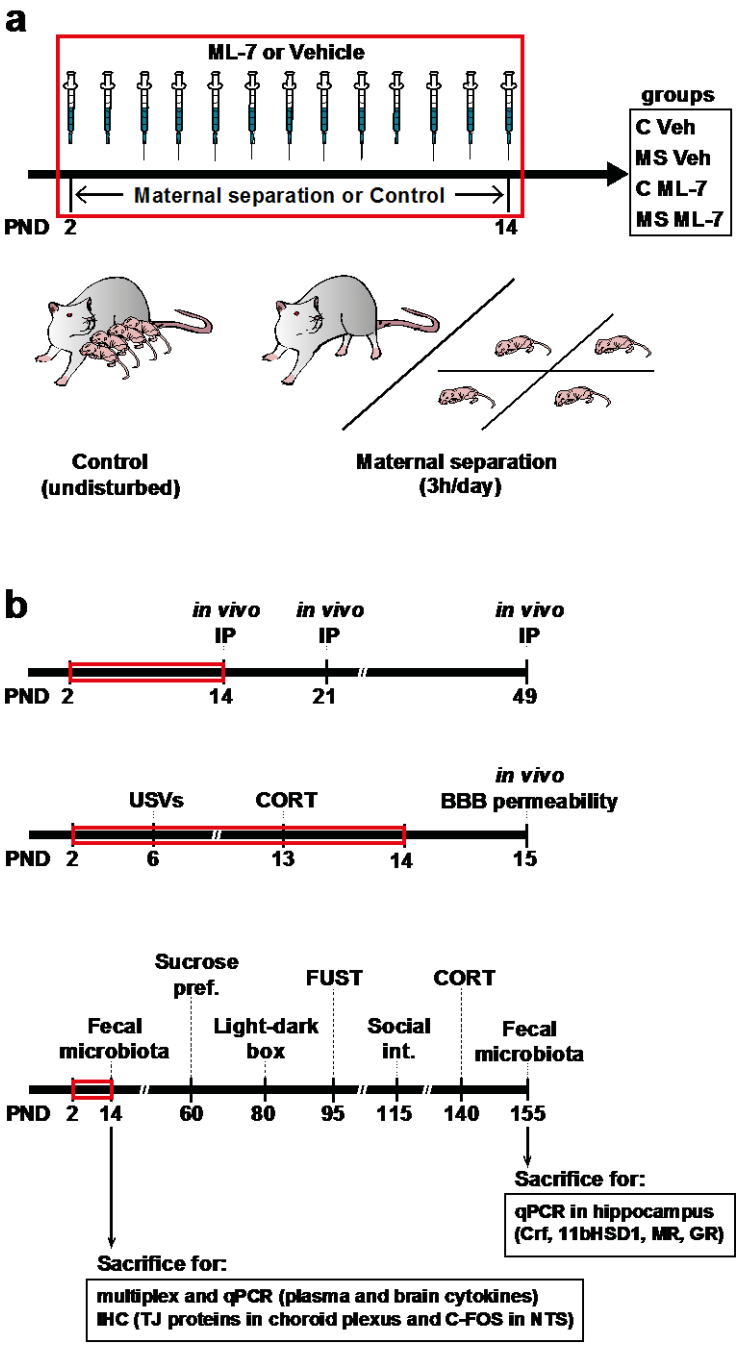

Fig 1. Experimental design. (a) Early manipulations were carried out daily from PND2-14. **(b)** MS consisted in 3h-separations of the entire litter with pups individually isolated, while controls remained undisturbed. Within each litter, 2 male pups from each litter received i.p. injections of ML-7 and 2 others received vehicle. Injections were performed immediately prior each separation session. **(c)** 3 cohorts were used in this study. Yellow frames represent the early manipulations. Behavior and gut microbiota assessment was carried out in the 3rd cohort (a maximum of two pups per litter was used to prevent litter effect). At PND21, male offspring were weaned and housed 6 per cage until PND40 and then 3 per cage (except during sucrose preference test) until the end of the experiment. 9 females from 7 different litters were kept for the female urine sniffing test. PND, post-natal day; Veh, vehicle; IP, intestinal permeability; USVs, ultrasonic vocalizations; CORT, plasma corticosterone; BBB, blood-brain barrier; FUST, female urine sniffing test; IHC, immunohistochemistry; TJ, tight-junction; NTS, nucleus tractus solitarius.

FIGURE 2

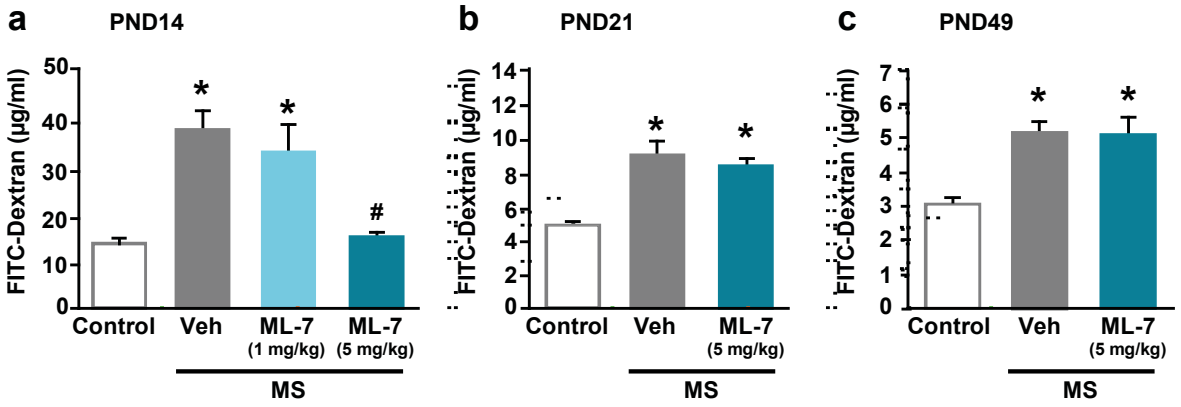

Fig 2. Chronic MLCK inhibition during early-life prevents MS-induced gut leakiness in PND14 pups. *In vivo* intestinal permeability to FITC-Dextran (µg/ml plasma) at PND14 **(a),** PND21 **(b)** and PND49 **(c).** MS increases gut permeability at all time points. At a dose of 5 mg/kg, ML-7 treatment prevents this effect specifically in PND14 rats. N=8 per group. Data are mean ± SEM. Student *t*-tests : **p*<0.05 *versus* Controls, # *p*<0.05 *versus* MS Veh.

FIGURE 3

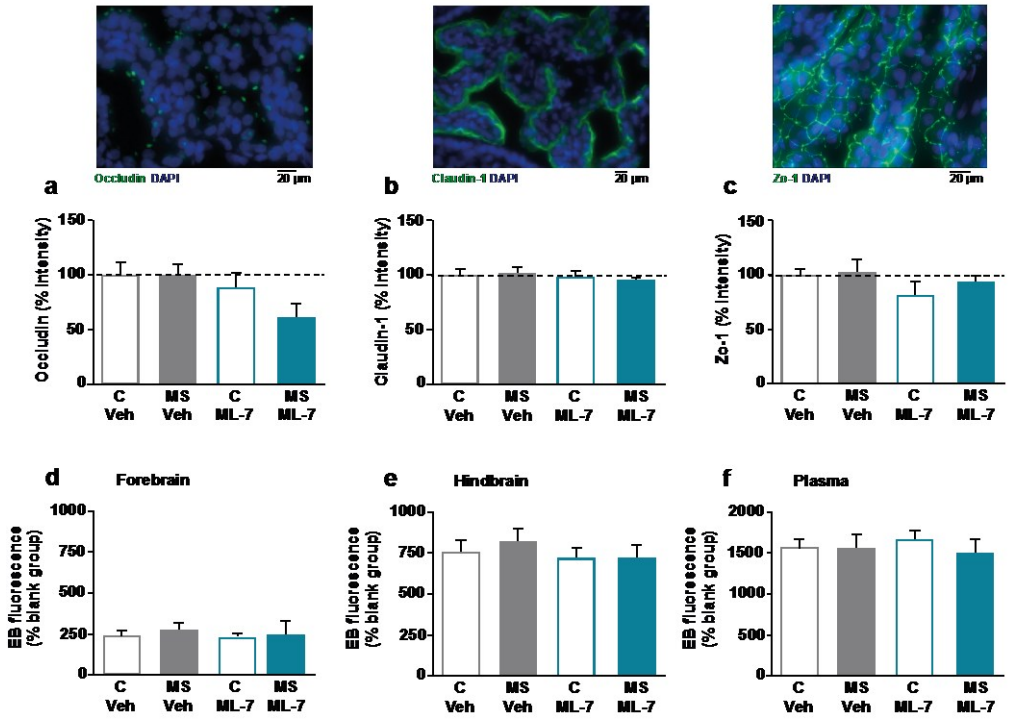

Fig 3. Effects of MS and ML-7 treatment on the BBB in juvenile rats. Relative quantification of occludin (N=4-6 per group) **(a)**, claudin-1 (N=5 per group) **(b)** and zo-1 (N=3-7 per group) **(c)** tight junction protein expression (% fluorescence intensity normalized to C Veh) in the choroid plexus of PND14 rats. *In vivo* BBB permeability to EB (% EB fluorescence) in the forebrain **(d)**, hindbrain **(e)** and plasma **(f)** of PND15 rats (N=5-9 per group). MS or ML-7 had no major impact on BBB integrity or permeability. Data are mean ± SEM.

FIGURE 4

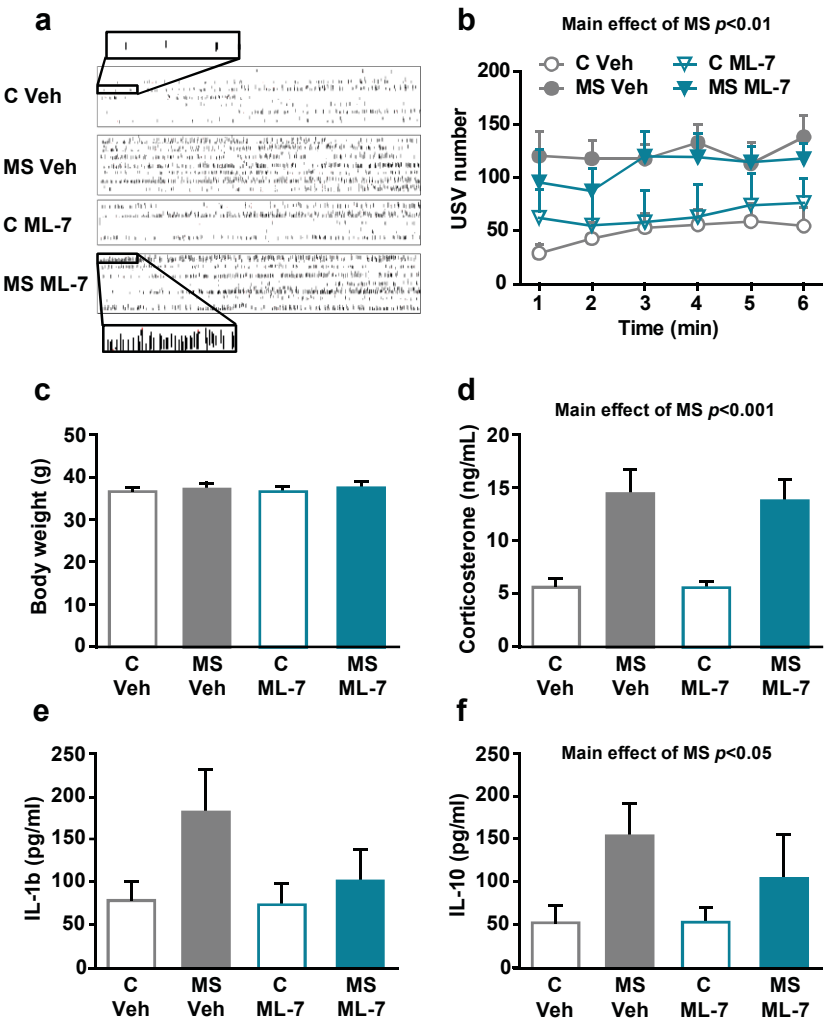

Fig 4. Effects of chronic MLCK inhibition during early-life on MS-induced endophenotypes in juvenile rats. (a) and **(b)** Ultrasonic vocalizations in response to a 6-min separation on PND6. In (a) each line corresponds to one animal and each dash represents one USV emission (N=5-7 per group). **(c)** Pups' body weight (g) at PND14 (N=13-15 per group). **(d)** Plasma corticosterone (ng/mL) following the 3hr-MS episode on PND13 (N=7-9 per group). **(e)** and **(f)** Plasma levels (pg/mL) of IL-1band IL-10 following the 3hr-MS episode on PND14 (N=10 per group). Neither MS or ML-7 affects pups' body weight. However, MS increases plasma corticosterone and cytokine levels as well as USVs. These effects are not affected by ML-7. Data are mean ± SEM.

FIGURE 5

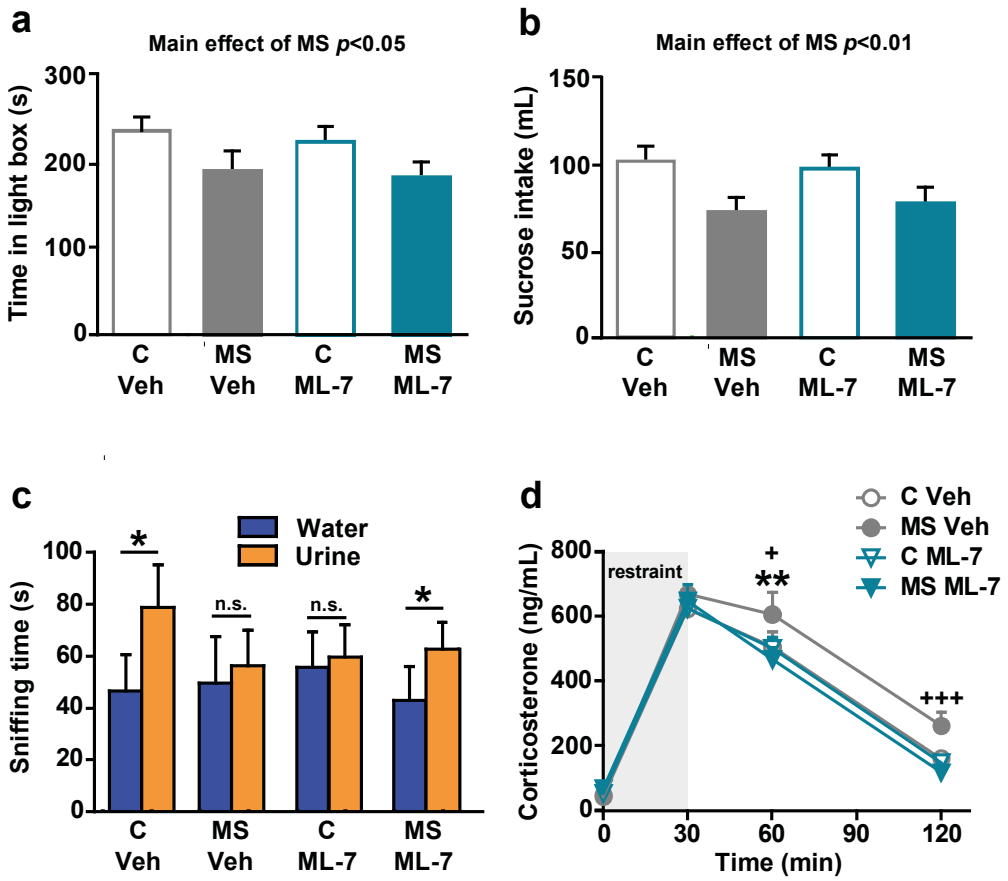

Fig 5. Chronic MLCK inhibition during early-life prevents MS-induced impairment in sexual reward seeking and HPA axis hyper-responsiveness to stress at adulthood. (a) Time spent in the light box (s) over 10 min (N=12 per group). **(b)** Sucrose intake (mL) in the 24hr sucrose preference test (N=12 per group). **(c)** Time (s) spent sniffing a Q-tip soaked with water or estrus female urine over 3 min (N=3-5 per group). MS induced hyperanxiety and loss of preference for sucrose or female urine *versus* water. ML-7 treatment prevented the MS-induced altered behavior in the female urine sniffing test. Plasma corticosterone (ng/mL) time course response to 30min of restraint stress **(c)** and area under the curve (AUC) (N= 12 per group) **(d)**. MS led to sustained corticosterone response after restraint stress. This effect was abolished by ML-7 treatment. In **c**: Paired *t*-tests, **p*<0.05; in **d**: Fisher LSD's *post-hoc*, ***p*<0.01 *versus* C Veh; +*p*<0.05, +++*p*<0.001 *versus* MS Veh. Data are mean ± SEM.

FIGURE 6

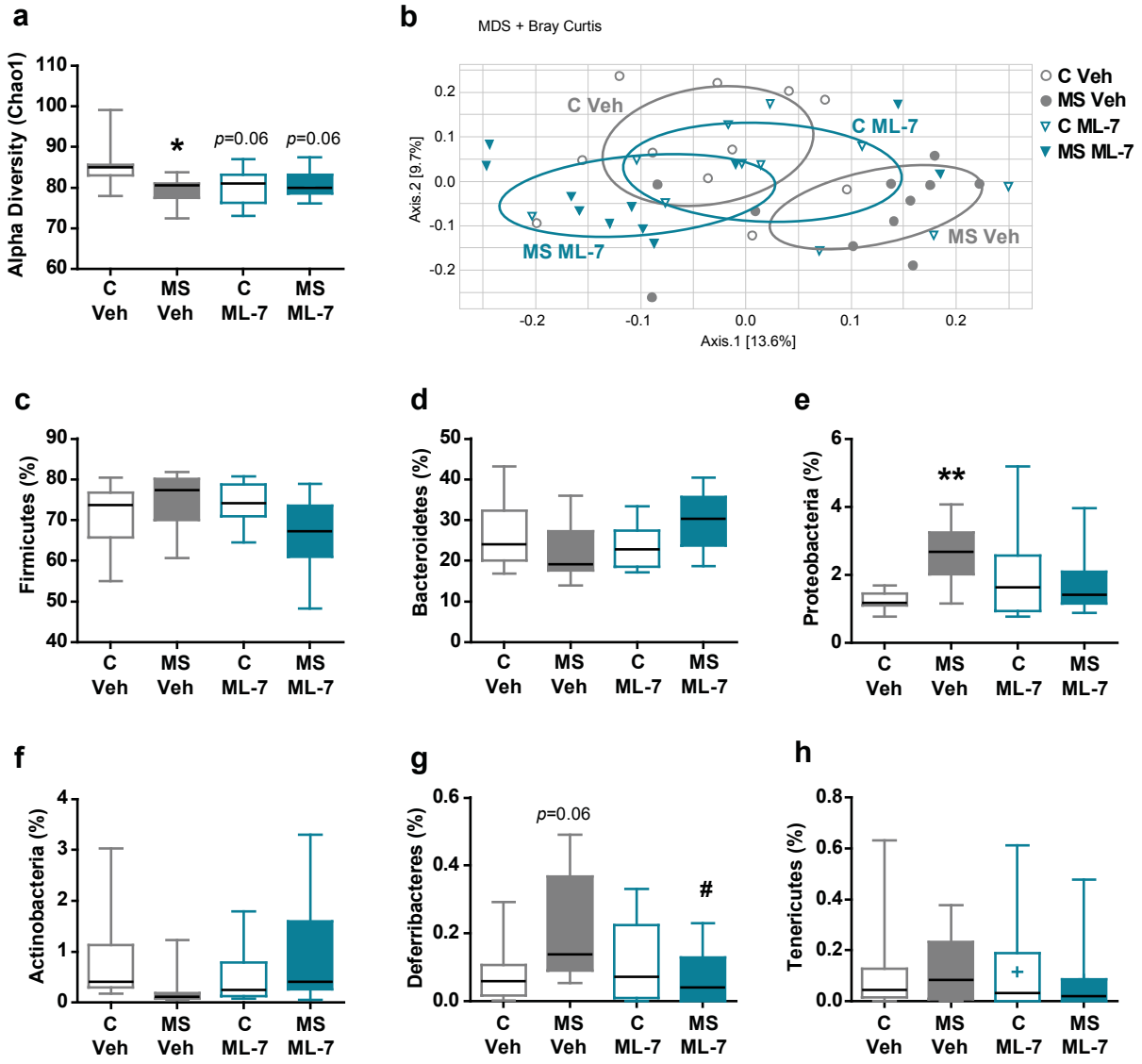

Fig 6. Impact of MS and ML-7 treatment during early life on composition and structure of fecal microbiota at adulthood. (a) Richness (Chao-1 indice of alpha diversity). **(b)** Bray-Curtis Multidimentional scaling (MDS) plots representing structural changes of microbiota composition between groups. **(c-h)** Relative abundance (%) per phylum. N=11 per group. Bars in boxplots represent medians. Two-way ANOVA with Holm-Sidak's post-tests: **p*<0.05 and ***p*<0.01 *versus* C Veh (other *p* values in **a** and **g** are also comparisons with C Veh); **#***p*<0.05 *versus* MS Veh.

FIGURE 7

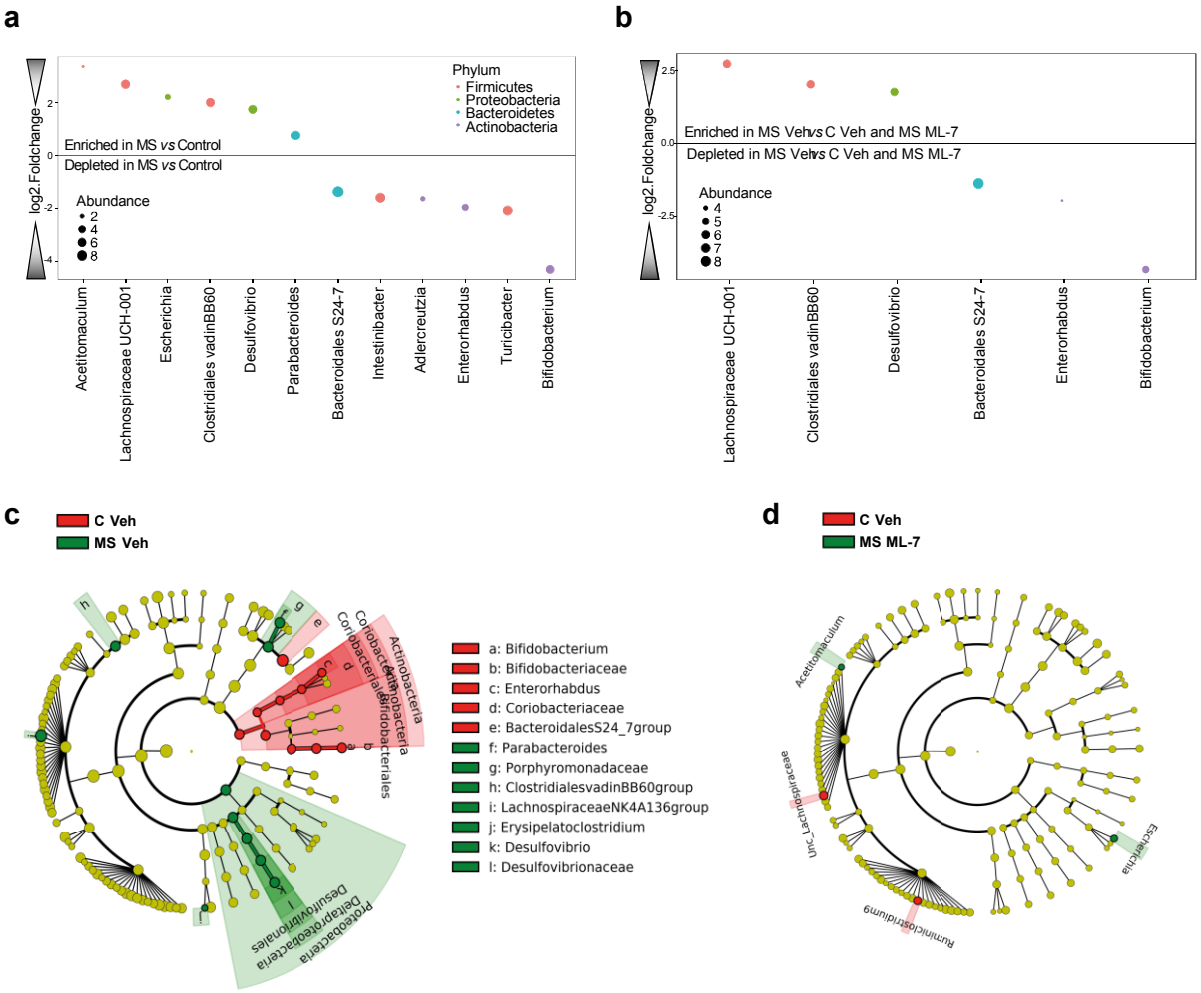

Fig 7. Impact of MS and ML-7 treatment during early-life on the abundance of fecal bacterial genera at adulthood. (a-b) Genera with differential abundance significantly affected by early-life stress: **(a)** early-life stress factor being the main effect and **(b)** normalized by ML-7 treatment (for which interaction term of the two-factor design was significant). Genera are classified by magnitude of effect. Phylum is indicated using color codes and circle size represents the mean normalized abundance of the genus across samples. Features were considered significant if their adjusted post-test *p*-value was less than 0.05 using a two-factor nested design. **(c-d)** Representative circular cladogram generated from LEfSe analysis showing the most differentially abundant taxa in MS Veh **(c)** or MS ML-7 **(d)** compared with C Veh. Taxa enriched in C Veh are shown in red whereas taxa enriched in MS Veh or MS ML-7 are shown in green. Only taxa with LDA scores higher than 3 and for which $p<0.01$ (Kruskal-Wallis test) are displayed. N=11 per group.

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SUPPLEMENTARY INFORMATION

SUPPLEMENTARY METHODS

C-Fos immunochemistry. Serial coronal sections (40µm) containing the nucleus tractus solitarius (NTS, anteroposteriority from bregma: -11.40 to -15.72) were obtained using a cryostat and stored in cryoprotectant solution (composition,) at -20°C until use. Free-floating sections (40µm, every 6th) were treated as previously described (Labrousse et al., 2009) (Labrousse et al., 2009). Briefly, Briefly, rabbit polyclonal antiserum raised against c-Fos (Santa Cruz Biotechnology, Santa Cruz, CA) was diluted 1:1000 in Tris Buffer Saline (TBS) containing 0.3% Triton X-100, 2% donkey serum and 1% bovine serum albumin (BSA), and sections were incubated overnight at room temperature before being incubated for 2 h with biotinylated goat anti-rabbit antibody (1:2000; Jackson 152, Jackson ImmunoResearch, Suffolk, UK) for 2 h with avidin-biotin peroxidase complex (1:1000; Vectastain ABC kit, Vector laboratories, Burlingame, CA), and finally revealed with diaminobenzidine via the nickel-enhanced glucose oxidase method. Sections were then slide-mounted onto gelatin-coated slides.

Quantification of C-Fos immunoreactivity. NDPI images at x20 magnification were obtained at the Bordeaux Imaging Center (CNRS-INSERM and Bordeaux University, France BioImaging) using a digital slide scanner (Nanozoomer, Hamamatsu Photonics, Massy, France) and converted into TIFF format using the ImageJ software (http://imagej.nih.gov.gate2.inist.fr/ij/) and NDPItools plugin (Deroulers et al., 2013). Regions of interest (ROI) were manually circumscribed using ROItools according to (Paxinos et al., 1985). The number of c-Fos-IR cells was automatically quantified in 8-bit thresholded images using the particle analysis function (size: 5-20 µm²; circularity: 0.5-1). The quantification of C-FOS-IR cells was carried out in 3-5 sections per animal for each area using ImageJ. Results are expressed as C-FOS-IR cells per mm². The experimenter remained blind to the treatment conditions throughout the analysis.

EB dose response. Rats pups (PND15-20) received i.p. injections of EB (80, 160 or 240 mg/kg, i.p.) and EB fluorescence in the plasma, hindbrain and forebrain was determined 24h later (see paragraph x in the method section). EB concentration in the samples was calculated according to a standard range made in 50% TCA.

Social interaction (3.5-4 months). Time spent in social interaction was evaluated using 3 different paradigms (conspecific, aggressor and juvenile) over 10 min, under dim light (30 lux) in a neutral arena (40×40 cm). For the conspecific test, pairs of weight-matched rats from the same experimental group were placed together in the arena. For the aggressor test, old breeder male Wistar rats (n=12, Janvier Labs, Le Genest Saint Isle,France) were isolated

for 2 weeks and placed first in the arena. Finally, for the juvenile test, 6 week-old Wistar rats (n=12, Janvier Labs, Le Genest Saint Isle, France) were placed first in the arena. Each aggressor and juvenile rat encountered a rat from the 4 experimental groups. Social behavior (sniffing, allogrooming and crawling over) was recorded using a digital camera and manually scored using an ethological software (The observer, Noldus Information Technology, Wageningen, The Netherlands) as previously described (Rincel et al., 2016).

Real-time quantitative PCR. PND14 pups (n=112 per group) or 5 month-old rats were deeply anesthetized with isoflurane and killed by decapitation. Whole brains were collected and the medial prefrontal cortex (mPFC, PND14) and hippocampus (PND14 and adults) were dissected and stored at -80°C until use. Total RNA was extracted using TRIzol (Invitrogen, Life Technologies, Carlsbad, CA, USA). RNA concentration and purity were determined using a ND-1000 spectrophotometer (Nanodrop Technologies, Wilmington, DE, USA). cDNA was synthetized from 1 µg of RNA using Superscript III reverse transcriptase (Invitrogen, Life Technologies) as previously described (Labrousse et al., 2012). Real time quantitative PCR was performed from 20 ng/ml cDNA samples using SYBR assays and the LightCycler 480 system (LC480, Roche diagnostics, Mannheim, Germany). All primers used were validated for selectivity and amplification efficiency (primer sequences are provided in **Supplementary Table 1**). All samples were performed in duplicates. Expression of target genes was calculated by the delta-delta Ct method and normalized to the housekeeping gene beta-2 microglobulin (β2m). All gene expression results are expressed relative to expression in the control group (folchange).

16S rRNA amplification and amplicon sequencing. The V3-V4 hypervariable region of the 16S rRNA gene was amplified by PCR. The forward PCR primer 5'CTTTCC CTA CAC GAC GCT CTT CCG ATC TAC GGR AGG CAG CAG3' was a 43-nuclotide fusion primer consisting of the 28-nt illumina adapter (designed by bold font) and the 14-nt broad range bacterial primer 343F. The reverse PCR primer 5'GGA GTT CAG ACG TGT GCT CTT CCG ATC TTA CCA GGG TAT CTA ATC CT3' was a 47-nuclotide fusion primer consisting of the 28-nt illumina adapter (designed by bold font) and the 19-nt broad range bacterial primer 784R. The PCR mix contained MTP Taq DNA polymerase (SIGMA, 0,05 U/µl), 200 µM of each DNTP (SIGMA, premix), and 0,5 µM of each primer. After initial denaturation at 94°C for 60 sec in CFX-96 Thermal Cycler (Bio-Rad), 30 cycles were run with 60 sec denaturation at 94°C, 60 sec annealing at 65°C and 60 sec at 72°C.Round ended with 10 min extension at 72°C. Amplification quality (length, quantity and specificity) was verified using the Agilent 2200 Tapestation system (High sensitivity D1000 ScreenTape Assay) and AATI Fragment Analyser at the GeT (Genomic and Transcriptomic, TRIX and PlaGe) platforms in Toulouse. Because MiSeq enables paired 250-bp reads, the ends of each read are overlapped and can

be stitched together to generate extremely high-quality, full-length reads of the entire V3 and V4 region in a single run. Single multiplexing was performed using home made 6 bp index, which were added to R784 during a second PCR with 12 cycles using forward primer (AAT GAT ACG GCG ACC ACC GAG ATC TAC ACT CTT TCC CTA CAC GAC) and reverse primer (CAA GCA GAA GAC GGC ATA CGA GAT-index-GTGACTGGAGTTCAGACGTGT). The resulting PCR products were purified and loaded onto the Illumina MiSeq cartridge according to the manufacturer instructions. The quality of the run was checked internally using PhiX, and then each pair-end sequences were assigned to its sample with the help of the previously integrated index. Each pair-end sequences were assembled using Flash sofware (Magoč and Salzberg, 2011) using at least a 10bp-overlap between the forward and reverse sequences, allowing 10% of mismatch. The lack of contamination was checked with a negative control during the PCR (water as template). The quality of the stitching procedure was controlled using 4 bacterial samples that are run routinely in the sequencing facility in parallel to the current samples.

Microbiome 16S data analysis. High quality filtered reads (6,820,034 reads) were further processed using FROGS pipeline to obtain OTUs and their respective taxonomic assignment thanks to Galaxy instance (http://sigenae-worbench.toulouse.inra.fr): Initial FROGS pre-process step allowed to select overlapped reads with expected length without N, yielding to 5,125,859 pass-filter reads (an average of 58,250 reads per sample). Swarm clustering method was applied by using a first run for denoising with a distance of 1 and then a second run for clustering with an aggregation maximal distance of 3 on the seeds of first Swarm (Mahé et al., 2014), yielding to 772,290 clusters (an average of 8,700 per sample at PND14 and 13,250 at PND155). Putative chimera were removed using vsearch combined to cross-validation (GitHub repository. Doi 10.5281/zenedo.15524), yielding to 487,412 clusters (an average of 6,510 per sample at PND14 and 5,980 at PND155).

Cluster abundances were filtered at 0,005% (Bokulich et al., 2013)and/or had to be present at least in 3 samples, yielding to 644 clusters (an average of 224 clusters per sample at PND 14 and 453 at PND 155) corresponding to 3,772,253 final valid reads (an average of 42,866 valid reads per sample whatever the age of rats).

100% of clusters were affiliated to OTU by using a silva123 16S reference database and a taxonomic multi-affiliation procedure (Blast+ with equal multi-hits (Camacho et al., 2009)). Since rarefaction has shown to result in high rates of false positive tests for differential abundance(McMurdie and Holmes, 2014), counts were not rarefied. OTU prevalence, abundance per phylum and rarefaction curves were plotted for each sample by using Phyloseq package (v 1.19.1) after a supplementary recommended prevalence filtering

(threshold prevalence as 5% of total sample, leading finally to 643 analysed OTUs (535 OTUs and 614 OTUs relative to PND14 and PND155 analysis respectively) accordingly to the Bioconductor workflow for microbiome data analysis proposed by Callahan et al., 2016.
Within sample community alpha diversity was assessed by Chao-1 diversity. Divergence in community composition between samples was quantitatively assessed by calculating weighted Unifrac (abundance and phylogenetic relation) distance matrice. Unconstrained ordination was visualized using multidimensional scaling (MDS) and hierarchical clustering (complete linkage combined with wUnifrac distance) and compared using Adonis test (9999 permutations).
In order to evaluate differential abundance in response to postnatal treatments and identify important taxa modulated by maternal separation and associated with ML-7administration, OTUs were agglomerated at the genus rank. Differentially abundant taxa were identified by characterizing the difference between two different postnatal treatments (Kruskall-Wallis non parametric pairwise comparisons) using LefSe algorithm (Segata et al., 2011)with an alpha value of 0.01 and a threshold on the logarithmic LDA score for discriminative features of 3. Univariate differential abundance of taxa was also tested using a negative binomial noise model for over dispersion as implemented in the R package DESeq2 (v 1.14.1)(Love et al., 2014; McMurdie and Holmes, 2014).A 2x2 factor design combined with a Wald test was applied in order to identify taxa for which maternal separation effect changed acrossML-7 exposition (interaction term). On the final taxa corresponding to the interaction term, 6 taxa were selected at PND155 because administration of ML-7 normalized their relative abundance in maternally separated rats. Taxa were considered significantly differentially abundant between treatments if their adjusted *p*-value was below 0.05.

SUPPLEMENTARY TABLES

Supplementary Table 1. Primer sequences used for qPCR

Gene name	Sense primer	Antisense primer
B2m	5'-CGTGCTTGCCATTCAGAAAA-3'	5'-GAAGTTGGGCTTCCCATTCTC-3'
Crh	5'-CAGCCGTTGAATTTCTTG-3'	5'-GACTTCTGTTGAGGTTCC-3' -3'
GR (Nr3c1)	5'-AACGCCCTTATAAATGTGAACTG -3'	5'-GGCTTCTCACCCAACTAGATCA C-3'
IL-1b	5'-CCGAAAGTTCCTCTTGACCTTTAG-3'	5'-GCACCAAATCGGTCATCCA-3'
IL-6	5'-GCATCTGCCGCTTGTTGAA-3'	5'-TCCTCGGAGATGCTGTCATG-3'
MR (Nr3c2)	5'- CTTTACGAAGTGTTTC TACTACTG-3'	5'- TGACACCCAGAAGCCTC ATCT-3'
TNFa	5'-GCGTTGTTGGGTGCCATAAT-3'	5'-CCGGATTGAGCAGGGAGTT-3'
11bHSD1	5'-GCGGTTTGTGAAATGGAAGT-3'	5'-CAGGGTCCACTCTTGGGTTA-3'

B2m, Beta 2 microglobulin; Crh, Corticotropin releasing hormone; GR, Glucocorticoid receptor; IL-1b, Interleukin 1-beta; IL-6, Interleukin 6; MR, Mineralocorticoid receptor; TNFa, Tumor necrosis factor alpha; 11bHSD1; 11 beta hydroxysteroid dehydrogenase 1; Nr3c, Nuclear receptor subfamily 3, group C.

Supplementary Table 2: Detailed statistics and taxonomic affiliation of clusters (agglomerated at the genus rank) significantly affected by early-life stress in adult animals.

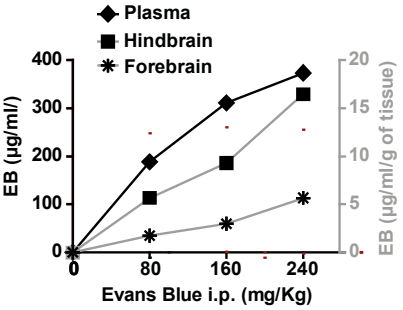
Phylum	Class	Order	Family	Genus	baseMean	log2FoldChange	lfcSE	stat	pvalue	padj
Actinobacteria	Actinobacteria	Bifidobacteriales	Bifidobacteriaceae	Bifidobacterium	193,25	-4,31	1,34	3,23	0,0012	0,0183
Actinobacteria	Coriobacteriia	Coriobacteriales	Coriobacteriaceae	Adlercreutzia	10,54	-1,64	0,59	-2,77	0,006	0,049
Actinobacteria	Coriobacteriia	Coriobacteriales	Coriobacteriaceae	Enterorhabdus	35,38	-1,97	0,54	3,64	0,0003	0,0062
Bacteroidetes	Bacteroidia	Bacteroidales	Bacteroidales S24-7 group		5189,43	-1,38	0,40	3,47	0,0005	0,0095
Bacteroidetes	Bacteroidia	Bacteroidales	Porphyromonadaceae	Parabacteroides	552,50	0,77	0,26	2,97	0,003	0,036
Firmicutes	Clostridia	Clostridiales	Clostridiales Vadin BB60 group		361,98	2,01	0,55	-3,66	0,0003	0,0062
Firmicutes	Clostridia	Clostridiales	Lachnospiraceae	Acetitomaculum	5,30	3,39	1,01	3,36	0,0008	0,025
Firmicutes	Clostridia	Clostridiales	Lachnospiraceae	Lachnospiraceae UCG-001	391,05	2,71	0,67	-4,04	0,0001	0,0024
Firmicutes	Clostridia	Clostridiales	Peptostreptococcaceae	Intestinibacter	1045,29	-1,60	0,48	-3,32	0,0009	0,019
Firmicutes	Erysipelotrichia	Erysipelotrichales	Erysipelotrichaceae	Turicibacter	829,35	-2,08	0,60	-3,45	0,0006	0,019
Proteobacteria	Deltaproteobacteria	Desulfovibrionales	Desulfovibrionaceae	Desulfovibrio	334,19	1,76	0,55	-3,20	0,0014	0,0183
Proteobacteria	Gammaproteobacteria	Enterobacteriales	Enterobacteriaceae	Escherichia-Shigella	14,66	2,23	0,67	3,35	0,0008	0,025

Supplementary Table 3: Detailed statistics and taxonomic affiliation of clusters (agglomerated at the genus rank) significantly affected at adulthood by early-life stress but for which ML-7 treatment prevented MS-induced alterations (Interaction term reach significance).

Phylum	Class	Order	Family	Genus	baseMean	log2FoldChange	lfcSE	stat	pvalue	padj
Actinobacteria	Actinobacteria	Bifidobacteriales	Bifidobacteriaceae	Bifidobacterium	193,25	-4,31	1,34	3,23	0,0012	0,0183
Actinobacteria	Coriobacteriia	Coriobacteriales	Coriobacteriaceae	Enterorhabdus	35,38	-1,97	0,54	3,64	0,0003	0,0062
Bacteroidetes	Bacteroidia	Bacteroidales	Bacteroidales S24-7 group		5189,43	-1,38	0,40	3,47	0,0005	0,0095
Firmicutes	Clostridia	Clostridiales	Clostridiales Vadin BB60 group		361,98	2,01	0,55	-3,66	0,0003	0,0062
Firmicutes	Clostridia	Clostridiales	Lachnospiraceae	Lachnospiraceae UCG-001	391,05	2,71	0,67	-4,04	0,0001	0,0024
Proteobacteria	Deltaproteobacteria	Desulfovibrionales	Desulfovibrionaceae	Desulfovibrio	334,19	1,76	0,55	-3,20	0,0014	0,0183

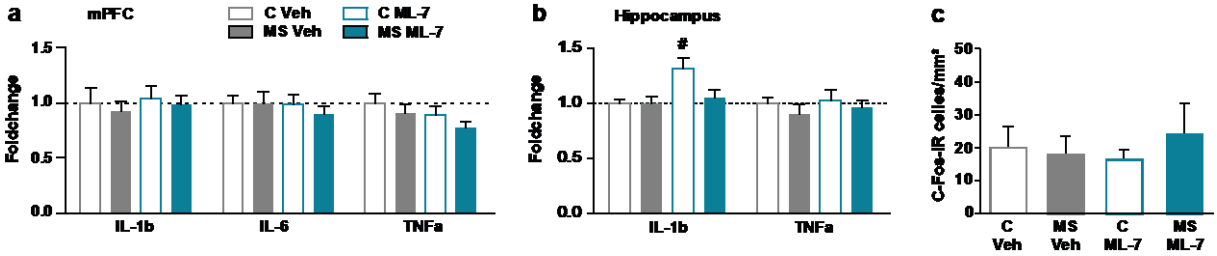
SUPPLEMENTARY FIGURES

SUPPLEMENTARY FIGURE 1



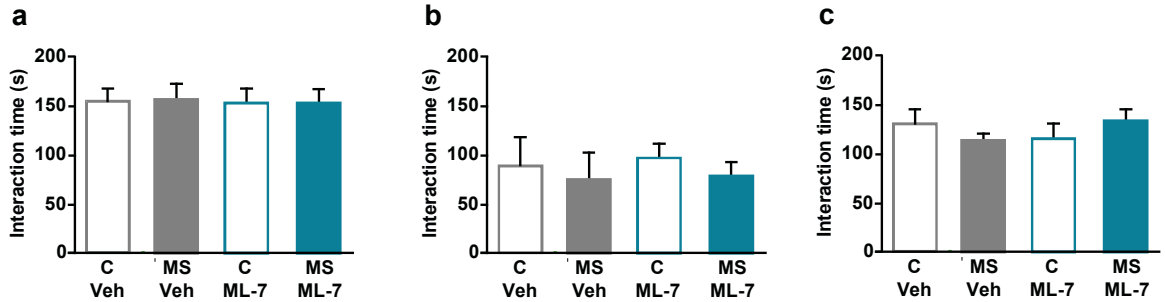
Supplementary Fig 1. EB dose-response curve after EB i.p. injection in PND15 pups. EB concentration in the plasma (µg/mL, left axis) and hindbrain/forebrain (µg/mL/g of tissue, right axis) 24 hr after EB i.p. injection.

SUPPLEMENTARY FIGURE 2



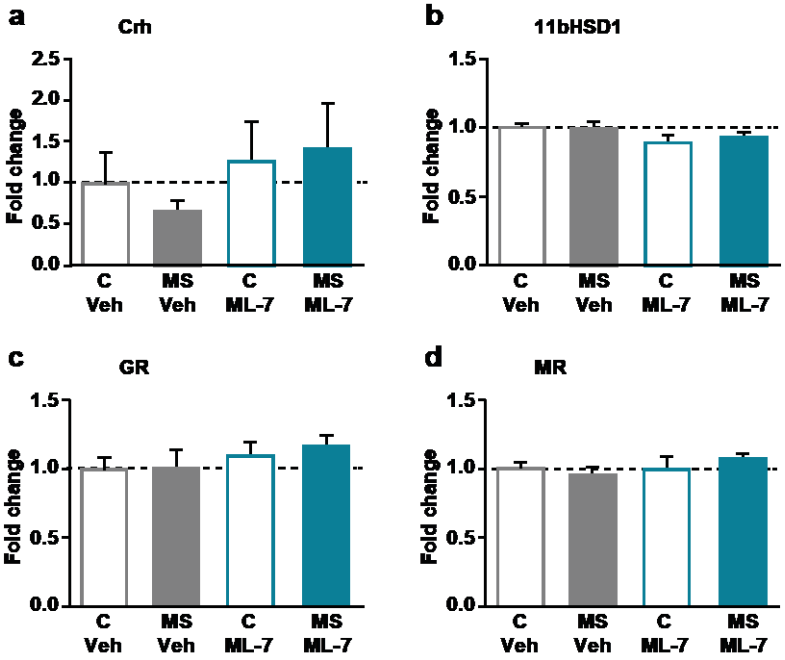
Supplementary Fig 2.Effects of MS and ML-7 treatment on brain cytokines expression and neuronal activation in the NTS of PND14 rats following 3hr of separation. mRNA expression of IL-1b, IL-6 and TNFa (foldchange) in the mPFC **(a)** and hippocampus **(b)** (N=7-9 per group). **(c)** Neuronal activation (C-Fos-IR cells/mm²) in the NTS (N=4-5 per group). MS or ML-7 had no major impact on brain cytokines expression or neuronal activation in the NTS following 3hr of MS. Data are mean ± SEM. Fisher LSD's *post-hoc*, # at least $p<0.05$ *versus* all other groups. IL-1b, Interleukin 1 beta; IL-6, Interleukin 6; TNFa, Tumor necrosis factor alpha.

SUPPLEMENTARY FIGURE 3



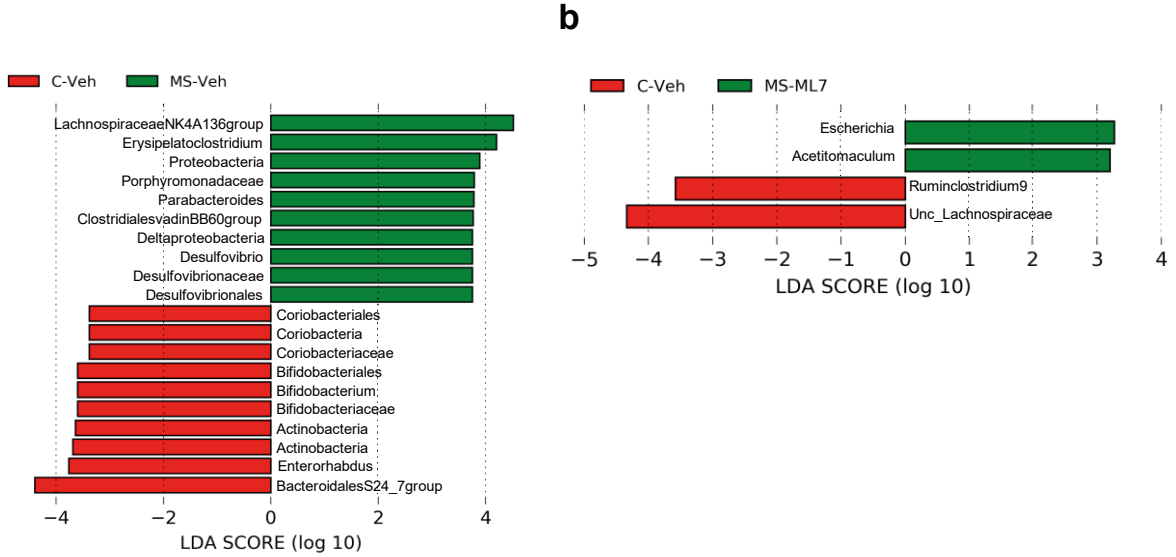
Supplementary Fig 3. Impact of MS and ML-7 treatment on social behavior in adult rats. Time spent in social interaction (s) with a male conspecific **(a)**, aggressor **(b),** or juvenile **(c)** over 10 min. Social interaction was not affected by the perinatal manipulations. N=10-11 per group. Data are mean ± SEM.

SUPPLEMENTARY FIGURE 4



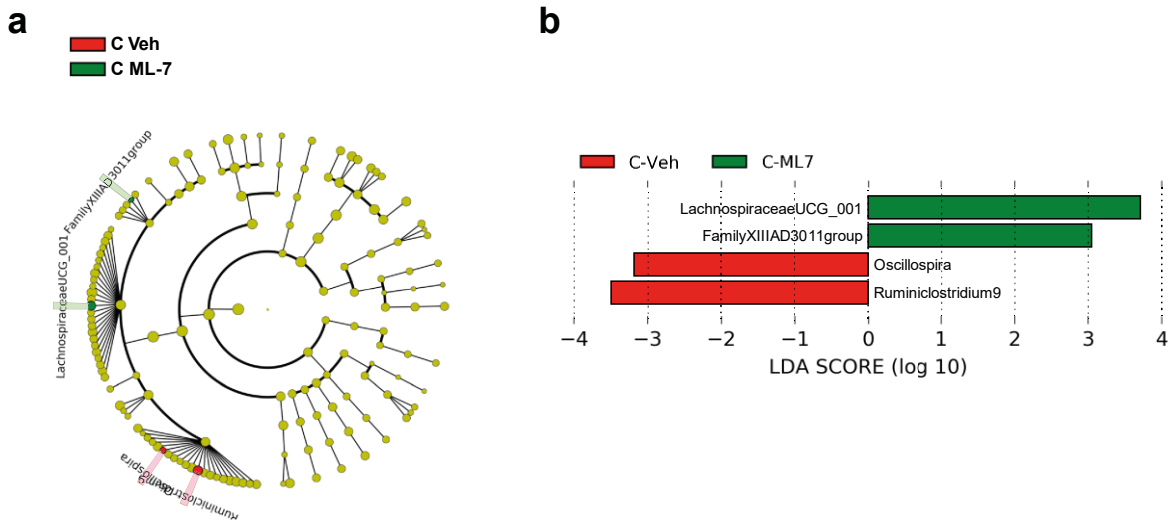
Supplementary Fig 4. Impact of MS and ML-7 treatment on hippocampal mRNA expression of stress-related genes in adult rats. Foldchange mRNA expression of Crh **(a),** 11bHSD1 **(b),** GR **(c)** and MR **(d).** N=8 per group. Data are mean ± SEM. Planned comparisons,Crh, Corticotropin-releasing hormone; 11bSHD1, 11β-Hydroxysteroid dehydrogenase type 1; GR, Glucocorticoid receptor; MR, Mineralocorticoid receptor.

SUPPLEMENTARY FIGURE 5



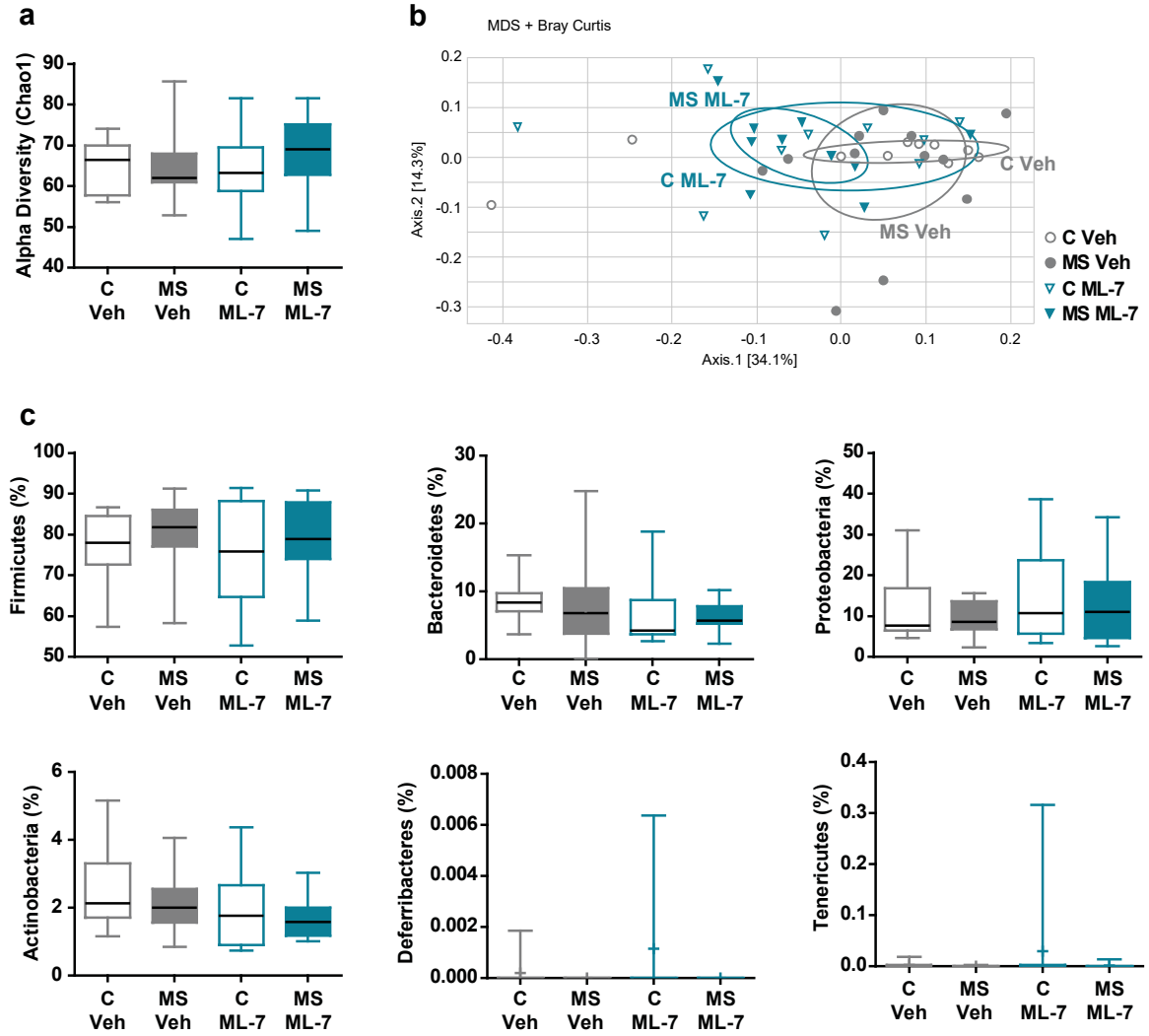
Supplementary Fig 5. Impact of the ML-7 treatment in non-stressed animals on the abundance of fecal bacterial genera at adulthood. Histograms generated from LEfSe analysis showing the most differentially abundant taxa in MS Veh **(a)** or in MS ML-7 **(b)** compared with C Veh rats. Taxa enriched in C Veh are shown in red whereas taxa enriched in MS Veh or MS ML-7 are shown in green. The magnitude of the LEfSe scores indicates the degree of consistency of the difference. Only taxa with LDA scores higher than 3 and for which $p<0.01$ (Kruskal-Wallis test) are displayed. N=11 per group.

SUPPLEMENTARY FIGURE 6



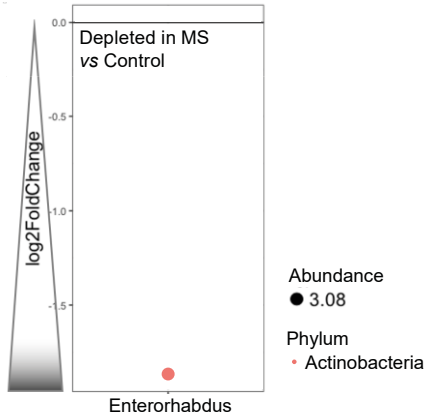
Supplementary Fig 6. Impact of the ML-7 treatment in non-stressed animals on the abundance of fecal bacterial genera at adulthood. (a) Representative circular cladogram and **(b)** histograms generated from LEfSe analysis showing the most differentially abundant taxa in C ML-7 compared with C Veh. Taxa enriched in C Veh are shown in red whereas taxa enriched in C ML-7 are shown in green. The magnitude of the LEfSe scores indicates the degree of consistency of the difference. Only taxa with LDA scores higher than 3 and for which $p<0.01$ (Kruskal-Wallis test) are displayed. N=11 per group.

SUPPLEMENTARY FIGURE 7



Supplementary Fig 7. Impact of MS and ML-7 treatment on the composition and structure of fecal microbiota in PND14 juveniles. (a) Richness (Chao-1 indice of alpha diversity). **(b)** Bray-Curtis Multidimentional scaling (MDS) plots representing structural changes of microbiota composition between groups. **(c)** Relative abundance (%) per phylum. Bars in boxplots represent medians. N=10-12 per group.

SUPPLEMENTARY FIGURE 8



Supplementary Fig 8. Impact of MS on the abundance of fecal bacterial genera in PND14 juveniles. Enterorhabdus was the only genus with differential abundance significantly affected by early-life stress. This effect is independent of the ML-7 treatment. Phylum is indicated using color codes and circle size represents the mean normalized abundance of the genus across samples. Features were considered significant if their adjusted post-test *p*-value was less than 0.05 using a two-factor nested design. N=10-12 per group.

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ARTICLE 3

Genetically-driven gut leakiness impairs emotional behavior, neuroendocrine response to stress and brain expression of stress-related genes in a sex-dependent manner

In preparation

ARTICLE 3 in preparation:

Genetically-driven gut leakiness impairs emotional behavior, neuroendocrine response to stress and brain expression of stress-related genes in a sex-dependent manner

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ABSTRACT

Substantial evidence points to a major role of the gut-brain-axis in the regulation of behavioral processes and stress response. In particular, gut dysbiosis has been associated with altered anxiety and depressive-like behaviors and microbiota-directed interventions (fecal transplantation, probiotics) normalize these behaviors. Critically, gut dysbiosis is often concomitant with gut permeability leakiness, but the primary contribution of gut barrier defects in the development of behavioral alterations remains unexplored. Intestinal epithelial tight junction permeability is regulated by the myosin light chain kinase (MLCK). Here, we used transgenic mice with tissue-specific expression of a constitutively active MLCK (CA-MLCK mice) within intestinal epithelia to explore the effects of gut leakiness *per se* on emotional behaviors and stress response. Male and female CA-MLCK transgenic mice exhibited similar increases in intestinal permeability and displayed exacerbated corticosterone response to stress and marked alterations in the expression of several stress-related genes in the medial prefrontal cortex, hippocampus and nucleus accumbens. Immunomapping of C-FOS expression within the brain indicates altered stress-induced connectivity in CA-MLCK mice. In addition, intestinal epithelial CA-MLCK expression impairs spatial memory and blunts sexual reward seeking in males and leads to anhedonia for sucrose and increased anxiety in females. These data reveal for the first time that increased intestinal paracellular permeability induces changes in the gut-brain axis that result in altered behavior.

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INTRODUCTION

There is increasing literature pointing to a key role of the microbiota-gut-brain axis in the regulation of brain function and behavior, particularly emotional processes and stress vulnerability (Dinan and Cryan, 2012; Foster and McVeyNeufeld, 2013; Burokas et al., 2015; Burokas et al., 2017). Studies in animals have shown that gut bacteria are critical for neuroendocrine stress response, anxiety-like behaviors, sociability and cognition (Luczynski et al., 2016). The mechanisms involved in this gut to brain communication remain unclear. Gut microbes are in close contact with the intestinal epithelium and have been repeatedly reported to influence gut barrier integrity and function (Zakostelska et al., 2011; Hsiao et al., 2013;.Nébot-Vivinus et al., 2014; Jakobsson et al., 2015; Reunanen et al., 2015). Consistently, gut dysbiosis is often accompanied by gut barrier dysfunction or leakiness. In addition to dysbiosis, gut leakiness has been reported in several psychiatric conditions (see Julio-Pieper et al., 2014 for review) including autism spectrum disorders (D'Eufemia et al., 1996; de Magistris et al., 2010; Fiorentino et al., 2016), schizophrenia (Cascella et al., 2011), major depressive disorder (Maes et al., 2008; Stevens et al., 2017) and alcohol dependence (Leclercq et al., 2014; de Timary et al., 2015). Moreover, in the study by Leclercq and coworkers, gut leakiness was found to be associated with higher scores of depression and anxiety in alcohol-dependent subjects (Leclercq et al., 2014). To date, however, most of the research has been focusing on the gut microbiota, and the intrinsic role of gut permeability in the regulation of emotional behavior remains poorly explored. Psychosocial stressors and childhood adverse experience (trauma or abuse) play a significant role in the onset and perpetuation of the irritable bowel syndrome (IBS), a functional gastrointestinal disorder frequently associated with gut leakiness (Bradford et al., 2012; Halland et al., 2014; see Chitkara et al., 2008 for review). Interestingly, this condition is highly comorbid with anxiety and mood disorders, and gastrointestinal symptoms severity in IBS patients is positively correlated with psychiatric symptoms (Van Oudenhove et al., 2016; Wilpart et al., 2017). However, it remains unclear whether there is a causal relationship between these disorders and if so, which comes first. Therefore, it is crucial to unravel the role of gut permeability in brain function and behavior. Furthermore, gut-brain literature suggests that the relationship between gut microbiota and behavior could be different in males *versus* females, although this aspect has not been studied extensively. *In vitro* and *in vivo* studies have shown that gut barrier function regulation involves cytoskeleton-mediated epithelial tight-junction opening, upon activation of the myosin II regulatory light chain kinase (MLCK) (Zolotarevsky et al., 2002; Clayburgh et al., 2005; Shen et al., 2006). High MLCK activity was recently reported in IBS (Wu et al., 2017) and other inflammatory bowel diseases (Blair et al., 2006), suggesting that this mechanism is relevant to human gut dysfunctions. We previously showed that prevention of gut leakiness by administration of a MLCK inhibitor (ML-7) in stressed rats

attenuates the increased neuroendocrine response to stress as well as the pro-inflammatory cytokine expression in the hypothalamus (Ait-Belgnaoui et al., 2012), suggesting a possible role of MLCK activity in stress-related behaviors. Transgenic mice with tissue-specific expression of a constitutively active MLCK within intestinal epithelia (CA-MLCK mice) exhibit gut leakiness (Su et al., 2009; Edelblum et al., 2017). Here, we aim to examine the consequences of CA-MLCK expression in the gut on emotional behavior and stress response in male and female mice. Furthermore, we explore stress-related gene expression and C-FOS activation upon acute stress in relevant brain areas according to the behavioral and endocrine results.

METHODS

Animals

All experiments were approved by the local Bioethical committees of our Universities (Bordeaux: N° 50120186-A; Toulouse: ToxCom/0031) and by the régions Aquitaine (ID: A33-063-920) and Midi-Pyrénées Veterinary Services (National Animal Care Committee ID: 86) according to the European legislation (Directive 2010/63/EU, 22 September 2010). Animals were maintained in a 12-h light/12-h dark cycle (lights on at 0800 hours) in a temperature-controlled room (22°C) with free access to food and water, unless otherwise stated. The breedings were carried out in Toxalim (Neuro-gastroenterology and Nutrition, INRA UMR 1331, Toulouse) and involved wild-type (WT) female and heterozygous CA-MLCK male parents (carrying the transgene encoding a constitutively active MLCK under the control of the villin promoter, specific of intestinal epithelial cells; C57/Bl6 genetic background) as previously described (Su et al., 2009). Four cohorts were used in this study. Heterozygous male and female CA-MLCK mice and WT control littermates were used for the experiments.

Genotyping

Mouse genomic DNA were prepared from mouse tail, digested for 10 minutes at 95°C in buffer (pH12.0) containing 25 mM NaOH and 0.2 mM EDTA. Then, samples were put on ice for 10 minutes and the reaction was stopped by adding 40 mM Tris-HCl (pH5.0). After centrifugation (6 min at 14000 rpm), PCR was performed using 2 µL of DNA, EconoTaq DNA polymerase (Euromedex, Souffelweyersheim, France) and primers for transgene detection.

In vivo intestinal permeability

In vivo intestinal permeability was assessed in 6-week-old mice using fluorescein-isothiocyanate (FITC, 396 Da, Invitrogen) as previously described (Edelblum et al., 2017). Briefly, mice were gavaged with 250 µL of 1 mg/mL solution of FITC dissolved in 10mM

NaOH. After 4 hours, 200 µL of blood was taken from the facial vein on heparinized tubes. Tubes were centrifuged for 10 minutes at 10000 g and FITC concentration was determined against a standard curve on a microplate reader (Tecan Infinite M200, Lyon, France).

Behavioral assessment

All experiments were performed during the light phase. For analyses involving manual quantifications, experimenters remained blind to the experimental groups. Animals were collectively housed except during sucrose preference and buried food tests.

Elevated plus maze (EPM, 2 months). The apparatus consisted of two opposing open arms (30 × 8 cm, brightness: 80 lux) and two opposing closed arms (30 × 8 × 15 cm, brightness: 20 lux) connected by a central platform (8 × 8 cm) and elevated 120 cm above the floor. During the light period (10:30 ± 1h), 2 month-old female mice were placed in the center of the maze facing an enclosed arm and allowed to explore for 5 min. Time spent in each arm was automatically quantified using videotracking (Smart software, Bioseb, Vitrolles, France). The percentage of time spent in open arms was calculated (open arms/(open arms+closed arms)x100). A reduction of the percent of exploration of the open arms is considered as an anxiety-like index (Belzung and Griebel, 2001).

Light-dark test (2,5 months). The apparatus was a two-compartment plexiglas box with a dark compartment wrapped with a cover (14 x 21 x 21 cm) and a light compartment exposed to intense light (30 x 21 x 21 cm; brightness: 200 lux). Both chambers were connected by a 7 x 5 cm opening. Mice were placed in the dark compartment and exploration of the light box was recorded with a digital camera during 10 min as previously described (de Cossío et al., 2017). Time spent in the light box was manually quantified using an ethological software (The observer, Noldus Information Technology, Wageningen, The Netherlands).

Sociability test (2.5 months). The apparatus was an open-field equipped with 2 perforated plastic beakers placed in opposite corners. Under dim light (brightness: 20 lux), mice were habituated to the setup for 5 min with empty beakers. They were transiently removed while a familiar mouse from the same cage was introduced in one of the beakers. Mice were put back in the open-field for 5 min and allowed to explore around either the empty or the mouse-containing beaker. Time spent exploring the empty beaker and the beaker with the social target was automatically quantified using videotracking (Smart, Bioseb, Vitrolles, France).

Y-maze (2-3.5 months). Spontaneous spatial recognition in the Y-maze was evaluated as previously described (Dinel et al., 2014). The apparatus was a Y-shaped maze made of dark grey plastic with three identical arms (34 x 8 x 14 cm). The floor was covered with corncob bedding and was mixed between each trial in order to remove olfactory cues. Visual cues were placed on the walls of the testing room and kept constant during the whole test. During

the acquisition phase, one of the three arms was closed with a plastic gate and male mice were placed in the depart arm (facing the wall) and allowed to explore the two open arms for 10 min. After an intra-trial interval (ITI) of 30 min (males and females) or 60 min (males only), mice were put back in the Y-maze with free access to all three arms (depart, familiar and novel arms) during 3 min (restitution phase). All these trials were conducted under dim light (brightness: 60 lux). The position of the novel arm was randomized to prevent from any position effect. The 30 min ITI trial was performed at 2 months. In males, 30 and 60 min ITI trials were carried out in the same animals, aged 2 and 3.5 months, respectively. To minimize bias, a different maze was used for the two experiments, and the novel arm position was changed. Quantification of time spent in the three arms was performed automatically using videotracking (Smart, Bioseb, Vitrolles, France or Ethovision, Noldus Information Technology, Wageningen, The Netherlands). The percentage of time spent in the new arm during the restitution phase was calculated (new arm/(new arm + familiar arm + depart arm)x100). A reduction of the percent of exploration of the new arm reflects impaired spatial memory (Conrad et al., 1996).

Tail suspension test (TST, 3months). The apparatus was a three-compartment box provided with 3 hooks (Bioseb). Mice were hanged by the tail using secure tape for a 6 min test session and their immobility was automatically assessed using movement detection software (Bioseb, Vitrolles, France).

Female urine sniffing test (4 months). Females from different litters were placed in a new cage without bedding for 3 hours and urine (approximately 500 µl per mouse) was collected and stored at -20°C until use. The cyclic status was determined by microscopic observation of vaginal smears and only urine from estrus females was used for the experiment. The test was conducted as previously described (Malkesman et al., 2010). Under dim light (5 lux), male mice were habituated to a dry Q-tip in the homecage (30 x 20 x 20 cm) for 60 min, and then presented with a Q-tip soaked with sterile water for 5 min. After 45 min without any Q-tip, mice were presented with a Q-tip soaked with estrus female urine for another 5 min. Q-tips were soaked with 200 µl of sterile water or urine and presented inside 1 ml plastic tips taped to the cage wall. 5 min trials were videorecorded using a digital camera and Q-tip sniffing time was manually quantified using the Observer software (Noldus Information Technology, Wageningen, The Netherlands).

Buried food test (2 months). Mice were food-deprived for 24h prior each day of testing. To assess motivation to eat, mice were transiently removed from their homecage and the feeder was filled back with food pellets (standard chow). To evaluate olfactory function, mice were placed in a new cage with 4 food pellets (standard chow) buried under 1 cm of bedding. During each test, the latency to eat was manually recorded (Yang and Crawley, 2009).

Sucrose preference. Female mice (4 month-old) were individually housed 4 days before the beginning of the experiment and underwent 48h of habituation with two bottles of tap water. On the first day of testing, animals were presented one bottle filled with 1% sucrose solution and one bottle of water during 24h. The day after, the 1% sucrose solution was replaced by 2 % sucrose solution for another 24h. Finally, 3% sucrose solution was tested on the next day. Water and sucrose solution intakes were measured after every 24h and sucrose preference was calculated as the percentage of sucrose intake over total fluid intake. Bottle side was randomized and switched between the two days of test to control for any side bias. Based on female results, the procedure was repeated in males of another cohort (1.5 month-old) with a 2 % sucrose solution.

Hypothalamus-pituitary-adrenal (HPA) axis reactivity to stress (4.5 months). Mice were placed in perforated 50 ml falcons for 30 min and blood samples were collected from the tail vein at the beginning of the restraint stress (t0), as well as 30 min (t30, end of restraint), 60 min (t60) or 120 min (t120) after the beginning of the stress. Blood samples were centrifuged (4000 rpm, 4°C) for 20 min and stored at -20°C until use.

Molecular and biochemical analyses

Corticosterone (CORT) radioimmunoassay. Total plasma CORT was measured with an in-house radio immunoassay, by competition between cold CORT (B) and 3H-B (B*) for a specific anti-CORT antibody, as previously described (Richard et al., 2010). The sensitivity of this assay is around 5 ng/ml. Intra- and inter assay variations were <15%.

RNA extraction from brain tissues. 5 month-old mice were deeply anesthetized with isoflurane and killed by decapitation. Whole brains were collected and stored at -80°C until used. Micropunches (1 mm of diameter) of the medial prefrontal cortex (mPFC) (from +2.22 to +1.42 mm; anterior-posterior from bregma (AP)), nucleus accumbens (NAc) (from +1.70 to +0.98 mm, AP) and dorsal hippocampus (dHPC) (from -1.46 to -2.3 mm, AP) were made in 200 µm slices obtained using a cryostat (Leica) at -14°C. Total RNA was extracted using TRIzol (Fisher Scientific, France) according to the manufacturer's protocol with slight modifications. The quality of total RNA was assessed on representative samples using RNA Nano chips on a Bioanalyzer 2100 (Agilent, Boeblingen, Germany). Samples had a RNA Integrity Number (RIN) score > 8.0. 300 ng of total RNA were reverse-transcribed using with Superscript III (Invitrogen, Cergy Pontoise, France) and random hexamers according to the manufacturer's protocol.

Stress-related gene expression. High throughput real time quantitative PCR was performed at the GeT-TRiX facility (GénoToul, Génopole Toulouse Midi-Pyrénées) using Biomark (Fluidigm) according to manufacturer's protocol. To avoid genomic DNA amplification, primer pairs were designed in two different exons (thus spanning an intron)

using the Primer Express software (PE APPLIED Biosystems, Courtaboeuf, France). Sequences of primers used are provided in **Supplementary Table 1**. The specificity of the PCR reaction was validated according to MIQE (Minimum Information for publication of Quantitative real time PCR Experiments) guidelines. Each cDNA was diluted (5 ng/µL) and used for target amplification. Primer pairs targeting all the genes of interest were pooled to a final concentration of 200nM for each primer pair. A multiplexed preamplification process was performed for the primers pool on every 1.25 µL of cDNA using 14 cycles of cDNA preamplification step (at 95°C for 15 s and at 60°C 4 min) and PreAmp Master Mix (Fluidigm) in a standard PCR thermocycler. Exonuclease I treatment (NEB) was performed to degrade excess primers then preamplified cDNA was diluted 1:5 in TE low EDTA (10 mMTris, 0.1 mM EDTA). Diluted cDNA (2 µL) was added to DNA Binding Dye Sample Loading Reagent (Fluidigm), EvaGreen (Interchim) and TE low EDTA to constitute Sample Mix plate. In Assay Mix plate, 2 µL of primer pairs (20 µM) were added to the Assay Loading Reagent (Fluidigm) and TE low EDTA to a final concentration of 5 µM. Following priming the chip in the Integrated Fluidic Circuit Controller, Sample Mix (5 µL) were loaded into the sample inlet wells, and Assay Mix (5 µl) were loaded into assay inlet wells. One well was loaded with water as a contamination control. To verify specific target amplification and qPCR process efficiencies, a sample control (human gDNA, Thermo Fisher) was treated, preamplified and quantified on assay control (RNasePTaqMan probe, Thermo Fisher) using the same process in same plate at the same time. The expected value of cycle quantification was around 13. The chip was placed into the IFC Controller, where 6.3nl of Sample Mix and 0.7nl of Assay Mix were mixed. Real-time PCR was performed on the Biomark System (Fluidigm) with protocol: Thermal Mix at 50 °C, 2 min; 70 °C, 30min; 25°C, 10min, UNG at 50°C, 2 min, Hot Start at 95°C, 10 min, PCR Cycle of 35 cycles at95 °C, 15 s; 60 °C, 60 s and Melting curves (to 60°C until 95°C). Results were analyzed using the Fluidigm Real-Time PCR Analysis software v.4.1.3. to control specific amplication for each primer then the raw results of the qPCR were analyzed using GenEx software (MultiD analyses AB) in order to choose the best reference gene to normalize mRNA expression and to measure the relative expression of each gene between groups. Beta-2 microglobulin (β2m) was found to be the best reference gene in this experiment and was thus used for normalization of gene expression. Expression of target genes was calculated by the delta-delta Ct method relative to expression in WT mice (foldchange).

Immunohistochemistry

Brain C-Fos immunomapping. Mice were killed either after 90 ± 20 min restraint stress or under basal conditions. After deep anesthesia (pentobarbital 500mg/kg, i.p.), they were intracardially perfused with 100 mL of 4% paraformaldehyde (PFA) and dissected brains

were stored in 4% PFA for 24h, transferred to 30% sucrose for 48h and finally stored in a cryoprotectant solution at -20°C. Serial coronal sections (40 µm) were obtained using a vibratome (Leica, Rueil Malmaison, France) and stored in a cryoprotectant solution at -20°C until use. Free-floating sections (40µm) were treated as previously described (Labrousse et al., 2009). The antibodies were a primary rabbit anti-c-Fos at 1:1000 (Sc520d, Santa Cruz Biotechnology, Santa-Cruz, CA, USA) and a secondary goat anti-rabbit at 1:2000 (Jackson 152, Jackson ImmunoResearch, Suffolk, UK). The glucose oxidase-DAB-nickel method was used for the detection of C-FOS immunoreactive (IR) cells.

Quantification of C-FOS-IR cell. NDPI images at x20 magnification were obtained at the Bordeaux Imaging Center (CNRS-INSERM and Bordeaux University, France BioImaging) using a digital slide scanner (Nanozoomer, Hamamatsu Photonics, Massy, France) and converted into TIFF format using ImageJ (http://imagej.nih.gov.gate2.inist.fr/ij/) and the NDPItools plugin (Deroulers et al., 2013). Regions of interest (ROI) were manually circumscribed using ROItools according to Paxinos and Franklin, 2001, as follows: mPFC (anterior cingulate dorsal, ACd; prelimibic, PrL and infralimibc, IL), bregma +1.98 to +1.18 mm; cingulate cortex (CG1/2), bregma +1.18 to -0.10 mm; NAc (shell/core), bregma 1.94 to 0.74 mm; paraventricular nucleus of the thalamus (PV), bregma-0.22 to -2.18 mm; paraventricular nucleus of the hypothalamus (PVN), bregma -0.70 to -1.22 mm; amygdala (basolateral, BLA and central, CeA), bregma -0.58 to -2.18 mm; dorsal hippocampus (cornu ammonis 1, CA1 and dentate gyrus, DG), bregma -1.22 to -2.18 mm; ventral hippocampus (CA1/DG), bregma -2.18 to -3.08 mm; nucleus tractus solitarius (NTS), bregma -7.92 to -8.24 mm (**Figure 5)**. The number of C-FOS-IR cells was automatically quantified in 8-bit thresholded images using the particle analysis function (size: 5-20 µm²; circularity: 0.5-1). The quantification of C-FOS-IR cells was carried out in 3-5 sections per animal for each area. Results expressed as C-FOS-IR cells per mm² were used for correlation analyses. Comparisons between groups were conducted on values normalized to the mean of WT basal animals (%). The experimenter remained blind to the treatment conditions throughout the analysis.

Statistics

All data were analyzed using Statistica 6.0 (Statsoft). Normality was assessed by Shapiro–Wilk tests. Student *t*-tests or Mann Whitney *U* tests were used to test the effects of genotype in males and in females according to the normality results. Two-way ANOVA with repeated measures (between-subjects factors: genotype; within-subject factor: beaker, urine or time) followed by Fisher's LSD *post-hoc* tests were used to analyze sociability, female urine sniffing test and HPA axis response to stress. The effects of acute stress and genotype on C-FOS immunoreactivity were analyzed by one-way Kruskal-Wallis ANOVA followed by Mann

Whitney *U* tests. C-FOS data for each group were also compared with chance level (100%). Spearman tests were used to examine C-FOS immunoreactivity correlations between brain areas. Statistical significance was set at $p<0.05$. Graphics were made using GraphPad Prism 5 and Adobe Illustrator CS5.1 was used for artwork. Data are expressed as means ± SEM.

RESULTS

Tissue-specific expression of a constitutively-active MLCK within intestinal epithelial cells induces gut leakiness in males and females.

Previous work has shown that female CA-MLCK mice exhibit gut leakiness compared with WT animals (Su et al., 2009; Edelblum et al., 2017). We first aimed to replicate this finding in both sexes **(Figure 1)**. Gut permeability was significantly higher in CA-MLCK mice of both sexes compared with WT animals (Mann-Whitney U tests, males: $U=20$, $p=0.0309$; females: $U=8.5$, $p=0.0340$). There was no difference in body weight between groups of the same sex (data not shown).

Tissue-specific expression of a constitutively-active MLCK within intestinal epithelial cells blunts sexual reward seeking and produces a hyper-response of the HPA axis to stress in males.

Adult male and female WT and CA-MLCK mice underwent a battery of behavioral tests **(Figure 2)**. We first explored anxiety-, despair- and anhedonia-like behaviors in WT and CA-MLCK males **(Figure 2a-d).** There was no effect of genotype on the time spent in the light compartment of the light-dark box or in the open arms of the EPM (Student *t* test, all $t(18)<1$, n.s., **Figure 2a,b**), the time spent immobile in the TST ($t(18)<1$, n.s., **Figure 2c**), or the sucrose preference index ($t(12)=1.32$, n.s., **Figure 2d**). We then assessed sociability in an adapted version of the three-chamber test **(Figure 2e).** When animals were placed in the open-field containing a social target (familiar mouse) and an empty beaker in opposite corners, both genotypes spent more time exploring the mouse (two-way ANOVA with repeated measures, genotype effect: $F(1,14)=0.04$, n.s.; beaker effect: $F(1,14)=17.00$, $p=0.0010$; genotype x beaker effect: $F(1,14)=0.56$, n.s.). Of note, there was no difference in the time spent exploring the two empty beakers during habituation (genotype effect, beaker effect, genotype x beaker effect, all $F(1.14)<1.5$, n.s.; data not shown). Overall, these data suggest that male CA-MLCK mice display normal emotional behavior. However, in the urine sniffing test, which involves sexual reward seeking behavior, the time spent sniffing a cotton Q-tip soaked with urine or water varied according to the genotype (two-way ANOVA with repeated measures, genotype effect: $F(1,18)=0.24$, n.s., urine effect: $F(1,18)=12.12$,

p=0.0027, genotype x urine effect: *F*(1,18)=5.53, *p*=0.0303, **Figure 2f)**. Only WT males exhibited a significant preference for estrus female urine *versus* water (Fisher LSD's *post-hoc*, WT-water *vs* WT-urine, *p*=0.0006), suggesting that CA-MLCK males are anhedonic as regards sexual rewards. This effect is likely not due to altered olfactory abilities as the latency to nose urine was similar between genotypes (WT: 12.4 ± 7.4 sec *vs* CA-MLCK: 18.1 ± 5.5 sec; Student *t* test, *t*(18)<1, n.s., data not shown). To further control for a potential olfactory bias, we conducted additional tests in 24h-fasted mice (**Figure 2g,h)**. Mice were food deprived and the latency to eat was measured upon food reintroducing (visible food, **Figure 2g)**. There was no difference between genotypes in the latency to eat (*t*(10)=1.50, n.s.), indicating that motivation to eat was not affected by intestinal CA-MLCK expression. Importantly, there was no difference in the latency to find food buried in their homecage bedding, providing additional evidence that CA-MLCK mice have normal olfaction (buried food, *t*(10)<1, n.s., **Figure 2h)**. Finally, mice were tested for spatial memory in the Y-maze task (**Figure 2i)**. Males of both genotypes spent significantly more time in the novel arm compared with chance levels after a 30 min inter-trial interval (ITI) (comparison with 33%, WT: *t*(9)=7.26, *p*<0.0001, CA-MLCK: *t*(9)=2.98, *p*=0.0154). However, after a 60 min ITI, only WT males displayed significant discrimination of the novel arm (WT: *t*(9)=3.62, *p*=0.0055, CA-MLCK: *t*(9)=1.10, n.s.), indicating that spatial memory is impaired in CA-MLCK mice in a more difficult task. These results suggest that gut permeability could play a role in the regulation of cognitive processes.

We next explored HPA axis responsiveness to stress by measuring plasma CORT levels at baseline and following 30min of restraint stress **(Figure 2j)**. Basal CORT levels were similar in both genotypes, whereas, at the end of restraint (T30min), plasma CORT was significantly higher in CA-MLCK *versus* WT males (two-way ANOVA with repeated measures, genotype x time effect: *F*(1,54)=3.54, *p*=0.0206, planned comparison WT *vs* CA-MLCK, T30, *p*=0.0458). This effect was not maintained after the end of the stress (T60 and T120, n.s.). Overall, the area under the curve (AUC) was larger in CA-MLCK versus WT males (Student *t* test, *t*(14)=2.27, *p*=0.0395) **(Figure 2k)**.

Tissue-specific expression of a constitutively-active MLCK within intestinal epithelial cells increases anxiety and produces anhedonia in females.

Female CA-MLCK mice spent significantly less time in the light box (*t*(18)=2.13, *p*=0.0474, **Figure 3a)** and the open arms of the EPM (*t*(18)=3.50, *p*=0.0026, **Figure 3b)** compared with WT, suggesting that they are more ranxious. These effects were accompanied by a decreased sucrose preference for a 2% sucrose solution, but not 1 or 3% (2%: *t*(17)=2.12, *p*=0.0491, **Figure 3d;** 1 and 3%, data not shown) but no difference in immobility time in the TST (*t*(18)<1, n.s., **Figure 3c)**. Surprisingly, in the sociability test, neither WT or CA-MLCK

females displayed significant preference for the social target *versus* the empty beaker (genotype effect, beaker effect, genotype x beaker effect: all $F(1,14)<4.5$, n.s., **Figure 3e)**. However, CA-MLCK mice spent less time exploring the two empty beakers during habituation (genotype effect: $F(1.14)=4.58$, $p=0.0505$; beaker effect: $F(1.14)<1$, n.s.; genotype x beaker effect: $F(1.14)=2.02$, n.s.; data not shown), possibly reflecting their increased anxiety. In the Y-maze task, neither WT or CA-MLCK females significantly discriminated the new arm after a 30 min ITI (comparison with 33%, WT: $t(9)=1.56$, $p=0.1528$, n.s., CA-MLCK: $t(9)=1.65$, $p=0.1330$, n.s., **Figure 3f)**. Together, our data suggest that the sociability test and the y maze task were not appropriate to assess social behavior and spatial memory in WT females under these conditions.

Plasma CORT of CA-MLCK females was not changed after 30min of restraint relative to WT, but remained higher after stress cessation, suggesting that HPA axis negative feedback is impaired in CA-MLCK females (genotype x time effect: $F(1,39)=1.02$, n.s.; planned comparison WT *vs* CA-MLCK, T30, $p=0.0539$), **(Figure 3g)**. Overall, the AUC tended to be increased in CA-MLCK versus WT females *(*$t(13)=1.95$, $p=0.0726$) **(Figure 3h)**.

Intestinal CA-MLCK expression alters stress-related gene expression in the mPFC, NAc and hippocampus in both sexes

In order to gain insight into the molecular mechanisms underlying these effects, we analyzed mRNA expression of stress-related genes (Datson et al., 2008) in brain areas involved in emotional processes and stress regulation **(Figure 4a,b,** see legend for genes full names). Among 92 genes, we found 19 up-regulated genes (see **Figure 3e** for details) in the mPFC of male CA-MLCK mice relative to WT, 2 up-regulated (Sirt1 and Dynll1) and 2 down-regulated (Ngf and Rheb) genes in the NAc and 1 down-regulated gene (Crhbp) in the dHPC (Mann-Whitney *U* tests, all *p* at least <0.05) **(Figure 4a)**. In CA-MLCK females, 3 genes (Mtor, Lasp1 and Hsf1) were up-regulated and 1 gene (Ttr) was down-regulated in the mPFC; 15 genes were down-regulated in the NAc; 1 gene (Reln) was up-regulated and 2 genes (Cox2 and Ttr) were down-regulated in the dHPC (all *p* at least <0.05) **(Figure 4b)**. These target genes could possibly be involved in the behavioral and neuroendocrine alterations reported here. Overall, the impact of intestinal CA-MLCK expression seems to be greater in the mPFC (males) and the NAc (females) than in the other brain regions studied. Interestingly, most of the impacted genes in males were up-regulated, and most impacted genes in females were down-regulated, indicating marked differential effects between sexes, as reported for the behavior. Moreover, Rheb expression was altered in both the mPFC and the NAc in males. In females, Cox2, Tnfa and Ttr expression was altered in two brain regions out of the three tested. Overall, our results suggest that these genes could be important targets for gut-brain communication.

Tissue-specific expression of a constitutively-active MLCK within intestinal epithelial cells is associated with altered neuronal activation patterns in both sexes

We then performed a brain mapping of neuronal activation after restraint stress in WT and CA-MLCK mice, using C-FOS immunolabeling **(Figure 5, 6 and 7)**. In males of both genotypes, there was a marked increase in the number of C-FOS-IR cells following stress in the majority of the brain areas examined (comparison with 100%, all *p* at least <0.05, except dDG and vCA1, see detailed between group comparisons in **Supplementary table 2**) (**Figure 6)**. However, WT males showed no increase of C-FOS expression in the dorsal DG and ventral CA1, whereas CA-MCLK males displayed neuronal activation in response to stress in these brain areas (Mann-Whitney U tests, CA-MLCK post-stress *vs* CA-MLCK basal, dDG p=0.0451 and vCA1 p=0.0635) **(Figure 6l,o)**. Although there is no significant difference between genotypes in the post-stress condition, these data suggest that the hippocampus is a selected target of the gut-brain axis under genetic manipulation of intestinal permeability in male mice. Similarly, in females, stress induced massive C-FOS expression in the brain of both genotypes compared with the basal condition (all *p* at least <0.05, except dCA1, vDG and vCA1; see detailed between group comparisons in **Supplementary table 3**) (**Figure 7)**. Importantly, WT females showed increased C-FOS response under stress in all the brain areas. In contrast, CA-MLCK females exhibited no increase of C-FOS expression in the dorsal and ventral DG (CA-MLCK post-stress *vs* CA-MLCK basal, dDG p=0.6991 and vDG p=0.4286) (**Figure 7l,n)**. Interestingly, C-FOS-IR cell numbers in basal conditions were increased in CA-MLCK mice relative to WT (CA-MLCK basal *vs* WT basal, dDG p=0.0931 and vDG p=0.0556). Furthermore, there was also greater basal C-FOS expression in the PV and PVN of CA-MLCK mice compared with WT (CA-MLCK basal *vs* WT basal, PV p=0.0649 and PVN p=0.0411) (**Figure 7h,i)**. Altogether with the male data, our findings suggest that the hippocampus, thalamus and hypothalamus are important targets of the gut-brain axis.

We further explored how C-FOS expression in response to stress correlates between the different brain areas in WT and in CA-MLCK mice **(Supplementary Figure 1, Supplementary Figure 2, Figure 6q,r and Figure 7q,r)**. In WT males, the vast majority of the brain regions studied correlate with each other, except the dDG and vCA1 **(Figure 6q)**. In contrast, in CA-MLCK mice, these areas correlate with most brain regions, but the vDG does not (**Figure 6r)**. In WT females, all areas except the NTS correlate with each other **(Figure 7q)**. Interestingly, the NTS only correlates with the vDG and vCA1. The latter correlations are lost in CA-MLCK females, while the NTS correlates with the other areas (**Figure 7r)**. Moreover, the connectivity between the dDG and the rest of the brain is lost and that of the vDG is also dramatically weakened. Overall, our data suggest that CA-MLCK mice exhibit

altered brain connectivity post-stress in both males and females. Changes in the connectivity between WT and CA-MLCK mice for dHPC, vHPC and NTS are illustrated in **Supplementary Figure 1** and **Supplementary Figure 2**.

DISCUSSION

There is mounting evidence that altered gut-brain communication could play a role in the onset and persistence of neuropsychiatric disorders. For instance, gastrointestinal symptoms are commonly found in major depressive disorder (Rios et al., 2017) or autism spectrum disorder (Buie et al., 2010). The role of gut microbiota in the neurobehavioral phenotypes associated with these pathologies has been elegantly demonstrated in animal models (Hsiao et al., 2013; De Palma et al., 2015; Kelly et al., 2016; Kim et al., 2017). Gut dysbiosis is often associated with gut leakiness, thus the behavioral alterations reported in a number of studies using germ-free animals or stress-induced dysbiosis could be mediated by impaired gut barrier function. Here, using transgenic mice with tissue-specific CA-MLCK expression within intestinal epithelia, we show for the first time that gut leakiness *per se* is sufficient to produce behavioral alterations in a sex-dependent manner. Specifically, we show that male CA-MLCK mice display reduced sexual reward seeking and spatial memory deficits while female CA-MLCK are more anxious and exhibit decreased sucrose preference relative to WT mice. These behavioral changes are accompanied by exaggerated HPA axis response to restraint stress, stress-related gene expression changes in the brain and altered brain C-FOS immunoreactivity in both sexes.

In previous studies, the link between manipulations of microbiota and anxiety-like behavior appeared as the most frequent and robust observation. However, most studies focus on males or pool males and females together for data analysis. Here, we report a causal effect of gut permeability on anxiety in females, but not in males. In addition, despite no change was observed in the TST, CA-MLCK females exhibited lower sucrose preference compared with WT. The exacerbated anxiety and depressive-like behavior in female CA-MLCK mice are particularly interesting with respect to the higher prevalence of anxiety and mood disorders, but also IBS, in women. On the other hand, our findings suggest that the impact of gut leakiness on male emotional behavior is limited. Decreased sexual reward seeking in the female urine sniffing test suggests that gut permeability and more generally gut-brain communication could play a role in sexual behaviors. A key finding in CA-MLCK males is the impaired spatial memory in the Y maze. Several studies have reported cognitive alterations in dysbiotic mice using spatial and non-spatial tasks (Gareau et al., 2011; Fröhlich et al., 2016; Hoban et al., 2016). Future studies are needed to better document the cognitive

outcomes of intestinal CA-MLCK expression, that might be affected in a sex-dependent fashion as well.

We sought for neurobiological correlates that might contribute to the behavioral changes induced by gut leakiness. Our findings reveal HPA axis disturbances in CA-MLCK mice of both sexes, although they are not affected in the same way. These results are consistent with previous findings showing that pharmacological MLCK inhibition prevents stress-induced neuroendocrine response to stress in rats (Ait-Belgnaoui et al., 2012). The sustained elevation in CORT levels observed in females suggests a deficit in negative feedback by glucocorticoids, while the increased CORT secretion at the end of the stress in males is likely independent of these feedback processes. Therefore, the molecular mechanisms underlying the altered HPA axis response to stress in males and females are presumably different. This hypothesis is supported by the cerebral gene expression and the C-FOS data reported here.

Again, the impact of gut leakiness on cerebral expression of genes relevant for stress response revealed sex differences. Overall, mRNA expression was greatly affected in the mPFC of males and NAc of females, suggesting an important contribution of these brain areas in the respective behavioral alterations. Notably, there was an opposite effect of gut leakiness on mRNA expression of Cox1, Cox2, Cox3, Glut1, Rheb, Tnfa, Serpina6, Hsp90aa1 and Ace, that were upregulated in males' mPFC but downregulated in females' Nac. We previously reported that pharmacological MLCK inhibition normalized the stress-induced increase in Crf and pro-inflammatory cytokine (interleukin (IL)-1b, IL-6 and tumor necrosis factor alpha (Tnfa)) mRNA expression in the hypothalamus in rats (Ait-Belgnaoui et al., 2012). Here, we show that MLCK overexpression in the gut produces mRNA expression changes, including Tnfa, in higher brain areas involved in the regulation of emotional behaviors. These data further document the causal link between MLCK activity and brain gene expression. Moreover, expression of the serotonin receptor Htr1a was increased in males' mPFC. Interestingly, upregulation of Htr1a in the PFC has been reported in male rodents submitted to chronic stress (Iyo et al., 2009; Rincel et al., 2016), suggesting that gut leakiness exerts stress-like effects in male MLCK. It has been shown that HTR1A shapes serotonergic modulation of a functional circuit between the amygdala and mPFC (Fisher et al., 2011). Accordingly, it would be interesting to assess stress-related gene expression in the amygdala.

In the present study, we have explored C-FOS immunoreactivity in several brain areas including the amygdala. No difference in neuronal activation was found in the mPFC or amygdala of CA-MLCK mice *versus* WT. Nevertheless, gut leakiness induced a shift in brain connectivity in both sexes. The hippocampus dDG and vCA1 subregions show very different profiles in males *versus* females, suggesting that this brain area could be involved in the differential effects of gut leakiness in males and females. Moreover, the effect of gut

leakiness on neuronal activation patterns is more pronounced in females than males. Especially, the increased basal C-FOS expression in the PVN, PV and DG in CA-MLCK females demonstrate that gut leakiness *per se*, without any exposure to stress, can alter brain function. It has been proposed that PVN overactivity in absence of stress may produce inadequate behaviors (Füzesi et al., 2016). Whether the observed patterns of neuronal activation could participate in the sex-dependent behavioral and endocrine effects of intestinal CA-MLCK expression remains to be demonstrated. Moreover, identification of the neuronal subpopulations involved in the C-FOS expression changes could provide insight into the molecular mechanisms underlying these changes as well as the possible link with the behavioral alterations.

Gut leakiness is often concomitant with dysbiosis. Owing to the observed sex-differences in microbiota composition (Markle et al., 2013; Fransen et al., 2017), one can hypothesize that gut leakiness has differential effects on gut bacterial communities in males and females. A limitation of the present study is the lack of data as regards gut microbiota composition. However, our work provides, to our knowledge, the first proof-of-concept that gut permeability can causally affect brain and behavior. Further studies are needed to unravel the molecular mechanisms underlying this gut-brain communication. Whether CA-MLCK mice exhibit gut dysbiosis or not, the increased gut permeability implies more passage of bacterial products in the lamina propria. Su and colleagues previously reported no increase in circulating endotoxin levels in CA-MLCK mice (Su et al., 2009). Nevertheless, these mice display mucosal immune activation including increased production of interferon-gamma (IFNg) and Tnf as well as increased T-bet/Gata-3 ratio indicative of type 1 T-helper cell polarization. Whether this local inflammation could in turn induce neuroinflammation in the brain remains to be determined. In line with this hypothesis, we report increased Tnfa mRNA in the mPFC of both male and female CA-MLCK in basal conditions. In contrast, Tnfa expression is decreased in the NAc of CA-MLCK females. It would be interesting to explore the effects of an immune challenge (e.g. LPS injection) on Tnfa and other neuroinflammatory markers including glial activity in the brain of CA-MCLK mice. In addition, intestinal CA-MLCK expression could lead to impaired blood-brain barrier function. Indeed, blood-brain barrier permeability has been shown to be influenced by the gut microbiota (Braniste et al., 2014).

Overall, our work provides new insight into the mechanisms of gut-brain communication and suggests that intestinal permeability should be taken into account in both animal and human studies. Our findings may have implications for the numerous conditions associated with gut leakiness including stress, psychiatric disorders and IBS, but also obesity, diabetes and other metabolic diseases (Bischoff et al., 2014; Slyepchenko et al., 2016), in which psychiatric comorbidities have been described (Bornstein et al., 2006; Luppino et al., 2010; Oladeji and Gureje, 2013; Holt et al., 2014).

FIGURE 1

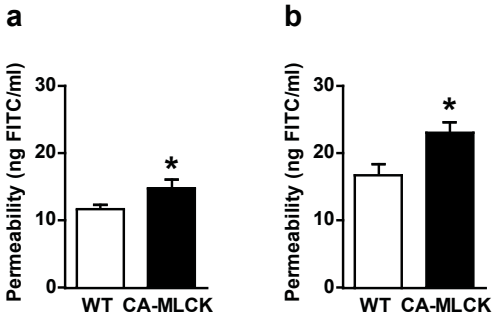


Fig 1. Increased gut permeability in male and female CA-MLCK mice. *In vivo* paracellular permeability to FITC (ng/ml) in males (N=14 WT and 8 CA-MLCK) **(a)** and females (N=5 WT and 10 CA-MLCK) **(b)**. FITC concentration is expressed per gram of body weight. Data are mean ± SEM. Mann-Whitney U tests: *p<0.05 versus WT.

FIGURE 2

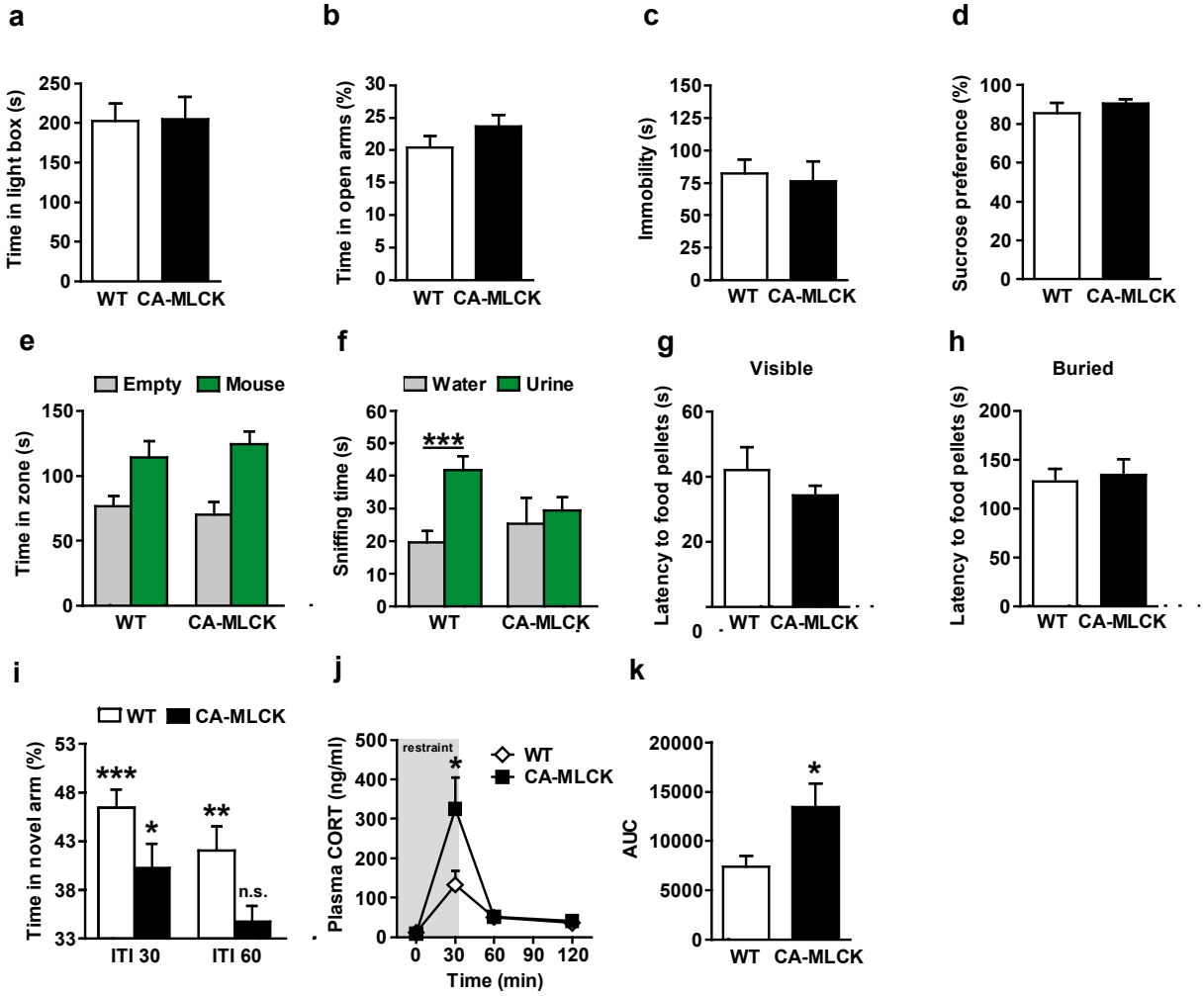


Fig 2. Male CA-MLCK mice display sexual reward-seeking impairment. (a) Time (s) spent in the light box of the light-dark apparatus over 10 min of test (N=10 per group). **(b)** Time (%) spent in the open arms of the EPM over 5 min of test (N=10 per group). **(c)** Time(s) spent immobile in the TST over 6 min (N=10 per group). **(d)** 24h-sucrose preference (%

sucrose intake over total fluid intake) in free choice condition with water and 2% sucrose solution (N=6-7 per group). **(e)** Time (s) spent exploring the empty beaker or the beaker containing a mouse during a 5 min three-chamber-like sociability test (N=8 per group). **(f)** Time (s) spent sniffing a water- or estrus-female-urine-filled Q-tip over 3 min in the female urine sniffing test (N=10 per group). Latency (s) to eat visible **(g)** or buried **(h)** food pellets after 24h of food-deprivation (N=6 per group). **(i)** Time (%) spent in the novel arm of the y maze during the restitution phase, 30 or 60 min following acquisition (N=10 per group). **(j)** Plasma CORT (ng/ml) at 0, 30, 60 and 120 min in response to 30 min of restraint stress and **(k)** area under the curve (N=8 per group). Data are mean ± SEM. **p*<0.05, ***p*<0.01 and ****p*<0.001 (Fisher LSD's *post-hoc* test in **f**; comparison with 33% in **i**; planned comparison in **j**; Student *t*-test in **k**). ITI, inter-trial interval.

FIGURE 3

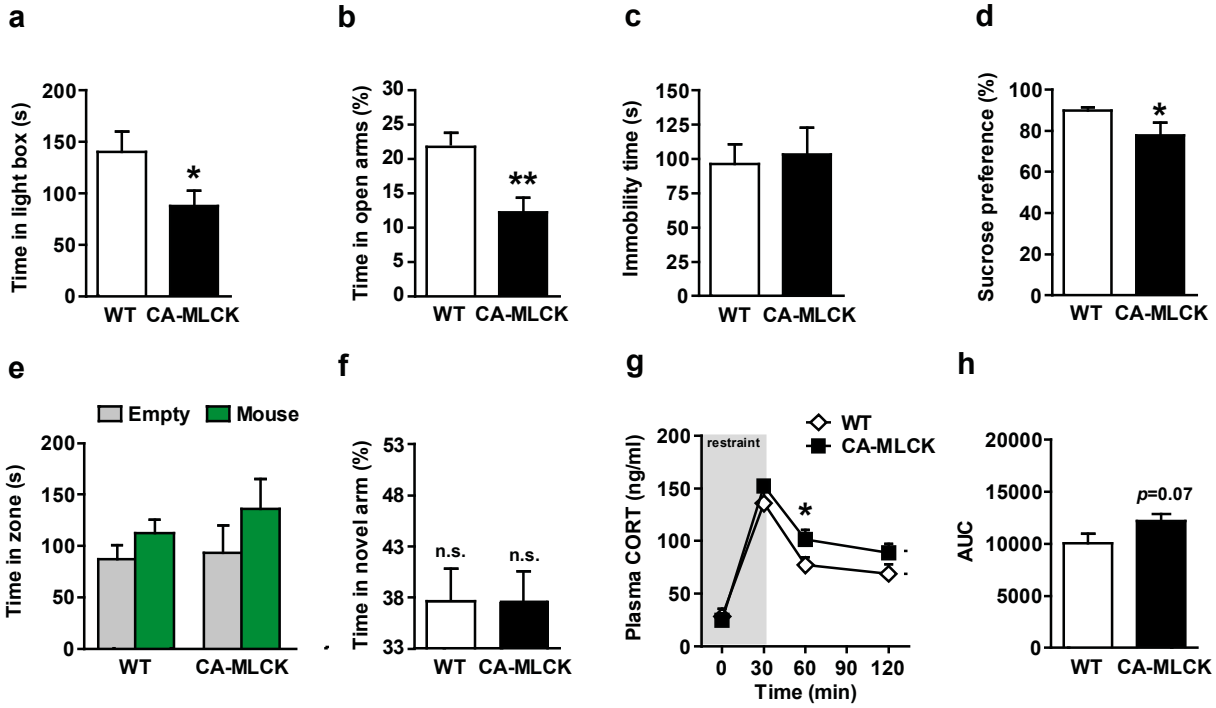


Fig 3. Female CA-MLCK mice exhibit increased anxiety and anhedonia. (a) Time (s) spent in the light box of the light-dark apparatus over 10 min of test (N=10 per group). **(b)** Time (%) spent in the open arms of the EPM over 5 min of test (N=10 per group). **(c)** Time(s) spent immobile in the TST over 6 min (N=10 per group). **(d)** 24h-sucrose preference (% sucrose intake over total fluid intake) in free choice condition with water and 2% sucrose solution (N=10 per group). **(e)** Time (s) spent exploring the empty beaker or the beaker containing a mouse during a 5 min three-chamber-like sociability test (N=8 per group). **(f)** Time (%) spent in the novel arm of the y maze during the restitution phase, 30 min following acquisition (N=7 per group). **(g)** Plasma CORT (ng/ml) at 0, 30, 60 and 120 min in response to 30 min of restraint stress and **(h)** area under the curve (N=7-8 per group). Data are mean ± SEM. **p*<0.05, ***p*<0.01 and ****p*<0.001 (Student *t*-test in **a**,**b**, **d** and **h**; comparison with 33% in **f**; planned comparison in **g**).

FIGURE 4

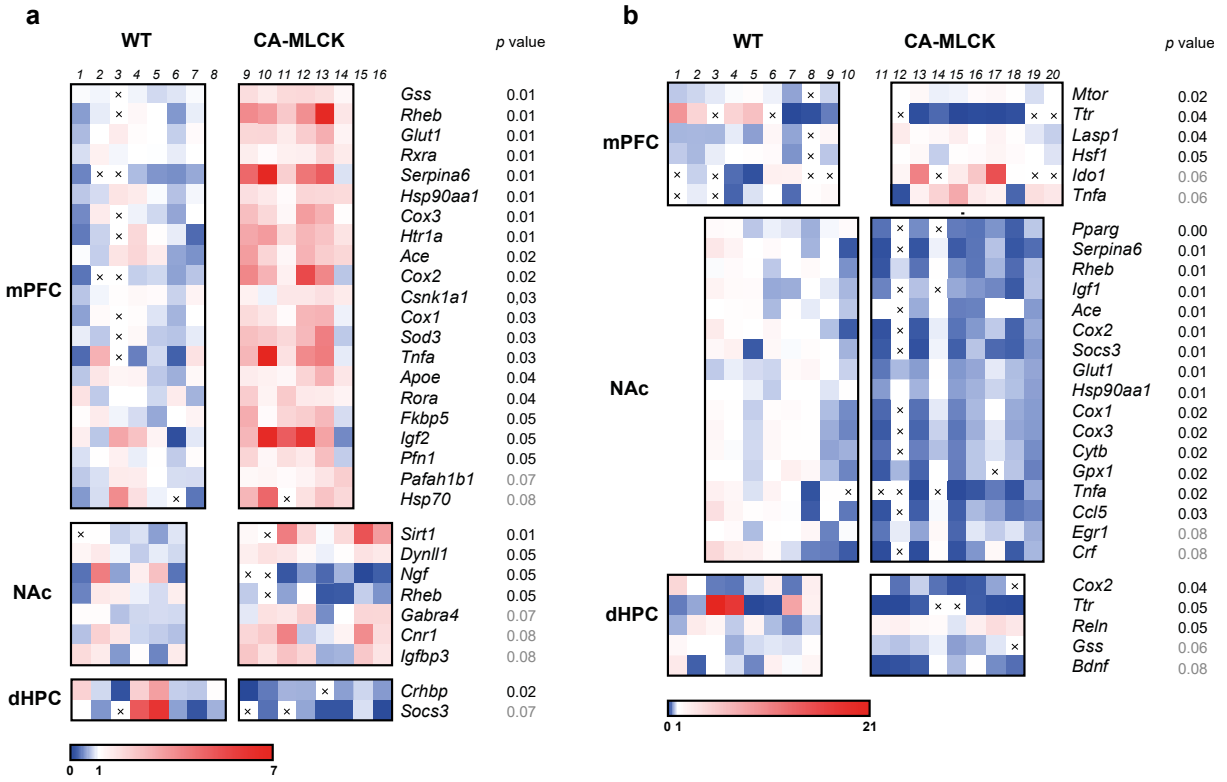


Fig 4. Tissue-specific CA-MLCK expression within intestinal epithelial cells leads to altered brain gene expression in males and females. mRNA expression (fold change) in the medial prefrontal cortex (mPFC), nucleus accumbens (NAc) and dorsal hippocampus (dHPC) of male **(a)** and female **(b)** mice. All data are expressed relative to the housekeeping gene Beta-2-microglobulin (B2m). Each row represents a single gene (n=6-9 per group).Down-regulation is shown in blue whereas up-regulation is shown in red; white color represents unaltered gene expression. Samples that failed the quality control as well as statistically significant outliers were excluded from analyses and are colored in black. Only significant changes are shown in this figure (Mann-Whitney *U* tests). Among the 92 genes tested, mRNA expression of 19 genes in the mPFC, 4 genes in the NAc and 1 gene in the dHPC was significantly altered by genotype in males; 4 genes in the mPFC, 15 genes in the NAc and 3 genes in the dHPCof females (at least $p<0.05$).*$p<0.05$ *versus* WT (in **a** and **c**: Planned comparisons, in **b** and **d**: Student *t*-tests). Ace, Angiotensin converting enzyme; Apoe, Apolipoprotein E; Bdnf, Brain-derived neurotrophic factor; Ccl5, C-C motif chemokine ligand 5; Cnr1, Cannabinoid receptor 1; Cox1, Cytochrome c oxidase subunit 1; Cox2, Cox subunit 2; Cox3, subunit 3; Crf, Corticotropin releasing factor; Crhbp, Crf-binding protein; Csnk1a1, Casein kinase 1 alpha 1; Cytb, Cytochrome B; Dynll1, Dynein light chain LC8-type 1; Egr1, Early growth response protein 1; Fkbp5, FK506 (tacrolimus)-binding protein 5; Gabra4, Gamma-aminobutyric acid (GABA) type A receptor alpha 4; Glut1, Glucose transporter 1; Gpx1, Glutathione peroxidase 1; Gss, Glutathione synthetase; Hsf1, Heat shock factor 1; Hsp70, Heat shock protein family A; Hsp90aa1, Heat shock protein 90 alpha family class A member 1; Htr1a, 5-hydroxytryptamine (serotonin) receptor 1A; Ido1, Indoleamine 2,3-dioxygenase 1; Igf1, Insulin like growth factor 1; Igf2, Insulin like growth factor 2; Igfbp3, Igf-binding protein 3; Lasp1, Lin11/Isl1/Mec3 (LIM) and sarcomatoid renal carcinoma (Src) homology 3 domain (SH3) protein 1; Mtor, Mechanistic target of rapamycin kinase; Ngf, Nerve growth factor; Pafah1b1, Platelet-activating factor acetylhydrolase,

isoform 1b, subunit 1; Pfn1, Profilin 1; Pparg, Peroxisome proliferator-activated receptor gamma; Reln, Reelin; Rheb, Ras homolog enriched in brain; Rora, Retinoic acid receptor (Rar) related orphan receptor A; Rxra, Retinoid X receptor alpha; Rxrb, Retinoid X receptor beta; Serpina6, Serpin family A member 6; Sirt1, Sirtuin 1; Socs3, Suppressor of cytokine signaling 3; Sod3, Superoxide dismutase 3; Tnfa, Tumor necrosis factor alpha; Ttr, Transthyretin. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

FIGURE 5.

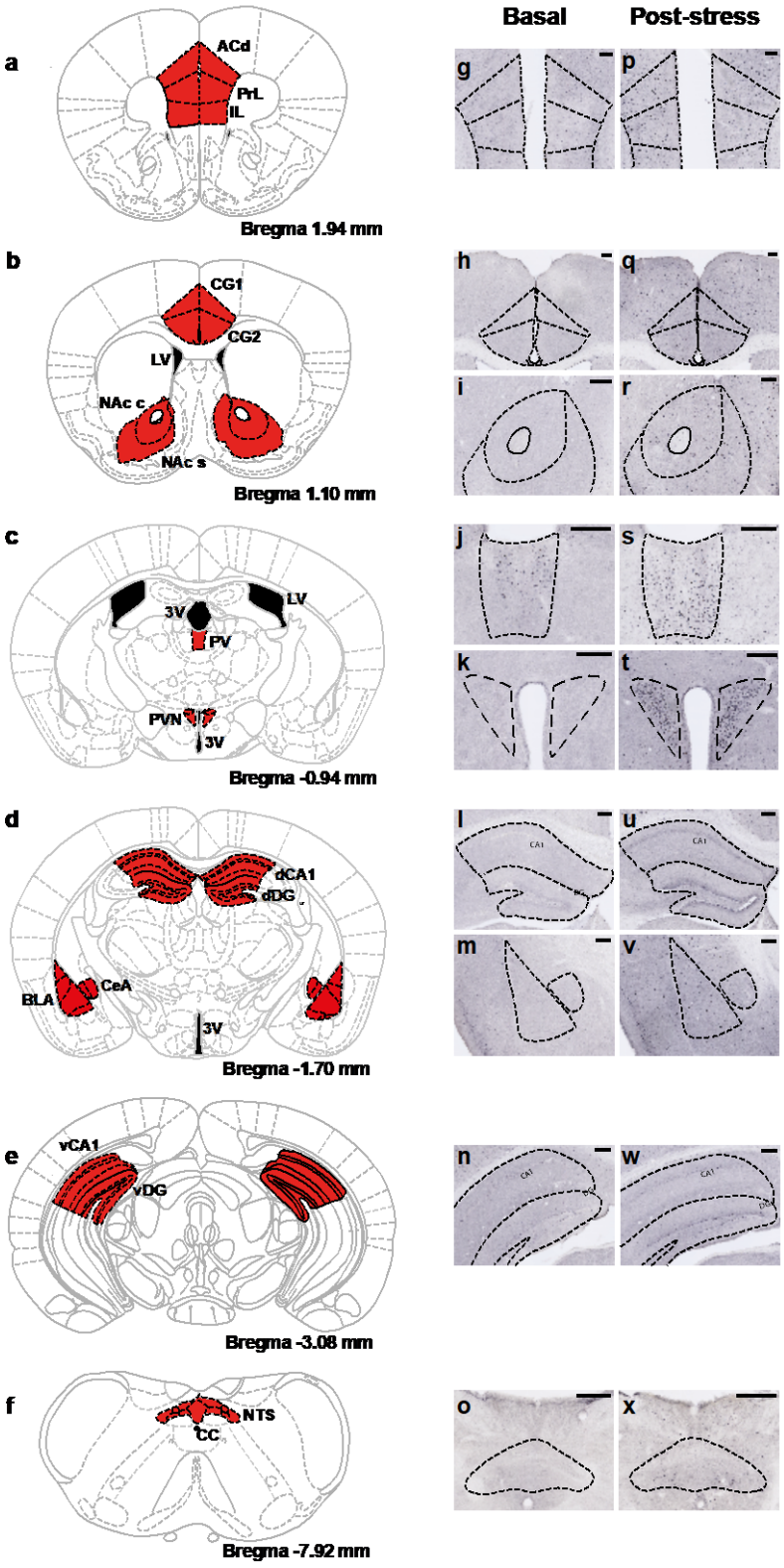
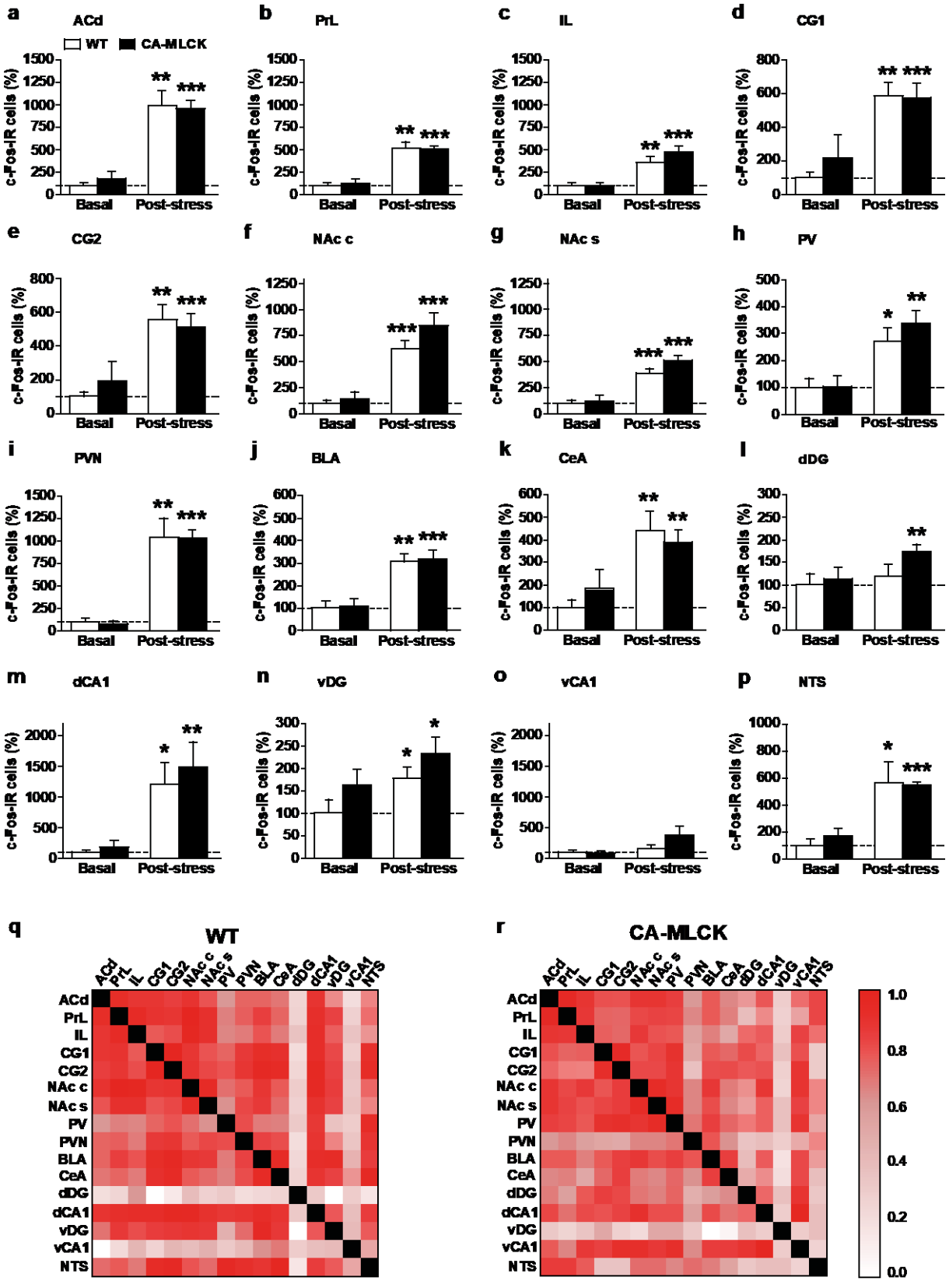


Fig 5. C-FOS immunomapping in the brain of WT and CA-MLCK mice. Regions of interest (ROI) (**a-f)** and representative images of C-FOS immunoreactivities in basal **(g-o)** and post-stress conditions **(p-x).** Acd, PrL and IL(mPFC) **(a,g,p)**, CG1 and CG2 **(b,h,q)**, NAccore and shell **(b,i,r)**, PV **(c,j,s)**, PVN **(c,k,t)**, dCA1 and dDG **(d,l,ur)**, BLA and CeA **(d,m,vr)**, vCA1 and vDG **(e,n,w)** and NTS **(f,o,x)**. ROI are colored in red and ventricles in black. All images are from male mice except for the NTS in **o** and **x**. Scale bars represent 200 µm. Acd, Anterior cingulate dorsal; PrL, Prelimbic cortex; IL, Infralimbic cortex; CG, Cingulate cortex; LV, Lateral ventricle; NAc c, Nucleus accumbens core; NAc s, Nucleus accumbens shell; PV, Paraventricular nucleus of the thalamus; 3V, 3rd ventricle; PVN, Paraventricular nucleus of the hypothalamus; BLA, Basolateral amydgdala; CeA, Central amygdala; dDG, dorsal dentate gyrus; dCA1, dorsal cornu ammonis of the hippocampus; vDG, ventral dentate gyrus; vCA1, ventral cornu ammonis of the hippocampus; NTS, Nucleus of the tractus solitaris; CC, Central canal.

FIGURE 6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BLA
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CeA
l
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dCA1
n
vDG
o
vCA1
p
NTS
c-Fos-IR cells (%)
Basal
Post-stress
q
WT
r
CA-MLCK
ACd
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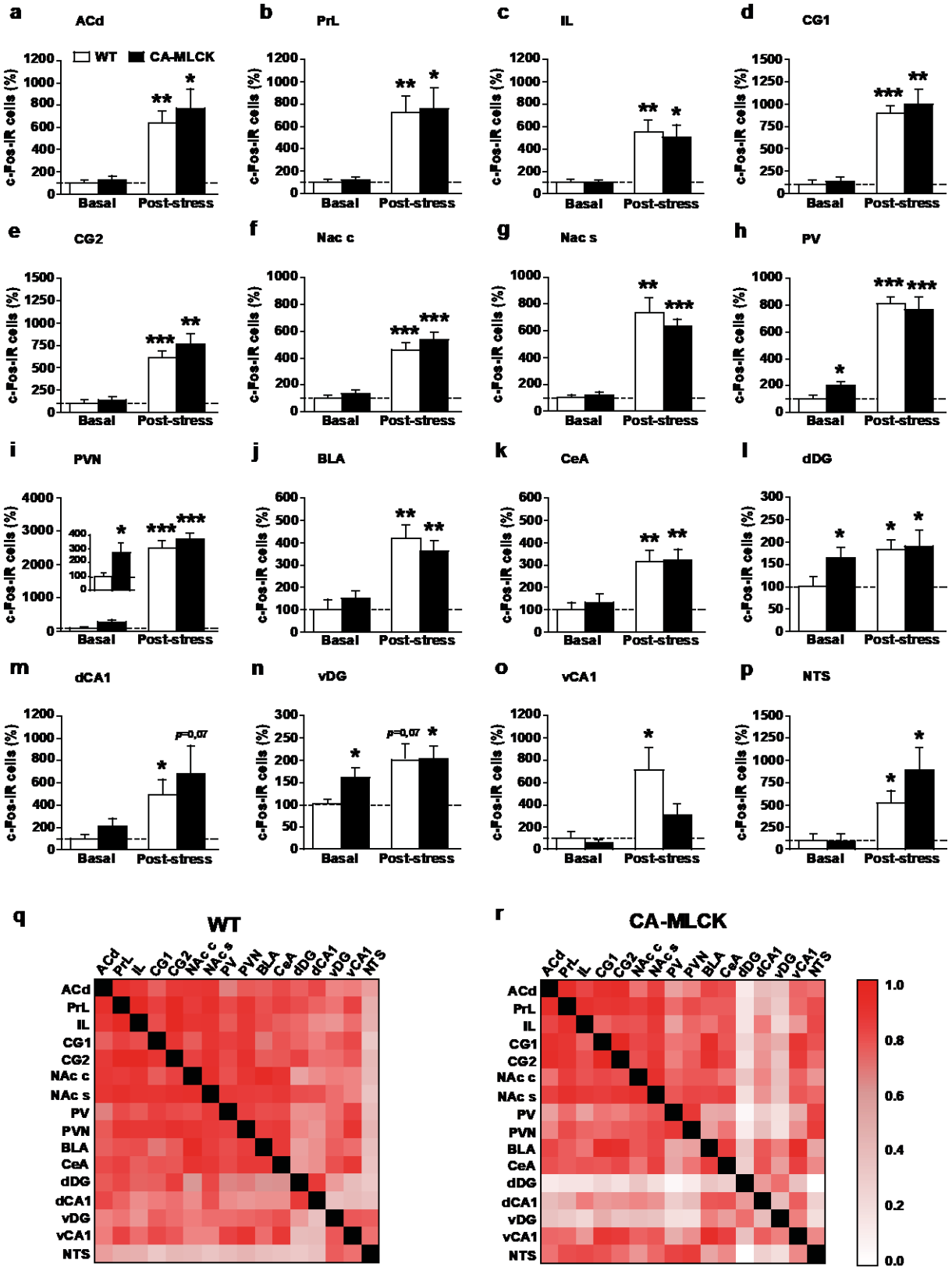
Fig 6. Intestinal CA-MLCK expression alters hippocampal c-fos expression following restraint stress in males. C-FOS expression (number of C-FOS-IR cells per mm²) in the mPFC **(a-c)**, CG **(d-e)**, NAc **(f-g)**, PV **(h)**, PVN **(i)**, amygdala **(j-k)**, hippocampus **(l-o)** and NTS **(p)** in basal conditions or following 1hr of restraint stress. Data are expressed as percentage of the WT basal group (dashed line).Overall, stress induces marked C-FOS expression in the brain of both genotypes. Interestingly, WT males show no increase of C-FOS expression in the dorsal DG and ventral CA1, whereas CA-MCLK males display neuronal activation in response to stress in these brain areas. Data are mean ± SEM. Comparison with 100%: *p<0.05, **p<0.01, ***p<0.001. Correlation heatmaps (Spearman *R* values) based on neuronal activation in the different regions of interest in WT **(q)** and CA-MLCK **(r)** males. Dark red represents strongest correlations (*R*=1) whereas white illustrates the absence of correlation (*R*=0). N=6 per group. Acd, Anterior cingulate dorsal; PrL, Prelimbic cortex; IL, Infralimbic cortex; CG, Cingulate cortex; NAc c, Nucleus accumbens core; NAc s, Nucleus accumbens shell; PV, Paraventricular nucleus of the thalamus; PVN, Paraventricular nucleus ofthe hypothalamus; BLA, Basolateral amydgdala; CeA, Central amygdala; dDG, dorsal dentate gyrus; dCA1, dorsal cornu ammonis of the hippocampus; vDG, ventral dentate gyrus; vCA1, ventral cornu ammonis of the hippocampus; NTS, Nucleus of the tractus solitarius. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

FIGURE 7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c-Fos-IR cells (%)
Basal
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Fig 7. Intestinal CA-MLCK expression alters basal C-FOS expression in the PV, the PVN and the DG in females. C-FOS expression (number of C-FOS-IR cells per mm²) in the mPFC **(a-c)**, CG **(d-e)**, NAc **(f-g)**, PV **(h)**, PVN **(i)**, amygdala **(j-k)**, hippocampus **(l-o)** and NTS**(p)** in basal conditions or following 1hr of restraint stress. Data are expressed as percentage of the WT basal group (dashed line). Overall, stress induces marked C-FOS expression in the brain of both genotypes. Interestingly, CA-MLCK females show no increase of C-FOS expression in the dorsal and ventral DG whereas WT females display neuronal activation in response to stress in these brain areas. In basal conditions, there is a greater C-FOS expression in the PV, PVN and dorsal and ventral DG of CA-MLCK mice compared with WT. Data are mean ± SEM. Comparison with 100%: *p<0.05, **p<0.01, ***p<0.001. Correlation heatmaps (Spearman *R* values) based on neuronal activation in the different regions of interest in WT **(q)** and CA-MLCK **(r)** females. Dark red represents strongest correlations (*R*=1) whereas white illustrates the absence of correlation (*R*=0). N=6 per group. Acd, Anterior cingulate dorsal; PrL, Prelimbic cortex; IL, Infralimbic cortex; CG, Cingulate cortex; NAc c, Nucleus accumbens core; NAc s, Nucleus accumbens shell; PV, Paraventricular nucleus of the thalamus; PVN, Paraventricular nucleus ofthe hypothalamus; BLA, Basolateral amydgdala; CeA, Central amygdala; dDG, dorsal dentate gyrus; dCA1, dorsal cornu ammonis of the hippocampus; vDG, ventral dentate gyrus; vCA1, ventral cornu ammonis of the hippocampus; NTS, Nucleus of the tractus solitarius. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

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SUPPLEMENTARY INFORMATION

Supplementary Table 1. Primer list for brain gene expression.

Genes	Forward Primers 5'-3'	Reverse Primers 5'-3'	Amplicon size (bp)
Ace	AGTTGCCCGGAATGAAACCC	ACTGGAACTGCAGCACAAAGC	104
Apoe	TGCGAAGATGAAGGCTCTGTG	GGTTGGTTGCTTTGCCACTC	111
B2m	AGTTAAGCATGCCAGTATGGCC	TCTCGATCCCAGTAGACGGTCT	51
Bdnf	CACAATGTTCCACCAGGTGAGA	GCCTTCATGCAACCGAAGT	81
Bdnf IV	CAGAGCAGCTGCCTTGATGTT	GCCTTGTCCGTGGACGTTTA	150
Cat	TGCCGTTCGATTCTCCACAG	AGGGGTGTTGTTTCCCACAAG	127
Ccl5	CACTCGGTCCTGGGAAAATG	TGCTGATTTCTTGGGTTTGCTG	70
Cd11c	AACCCCGTCCCTCTTATCGT	GGGTCCCGTCTGAGACAAAC	151
Cnr1	CCTGGGAAGTGTCATCTTTGTCT	GGTAACCCCACCCAGTTTGA	99
Cox1	CAAAAGCCCACTTCGCCATC	GAAAGGCCCAGGAAATGTTGAG	73
Cox2	CCGAGTCGTTCTGCCAATAGAA	TGATTTAGTCGGCCTGGGATG	123
Cox3	AACCCTTGGCCTACTCACCAA	TGGCCTTGGTAGGTTCCTTCA	81
Crebbp	TCTCCGCGAATGACAACACAG	ACGCAGCATCTGGAACAAGG	126
Crf	AAAGCAGATGGGAGTCATCCA	TCTTCCACTGCAGCTCCAAA	51
Crhbp	TGGTTCCATACCAGCACCAAAAC	AGCTCCACAAAGTCACCAGTCC	148
Csnk1a1	CGTCGGTGGAAAATACAAACTGG	TCTCGTACAGCAACTGGGGATG	146
Cx3cr1	CCCATCTGCTCAGGACCTCACC	CGCCCAAATAACAGGCCTCAGC	98
Cytb	CCCGATTCTTCGCTTTCCAC	CCTGTTGGGTTGTTTGATCCTG	103
Dncic1	AACTTCGTGGTTGGCAGTGAG	ACCGATGCCTGCTTTGCTTC	72
Dynll1	ATCGAGAAGGATATTGCGGCCC	TTTCGGCCCACAATGCAGTG	83
Erk1	TCCCCATAGCCTGAGTGATGAG	CCATTCCAGAACGGTCTACCAGA	102
Fkbp1a	TCCTCTCGGGACAGAAACAAGC	AGTTTGGCTCTCTGACCCACAC	110
Fkbp5	CAAACCCAAACGAAGGAGCAAC	TCCCCAACAACGAACACCAC	97
Fos B	GGGATCTTGCAGAGGGAACTTG	TGGCCGAGTGGAATGAGATG	130
G6pd	GCAGTCACCAAGAACATTCAAGAG	CACGATGATGCGGTTCCA	63
Gabra4	TCCCCAGGACAGAACTCAAAGG	AACAGGACCCCCAAATCCAG	117
Galnt9	GAACGTGTACCCCGAGATGAG	GCCGCTAGCTTTGCTGTTTC	76
Glut1	TCGTCGTTGGCATCCTTATTG	TGCATTGCCCATGATGGA	59
Glut4	TCCCTTCAGTTTGGCTATAACATTG	CTACCCAGCCACGTTGCATT	86
Gpm6a	ACTGCTGGAGACACACTGGATG	AAGAAAGCAGCCGCAATGCC	80
Gpx1	TCGGACACCAGAATGGCAAG	AGGAAGGTAAAGAGCGGGTGAG	142
Gss	CCGACACGTTCTCAATGTCCTG	TCCCTTGCTGGGGTTATTGG	73
Hacd2	GTCATTGCCTGGACAATCACGG	TCCCATTGGGTACAGCACGATG	126
Hsd11b1	GGAAGGTCTCCAGAAGGTAGTGTC	GAGGCTGCTCCGAGTTCAAG	51
Hsf1	TGGCCATGAAGCACGAGAAC	TTTGCTGCTGGGCATGCTTC	75
Hsf2	CCGGGCTAACAATGAAGCAG	TCGTTGGTGTGGGTTTCCTC	85
Hsp70	GGGCATCGACTTCTACACATCC	TCTGCGCCTTGTCCATCTTG	125
Hsp90aa1	TGGCAGTAAAGCATTTTTCTGTTG	AGCGCGTCTTGGGACAAA	71
Htr1a	TCACTTGGCTCATTGGCTTTC	GCGCCAGCCCAGCAT	53
Htr2c	TGCTGATATGCTGGTGGGACTA	CTAGGTAAAGGCCAGACATAATCATAAA	81
Ido1	ACGACATAGCTACCAGTCTGGAGAA	AAACGTGTCTGGGTCCACAAA	77
Igf1	ACTTCAACAAGCCCACAGGC	TCTGAGGTGCCCTCCGAAT	51
Igf2	TCGGAGGCCACAAAAGATGG	TCCCCCAAATGCTCAGAAGG	127
Igfbp3	TAAGAAGAAGCAGTGCCGCC	ACGCTGAGGCAATGTACGTC	126
Itpr1	ATCGGCCACCAGTTCCAAAG	AGCCAAGTAATGCCCTGTAGCC	127
Kif5c	ATGTAAAGGGGTGCACCGAGAG	ACGTGTCGGTTTGCTTTGCC	85
Lasp1	TCAAGCAACAGAGCGAGCTG	ACCACGCTGAAACCTTTGCC	82
Limk1	TCCGAGCACATCACCAAAGG	AGGCGAGGCAGATGAAACAC	79
Maoa	TGAGGTATCTGCCCTGTGGTTC	CCCCAAGGAGGACCATTATCTG	146
Map1b	TCAGATGAAGCCGTCAGCACTG	AGCACCAGCAGTTTATGGCG	71
Mapk1	AGCTAACGTTCTGCACCGTG	TGATCTGGATCTGCAACACGGG	108
Me1	TGACCAAGGGACGTGCAA	GGGAGAGTGACTGGATCAAAAGG	58

Me3	GACAAGGGCCACATTGCAA	ACCACCACAGCCTTAATATTGTCTT	65
Mhc II	GCTCTCGGAGACCTATGACG	ACAGGCAAACCTCTGGACAC	210
Mtor	TGGGTGCTGACCGAAATGAG	TCCCACACAGCCACAAAAATG	133
Nfkb1	GCCAGCTTCCGTGTTTGTTC	GGCGTTTCCTTTGCACTTCC	116
Nfkb2	TGCGCTTCTCAGCTTTCCTTC	AACCCGCTGTCTTGTCCATTC	141
Ngf	ATCAAGGGCAAGGAGGTGACAG	GAGTTCCAGTGTTTGGAGTCGATG	143
Ngf1a	AGCCGAGCGAACAACCC	TGTCAGAAAAGGACTCTGTGGTCA	51
Nr3c1	GTGGAAGGACAGCACAATTACCT	GCGGCATGCTGGACAGTT	87
Nr3c2	GCCGTGGAAGGACAACACA	CCTAAGTTCATGCCGGCTTG	125
Nr4a1	CTGCCTTCCTGGAACTCTTCA	CGGGTTTAGATCGGTATGCC	51
Nsf	GCATTTGCTTCTCGGGTGTTC	TGCCAATCTGTCGAGCCAAG	124
Pafah1b1	GATGTGGGAAGTGCAAACTGG	CTGATTTGGCCGCACCATAC	82
Per1	TGTCCTGCTGCGTTGCAAAC	TTGAGACCTGAACCTGCAGAGG	150
Per2	GCTGGCAACCTTGAAGTATGC	TGGTAGTACTCCTCATTAGCCTTCAC	66
Pfn1	ATCGTAGGCTACAAGGACTCGC	AACCTCAGCTGGCGTAATGC	81
Pgd	CATCTCGGCGCTGGAGTAC	GCACCGAGCAAAGACAGCTT	64
Pparg	CAAGAATACCAAAGTGCGATCAAA	AGCTGGGTCTTTTCAGAATAATAAGG	68
Ppargc1a	GTCTTAGCACTCAGAACCATGCA	CCATGAATTCTCGGTCTTAACAATG	83
Ppargc1b	TGAGGTGTTCGGTGAGATTGTAGA	AAGGTGATAAAACCGTGCTTCTG	78
Prex1	TCCGACAAGCAGGACAAACTTC	TCATGGGGAAATGCCTGGTC	127
Rarb	CCGCCTGCTTGGATATCTTG	GTGTAAGGCCATCAGAGAAAGTCA	87
Rarg	CCCAAGGATGCTGATGAAAATC	GCCCTTTCTGCTCCCTTAGTG	63
Reln	AGTACTCAGATGTGCAGTGGGCAA	AG CGCTCCTTCAGGAAAGTCTTCA	171
Rheb	CACCAAGATGCCTCAGTCCAAG	ATGAGGACTTTCCCACAGACCG	71
Rora	GGAATCCATTATGGTGTCATTACG	GTGGCATTGCTCTGCTGACTT	74
Rxra	CCATCTTTGACAGGGTGCTAACA	ATCTGCATGTCACGCATCTTAGAC	55
Rxrb	GCGCCAGCGGAATCG	AACCGCCTCCCTTTTCATG	73
Serpina6	AGCAGACGACCTGGTCAACC	GACAGGTATACAGGGCAAGCG	51
Sgk1	CGTCAAAGCCGAGGCTGCTCGAAGC	GGTTTGGCGTGAGGGTTGGAGGAC	279
Sgk1.1	GAAGGCGGATCGGGATACAGATGCAGTAA	GGTTTGGCGTGAGGGTTGGAGGAC	644
Sirt1	AGACCCTCAAGCCATGTTTG	ACACAGAGACGGCTGGAAC	105
Sirt2	TCCACTGGCCTCTATGCAAACC	TTGGCAAGGGCAAAGAAGGG	110
Slc6a4	TCGCCAGAGCACATCCAAAG	TTTTGCCCGTTCCAAGAGAAG	131
Socs3	AGCAGATGGAGGGTTCTGCTTTGT	ATTGGCTGTGTTTGGCTCCTTGTG	112
Sod1	TTGGCCGTACAATGGTGGTC	GCAATCCCAATCACTCCACAG	118
Sod3	TTCCCAGTGAGCACCTTTGAG	AAGGGGGCAGAAGGAAATGG	77
Stat3	GAAACTTAATGAAGAGTGCCTTCGT	CCGGTCCGGGTGCAT	65
Tgfb1	TTGCTTCAGCTCCACAGAGA	TGGTTGTAGAGGGCAAGGAC	183
Tnfa	ATGATCCGCGACGTGGAA	ACCGCCTGGAGTTCTGGAA	73
Ttr	CACTTGGCATTTCCCCGTTC	TCTCAATTCTGGGGGTTGCTG	145
Ttyh1	TGGCAAAGCAGAGCAAATGG	TAGAACCCCAGCTCAGGACAAG	78

Ace, Angiotensin converting enzyme; Apoe, Apolipoprotein E; B2m, Beta-2-microglobulin; Bdnf, Brain-derived neurotrophic factor; Bdnf IV, Bdnf exon IV; Cat, Catalase; Ccl5, C-C motif chemokine ligand 5; Cd11c, Complement component 3 receptor 4 subunit (Itgax); Cnr1, Cannabinoid receptor 1; Cox1, Cytochrome c oxidase subunit 1; Cox2, Cox subunit 2; Cox3, Cox subunit 3; Crebbp, C-adenosine monophosphate (AMP) response element-binding protein-binding protein; Crf; Corticotropin releasing factor; Crhbp; Crf-binding protein; Csnk1a1, Casein kinase 1 alpha 1; Cx3cr1, C-X3-C motif chemokine receptor 1; Cytb, Cytochrome B; Dncic1, Dynein cytoplasmic 1 intermediate chain 1 (Dync1i1); Dynll1, Dynein light chain LC8-type 1; Erk1, mitogen-activated protein kinase 3 (Mapk3); Fkbp1a, FK506 (Tacrolimus)-binding protein 1a; Fkbp5, FK506-binding protein 5; Fos B, Finkel–Biskis–Jinkins (FBJ) murine osteosarcoma viral oncogene homolog B; G6pd, Glucose-6-phosphate dehydrogenase; Gabra4, Gamma-aminobutyric acid (GABA) type A receptor alpha 4; Galnt9, Polypeptide N-acetylgalactosaminyltransferase 9; Glut1, Glucose transporter 1; Glut4, Glucose transporter 4; Gpm6a, Glycoprotein M6A; Gpx1, Glutathione peroxidase 1; Gss, Glutathione synthetase; Hacd2, 3-hydroxyacyl-CoA dehydratase 2; Hsd11b1, Hydroxysteroid 11-beta dehydrogenase 1; Hsf1, Heat shock factor 1; Hsf2, Heat shock factor 2; Hsp70, Heat shock protein family A; Hsp90aa1, Heat shock protein 90 alpha family class A member 1; Htr1a, 5-hydroxytryptamine (serotonin) receptor 1A; Htr2c, Serotonin receptor 2C; Ido1, Indoleamine 2,3-dioxygenase 1; Igf1, Insulin like growth factor 1; Igf2, Insulin like growth factor 2; Igfbp3, Igf-binding protein 3; Itpr1, Inositol 1,4,5-trisphosphate receptor, type 1; Kif5c, Kinesin family member 5C; Lasp1, Lin11/Isl1/Mec3 (LIM)

and sarcomatoid renal carcinoma (Src) homology 3 domain (SH3) protein 1; Limk1, LIM domain kinase 1; Maoa, Monoamine oxidase A; Map1b, Microtubule-associated protein 1B; Mapk1, mitogen-activated protein kinase 1; Me1; Malic enzyme 1; Me3, Malic enzyme 3; Mhc II, Major histocompatibility complex, class II; Mtor, Mechanistic target of rapamycin kinase; Nfkb1, nuclear factor kappa B subunit 1; Nfkb2, Nfkb subunit 2; Ngf, Nerve growth factor; Ngf1a, Early growth response protein 1 (Egr-1); Nr3c1, Nuclear receptor subfamily 3 group C (glucocorticoid receptor) member 1; Nr3c2, Glucocorticoid receptor member 2; Nr4a1, Nuclear receptor subfamily 4 group A member 1; Nsf, N-ethylmaleimide sensitive factor; Pafah1b1, Platelet-activating factor acetylhydrolase, isoform 1b, subunit 1; Per1, Period circadian regulator 1; Per2, Period circadian regulator 2; Pfn1, Profilin 1; Pgd, Phosphogluconate dehydrogenase; Pparg, Peroxisome proliferator-activated receptor gamma; Ppargc1a, Pparg coactivator 1 alpha; Ppargc1b, Prex1, Phosphatidylinositol-3,4,5-trisphosphate dependent Ras-related C3 (Rac) exchange factor 1; Rarb, Retinoic acid receptor beta, Rarg, Retinoic acid receptor gamma; Reln, Reelin; Rheb, Ras homolog enriched in brain; Rora, Rar related orphan receptor A; Rxra, Retinoid X receptor alpha; Rxrb, Retinoid X receptor beta; Serpina6, Serpin family A member 6; Sgk1, Serum/Glucocorticoid Regulated Kinase 1; Sgk1.1, Serum/glucocorticoid regulated kinase 1, brain isoform; Sirt1, Sirtuin 1; Sirt2, Sirtuin 2; Slc6a4, solute carrier family 6 member 4 (serotonin transporter); Socs3, Suppressor of cytokine signaling 3; Sod1, Superoxide dismutase 1; Sod3, Superoxide dismutase 3; Stat3, Signal transducer and activator of transcription 3; Tgfb1, Transforming growth factor beta 1; Tnfa, Tumor necrosis factor alpha; Ttr, Transthyretin; Ttyh1, Tweety family member 1.

Supplementary Table 2. Detailed statistics for C-Fos data in males. One-way Kruskal-Wallis ANOVAs were followed by Mann-Whitney *U* between group tests. N=6 per group.

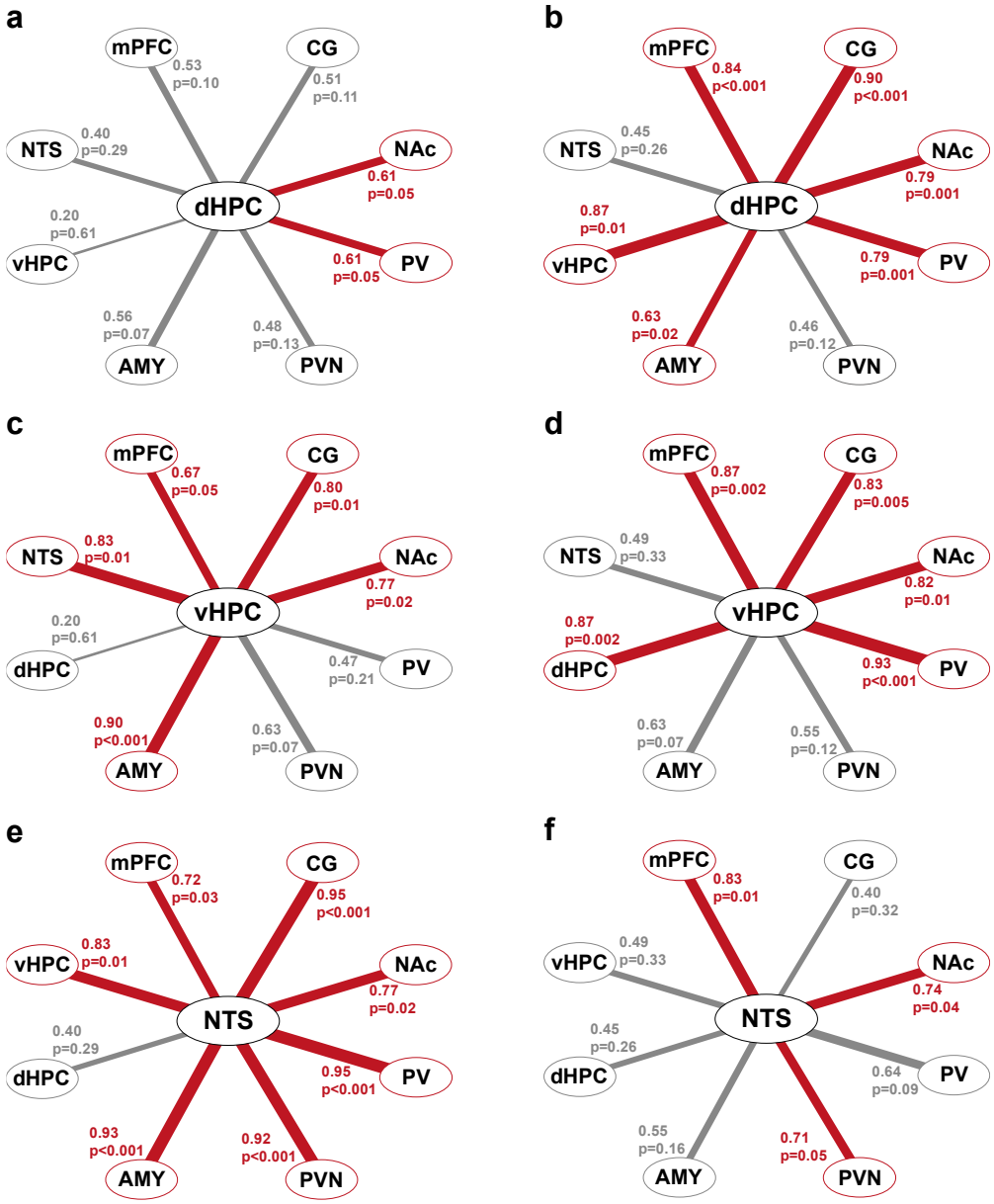
Brain area	N	Kruskal-Wallis *H*	Kruskal-Wallis *p*	WT basal *versus* WT post-stress	CA-MLCK basal *versus* CA-MLCK post-stress	WT basal *versus* CA-MLCK basal	WT post-stress *versus* CA-MLCK post-stress	WT basal *versus* CA-MLCK post-stress	CA-MLCK basal *versus* WT post-stress
Acd	24	16.81	*p*<0.001	*p*=0.0043	*p*=0.0016	n.s.	n.s.	*p*=0.0016	*p*=0.0043
PrL	24	16.34	*p*=0.001	*p*=0.0043	*p*=0.0016	n.s.	n.s.	*p*=0.0016	*p*=0.0087
IL	24	16.47	*p*<0.001	*p*=0.0087	*p*=0.0016	n.s.	n.s.	*p*=0.0016	*p*=0.0087
CG1	24	12.52	*p*=0.05	*p*=0.0043	*p*=0.0451	n.s.	n.s.	*p*=0.0016	*p*=0.0823
CG2	24	13.09	*p*=0.04	*p*=0.0043	*p*=0.0451	n.s.	n.s.	*p*=0.0016	*p*=0.0303
NAC c	24	17.79	*p*<0.001	*p*=0.0043	*p*=0.0016	n.s.	*p*=0.1079	*p*=0.0016	*p*=0.0043
NAC s	24	16.22	*p*=0.001	*p*=0.0043	*p*=0.0031	n.s.	*p*=0.1812	*p*=0.0016	*p*=0.0173
PV	24	13.89	*p*=0.003	*p*=0.0173	*p*=0.0062	n.s.	n.s.	*p*=0.0031	*p*=0.0173
PVN	24	16.86	*p*<0.001	*p*=0.0043	*p*=0.0016	n.s.	n.s.	*p*=0.0016	*p*=0.0043
BLA	24	14.06	*p*=0.002	*p*=0.0087	*p*=0.0062	n.s.	n.s.	*p*=0.0062	*p*=0.0087
CeA	24	11.00	*p*=0.01	*p*=0.0173	*p*=0.0653	n.s.	n.s.	*p*=0.0031	*p*=0.0823
dDG	24	6.45	*p*=0.09	*p*=0.7922	*p*=0.0451	n.s.	*p*=0.1419	*p*=0.0295	n.s.
dCA1	24	15.45	*p*=0.01	*p*=0.0043	*p*=0.0062	n.s.	n.s.	*p*=0.0016	*p*=0.0087
vDG	18	5.96	*p*=0.11	*p*=0.0635	*p*=0.2857	n.s.	n.s.	*p*=0.0635	n.s.
vCA1	18	5.61	*p*=0.13	*p*=0.7302	*p*=0.0635	n.s.	n.s.	*p*=0.0635	n.s.
NTS	17	9.83	*p*=0.02	*p*=0.0476	*p*=0.0476	n.s.	n.s.	*p*=0.0571	*p*=0.0667

Supplementary Table 3. Detailed statistics for C-Fos data in females. One-way Kruskal-Wallis ANOVAs were followed by Mann-Whitney *U* between group tests. N=6 per group.

Brain area	N	Kruskal-Wallis *H*	Kruskal-Wallis *p*	WT basal *versus* WT post-stress	CA-MLCK basal *versus* CA-MLCK post-stress	WT basal *versus* CA-MLCK basal	WT post-stress *versus* CA-MLCK post-stress	WT basal *versus* CA-MLCK post-stress	CA-MLCK basal *versus* WT post-stress
Acd	24	15.17	*p*=0.01	*p*=0.0022	*p*=0.0087	n.s.	n.s.	*p*=0.0865	*p*=0.0022
PrL	24	17.45	*p*<0.001	*p*=0.0022	*p*=0.0022	n.s.	n.s.	*p*=0.0022	*p*=0.0022
IL	24	15.58	*p*=0.001	*p*=0.0022	*p*=0.0087	n.s.	n.s.	*p*=0.0022	*p*=0.0022
CG1	24	17.61	*p*<0.001	*p*=0.0022	*p*=0.0022	n.s.	n.s.	*p*=0.0022	*p*=0.0022
CG2	24	18.06	*p*<0.001	*p*=0.0022	*p*=0.0022	*p*=0.1797	n.s.	*p*=0.0022	*p*=0.0022
NAC c	24	17.76	*p*<0.001	*p*=0.0022	*p*=0.0022	n.s.	n.s.	*p*=0.0022	*p*=0.0022
NAC s	24	17.45	*p*<0.001	*p*=0.0022	*p*=0.0022	n.s.	n.s.	*p*=0.0022	*p*=0.0022
PV	24	18.27	*p*<0.001	*p*=0.0022	*p*=0.0022	*p*=0.0649	n.s.	*p*=0.0022	*p*=0.0022
PVN	24	18.57	*p*<0.001	*p*=0.0022	*p*=0.0022	*p*=0.0411	n.s.	*p*=0.0022	*p*=0.0022
BLA	24	14.77	*p*=0.002	*p*=0.0087	*p*=0.0087	n.s.	n.s.	*p*=0.0043	*p*=0.0087
CeA	24	12.78	*p*=0.005	*p*=0.0087	*p*=0.0260	n.s.	n.s.	*p*=0.0043	*p*=0.0260
dDG	24	6.27	*p*=0.10	*p*=0.0260	*p*=0.6991	*p*=0.0931	n.s.	*p*=0.0931	n.s.
dCA1	24	10.90	*p*=0.01	*p*=0.0087	*p*=0.0649	n.s.	n.s.	*p*=0.0865	n.s.
vDG	20	8.00	*p*=0.05	*p*=0.0635	*p*=0.4286	*p*=0.0556	n.s.	*p*=0.0173	n.s.
vCA1	20	11.64	*p*=0.008	*p*=0.0159	*p*=0.0173	n.s.	*p*=0.1143	n.s.	*p*=0.0159
NTS	22	12.17	*p*=0.006	*p*=0.0152	*p*=0.0159	n.s.	n.s.	*p*=0.0173	*p*=0.0303

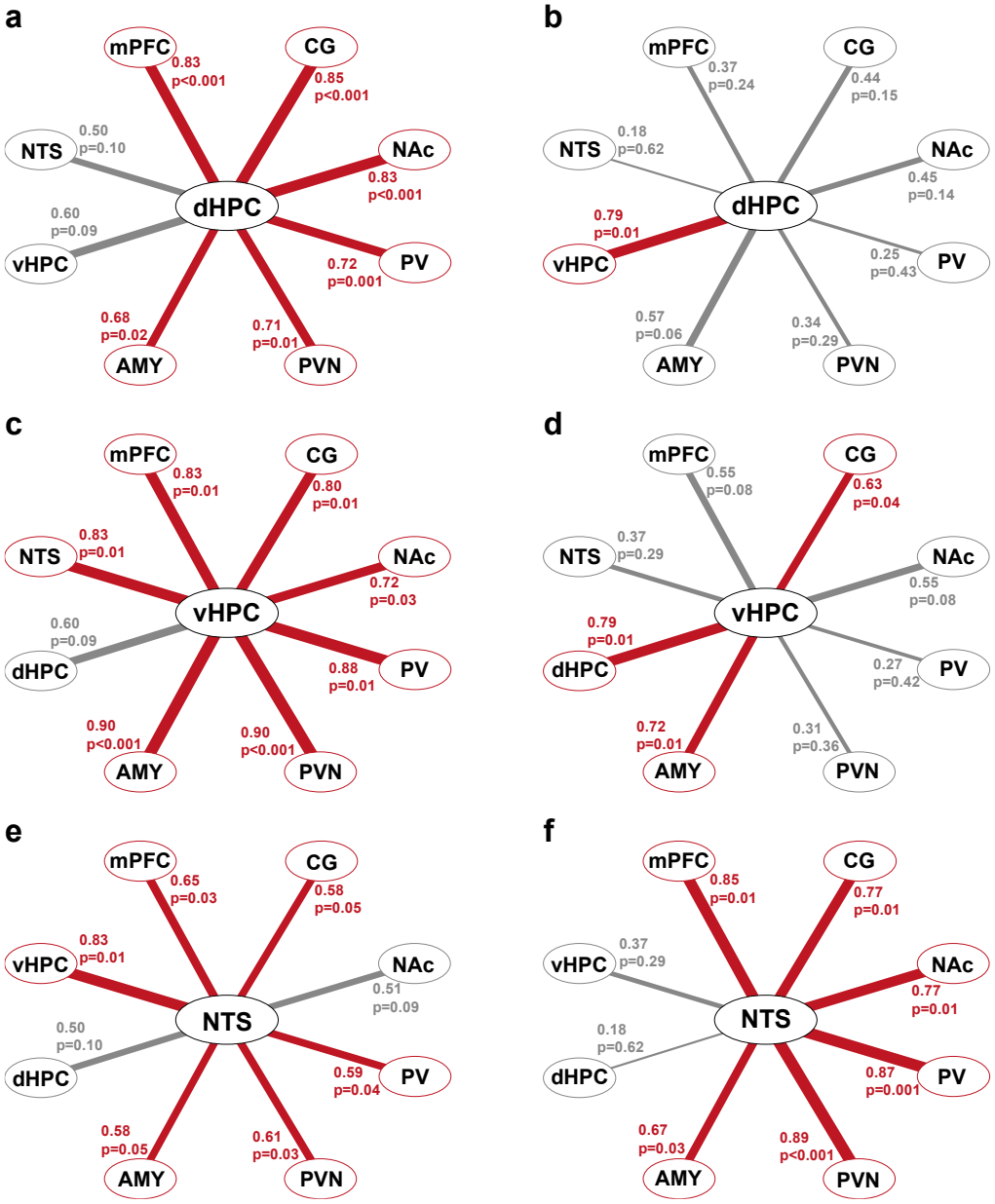
SUPPLEMENTARY FIGURES

Supplementary Figure 1



Supplementary Fig 1. Shift in coactivated brain areas in male CA-MLCK mice. Correlation patterns (Spearman *R* values) based on neuronal activation (C-Fos-IR cells) in the different regions of interest in WT **(a,c-e)** and CA-MLCK **(b,d,f)** males. N=6 per group. Dark red represents strongest correlations (*R*=1) whereas white illustrates the absence of correlation (*R*=0). Correlation strength between neuronal activation in the dorsal hippocampus and the other brain areas in WT **(c)** and CA-MLCK **(d)** males. Correlation strength between neuronal activation in the ventral hippocampus and the other brain areas in WT **(e)** and CA-MLCK **(f)** males. Correlation strength between neuronal activation in the NTS and the other brain areas in WT **(g)** and CA-MLCK **(h)** males. Red color is used when the correlation is statistically significant (*p* value); grey color is used for non significant correlations. Thickest segments represents strongest correlations (*R* value). In WT males, the dHPC only correlates with the NAc and PV, whereas in CA-MLCK mice it also correlates with the mPFC, CG, AMY and vHPC. In contrast, the vHPC correlates with the NTS, mPFC, CG, NAc and AMY in WT, but NTS and AMY correlations are lost in CA-MLCK, whereas the PV and dHPC are recruited in the circuit. Finally, the NTS correlates with all except the dHPC in WT, but only with the mPFC, NAc and PVN in CA-MLCK**.** mPFC, medial prefrontal cortex; CG, Cingulate cortex; NAc, Nucleus accumbens; PV, Paraventricular nucleus of the thalamus; PVN, Paraventricular nucleus ofthe hypothalamus; AMY, Amydgdala; dHPC, dorsal hippocampus; vHPC, ventral hippocampus; NTS, Nucleus of the tractus solitarius. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Supplementary Figure 2



Supplementary Fig 2. Shift in coactivated brain areas in female CA-MLCK mice. Correlation heatmaps (Spearman *R* values) based on neuronal activation (C-Fos-IR cells) in the different regions of interest in WT **(a,c,e)** and CA-MLCK **(b,d,f)** females. N=6 per group. Dark red represents strongest correlations (*R*=1) whereas white illustrates the absence of correlation (*R*=0). Correlation strength between neuronal activation in the dorsal hippocampus and the other brain areas in WT **(a)** and CA-MLCK **(b)** females. Correlation strength between neuronal activation in the ventral hippocampus and the other brain areas in WT **(c)** and CA-MLCK **(d)** females. Correlation strength between neuronal activation in the NTS and the other brain areas in WT **(e)** and CA-MLCK **(f)** females. Red color is used when the correlation is statistically significant (*p* value); grey color is used for non significant correlations. Thickest segments represents strongest correlations (*R* value). In WT females, the dHPC correlates with all except the vHPC and NTS, whereas it only correlates with the vHPC in CA-MLCK. Similarly the vHPC correlates with all except the dHPC in WT but NTS, mPFC, NAc, PV and PVN are lost in CA-MLCK, whereas thedHPCis recruited. Finally, the NTS correlates with all except the dHPCand NAcin WT, whereas the vHPC is lost and the NAc is recruited in CA-MLCK. mPFC, medial prefrontal cortex;CG, Cingulate cortex; NAc, Nucleus accumbens; PV, Paraventricular nucleus of the thalamus; PVN, Paraventricular nucleus ofthe hypothalamus; AMY, Amydgdala; dHPC, dorsal hippocampus;vHPC, ventral hippocampus; NTS, Nucleus of the tractussolitarius. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

ARTICLE 4

Sex-specific behavioral alterations are associated with gut dysbiosis in mice exposed to multifactorial early-life adversity

In preparation

ARTICLE 4 in preparation:

Sex-specific behavioral alterations are associated with gut dysbiosis in mice exposed to multifactorial early-life adversity

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ABSTRACT

The accumulation of adverse early-life events during brain development can exacerbate the vulnerability to neuropsychiatric diseases such as autism, schizophrenia, anxiety disorders or depression. Gut microbiota alterations have been recently hypothesized to affect brain development, and dysfunction of the gastrointestinal tract is a common feature of numerous neuropsychiatric diseases. However, the long lasting impact of early-life adversity on gut microbiota, notably with regards to sex differences, remains underexplored. This question is of particular importance since there are marked sex differences for both the prevalence of psychiatric disorders and the composition of gut microbiota. Here, we examined the effects of early-life adversity on behavior, gut permeability and microbiota composition in males and females using a multifactorial animal model in C3H/HeN mice combining maternal immune activation (120 µg/kg Lipopolysaccharide i.p. injection at embryonic day 17), maternal separation (3hr per day from postnatal day (PD)2 to PD14) and maternal unpredictable chronic mild stress during separation sessions. During infancy, ultrasonic vocalizations in response to isolation were analyzed in PND7 pups. At adulthood, animals underwent a battery of behavioral tests for anxiety, social behavior and depressive-like behaviors. In *vivo* gut permeability and visceral sensitivity to colorectal distension were also evaluated. Finally, the impact of multifactorial early adversity on gut microbiota composition and gene expression in the medial prefrontal cortex (mPFC) was studied in the same animals. Our results reveal that multifactorial early adversity impaired ultrasonic vocalization in isolated pups of both sexes. At adulthood, offspring exposed to early adversity displayed sex-specific behavioral and intestinal phenotypes. Males exposed to early adversity showed decreased social interaction but intact anxiety-like behavior. In contrast, social interaction was not affected in females, but they exhibited greater anxiety relative to controls. Depressive-like behaviors were unaffected in both sexes. Microarray analysis reveal that early adversity specifically upregulated Krüppel-like factor 2 (Klf2) mRNA in the mPFC of female mice, but had no effect in males. Gut permeability was exacerbated in males, but not in females. Finally, 16S-based microbiota profiling revealed higher proportions of *Bacteroides, Lactobacillus*, *Porphyromonas, Alloprevotella* and *unclassified Firmicutes* spp. and lower

proportions of *unclassified Lachnospiraceae* and *unclassified Porphyromonadaceae* spp. in male mice submitted to early adversity compared with controls. In females, early adversity decreased the abundance of *Mucispirillum and Lactobacillus* spp. In conclusion, our work highlights sex differences in a multifactorial model of early-life adversity, for both behavior and gut parameters. Further studies are needed to unravel the role of the gut dysbiosis reported here in the expression of the behavioral phenotypes associated with early-life adversity.

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INTRODUCTION

The risk of developing psychiatric symptoms is enhanced by early-life adverse events occurring during prenatal or early postnatal periods, when brain development is ongoing (McDonald and Murray, 2000; Opler and Susser, 2005). The multi-hit hypothesis (or cumulative stress hypothesis) proposes that neuropsychiatric disorders may be precipitated by a combination of two or more major adverse events in particular during development. (McEwen, 1998; Maynard et al., 2001; Nederhof and Schmidt, 2012). For instance, women exposed to one or more childhood adversities were more likely to become depressed following exposure to moderate stress at adulthood than women without early adversity (Hammen et al., 2000). Maternal infection and postnatal exposure to psychological stress or trauma are two environmental risk factors for several psychiatric disorders including autism, schizophrenia and depression (Phillips et al., 2005; Herbert, 2010; McLaughlin et al., 2010; Brown, 2011, 2014; Mayer et al., 2014; Ornoy et al., 2015; Flinkkilä et al., 2016). According to the multiple-hit hypothesis, it has been proposed that initial exposure to prenatal infection can render the offspring more vulnerable to the deleterious effects of a second postnatal stimulus, such as stress (Maynard et al., 2001). Previous findings support this hypothesis in animal models combining prenatal inflammation and adolescent or adult stress exposure (Deslauriers et al., 2013; Giovanoli et al., 2013; Monte et al., 2017). In contrast, the impact of prenatal inflammation combined with an early post-natal stress remains underexplored. Interestingly, even if a large majority of the literature on early stress is conducted in males, an increasing body of evidence suggests that early adversity does not equally affect emotional vulnerability in males and females (Slotten et al., 2006; Mourlon et al., 2010; Klug and van den Buuse, 2012; Foley et al., 2014a, 2014b, 2015; Hill et al., 2014; Farrell et al., 2016; Lundberg et al., 2017; Monte et al., 2017). This issue is of particular importance with respect to the gender differences observed in the prevalence of psychiatric disorders. Indeed, autism spectrum disorders are more prevalent in men (Werling and Geschwind,

2013), whereas women are more susceptible to anxiety and depression (Altemus, 2006; Steel et al., 2014). The mechanisms underlying the long-term behavioral effects of early adversity remain unclear. Recent studies suggest that gastrointestinal alterations during early-life, notably gut dysbiosis and loss of barrier function, can affect brain development and lastingly impair gut-brain communication (Hsiao et al., 2013; Kim et al., 2017; see Borre et al., 2014 for review). In particular, maternal immune activation and maternal separation (MS) are among the most used experimental models of early adversity producing short and long-term intestinal defects in association with behavioral outcomes (O'Mahony et al., 2011; Labouesse et al., 2015a). Numerous studies using either adversity have shown that microbiota-directed interventions such as probiotic treatments or fecal transplantation modulate brain and behavior, especially stress-related behaviors (Hsiao et al., 2013; Mattei et al., 2014; De Palma et al., 2015; Giovanoli et al., 2016; Kim et al., 2017; Moya-Pérez et al., 2017). Importantly, it has been reported that gut microbiota composition differs according to sex both in animals and humans (Markle et al., 2013; Hollister et al., 2014; Dominianni et al., 2015; Jašarević et al., 2016; Fransen et al., 2017). However, to our knowledge, sex differences in gut microbiota in a context of early adversity remain unexplored. Indeed, most of the studies use males and the few studies involving males and females often pool both sexes together for data analysis (Hsiao et al., 2013; De Palma et al., 2015; El Aydi et al., 2017). In the present study, we hypothesized that early adversity differentially affects the gut microbiota in males and females and that these differential effects may underlie the sex differences observed at the behavioral level. To test this hypothesis, we developed a mouse model of multifactorial early adversity combining prenatal inflammation (lipopolysaccharide (LPS) injection), post-natal MS and unpredictable chronic mild stress (UCMS) in dams and we investigated emotional behavior, intestinal function and gut microbiota in adult male and female offspring.

METHODS

Animals

All experiments were approved by the Bioethical committee of our University (N° 50120186-A) and région Aquitaine Veterinary Services (Direction Départementale de la Protection des Animaux, approval ID: A33-063-920) according to the European (Directive 2010/63/EU, 22 September 2010) legislation. Mice were maintained in a 12-h light/12-h dark cycle (lights on at 0800 hours) in a temperature-controlled room (22 °C) with free access to food and water, unless otherwise mentioned. Gestant female C3H/HeNRj mice (n=30) purchased (Janvier

Labs, Le Genest Saint Isle, France) at gestational day (G) 2 were individually housed throughout gestation and lactation and assigned to either early adversity or control groups.

Early life adversity

The multiple-hit early-life adversity consisted in maternal immune activation during gestation, chronic MS and maternal exposure to UCMS during lactation. On embryonic day 17 (E17), dams of the early adversity group (n=14) received LPS injection (E. Coli O127B8, 120 µg/kg, i.p.), while dams of the control group (n=14) received saline (Golan et al., 2005; Zager et al., 2014; Zager et al., 2015). Maternal body temperature was determined immediately before and 3hr after the injection. 5 litters were delivered at G18 (all from LPS-injected dams), and 23 litters at G19. None of the 5 litters born the day after LPS injection survived. MS was carried out from post-natal day (PND)2 to PND14 (180 min daily) (Rincel et al., 2016; 2017). To minimize habituation, MS started randomly at 8:30, 9:00, 9:30, 10:15, 10:30 or 11:00. Pups were individually separated and kept at 32°C ±2. During separation sessions, dams were placed in a new cage and UCMS (no bedding, wet bedding, 45° tilted cage or defiled rat bedding in random order). Control litters (born to saline injected dams) were housed in an adjacent room and remained undisturbed until weaning. At PND21, male and female offspring from litters with equilibrated sex-ratio (n=9 control litters and 8 early adversity litters) were weaned and kept for long-term analyses. Two batches of animals were used for the different experiments (batch 1 n=12 per group, batch 2 n=7-8 per group). Within each batch, a maximum of 2 siblings per dam was used to minimize the litter effect. The experimental design is depicted in **Figure 1**.

Ultrasonic vocalizations (USVs) in pups

USVs were assessed in response to a 6min isolation on PND7 (in the afternoon, 4 hours after the end of the MS episode). Pups were gently removed from the homecage and placed in a glass crystallizer bedded with thick cotton in a different room. USVs emissions (range 60-80 kHz, threshold -50 dB) were recorded using an ultrasound microphone coupled with the Recorder USGH software (Avisoft, Glienicke, Germany) and automatically quantified using the whistle tracking mode of the SAS LabPro software (Avisoft Bioacoustics).

Behavioral assessment in adult offspring

All experiments were performed during the light phase (8:00-14:00, except for the resident-intruder test: 16:00-17:00). For analyses involving manual quantifications, experimenters remained blind to the experimental groups. Animals were collectively housed except for visceral sensitivity and gut permeability assessment (batch 2).

Elevated plus maze (EPM) (3 months). The apparatus consisted of two opposing open arms (30 × 8 cm, light intensity: 80 lux) and two opposing closed arms (30 × 8 × 15 cm, light intensity: 20 lux) connected by a central platform (8 × 8 cm) and elevated 120 cm above the floor. Mice were placed in the center of the maze facing an open arm and allowed to explore for 10 min. Distance traveled and time spent in each arm were automatically quantified using videotracking (Smart software, Bioseb, Vitrolles, France). The percentages of distance traveled and time spent in open arms were calculated (open arms/(open arms+closed arms)x100). A reduction of the percent of exploration of the open arms is considered as an anxiety-like index (Walf and Frye, 2007).

Marble burying test (4 months). Mice were individually housed in large cages (30 x 20 cm) filled with 3L of wood chip bedding (5 cm deep). On the next day, they were transiently removed from the cages and 20 marbles were evenly placed on top of the bedding as previously described (Deacon, 2006). Mice were put back in the cage and their behavior was videorecorded during 20 min (light intensity: 80 lux). The number of buried marbles was manually quantified every 4 min. A marble was considered buried when at least 2/3 of its volume was coated.

Social interaction (4.5 months). Time spent in social interaction was evaluated over 6 min, under dim light (15 lux) in a new cage (30 x 20 cm) filled with fresh bedding. Pairs of weight-matched mice from the same experimental group (batch 1) were placed together in the cage. In another subset of males (batch 2), an additional test was carried out using aggressor CD1 mice (old breeders previously used for social defeat protocols, n=6). Aggressors were isolated for 2 weeks and experimental mice were introduced in their homecage for 6 min (resident-intruder paradigm). Each aggressor encountered a control mouse and a mouse from the early adversity group to avoid bias. Mice behavior was recorded using a digital camera and social interaction (sniffing, allogrooming and crawling over), aggression and submission were manually scored using an ethological software (The observer, Noldus Information Technology, Wageningen, The Netherlands).

Tail suspension test (TST) (5 months). Mice were hanged by the tail to a hook placed 30cm above the floor of the apparatus (Bioseb) using adhesive tape (and plastic pipes to prevent from climbing). Their behavior was recorded with a digital camera during 6min. Mice demonstrated several escape attempts interspersed with immobility periods during which they hung passively and completely motionless. Time spent immobile was manually quantified using the Observer software (Noldus) (Dinel et al., 2011).

HPA axis reactivity to stress (5 months). Mice were restrained in perforated 50 ml falcons for 30 min. Blood samples were collected before the restraint stress (t0, facial vein) and at the end of the stress by tail nick (t30) using EDTA-coated tubes. Mice returned to their home

cage and blood samples were collected 60 min later (t90). Blood samples were centrifuged (4000 rpm, 4°C) for 20 min and stored at -20°C until use. Plasma corticosterone was determined with an in-house radioimmunoassay using a highly specific antibody as previously described (Richard et al., 2010). Cross reactivity with related compound such as cortisol was less than 3%. Intra- and inter-assay variations were less than 10% and less than 15%, respectively.

Intestinal phenotype of adult offspring

In vivo intestinal permeability. Mice (2.5 month-old, males and females, or 5.5 month-old, only males) were individually housed 24h prior to the experiment. On the day of the test, they were gavaged with 120 µL of a solution containing Fluorescein–5.6 sulfonic acid (FSA, (478.32 D, Thermo Fisher Scientific, Saint Aubin, France, paracellular permeability), horse radish peroxidase (HRP, 44 kDa, Sigma Aldrich, France, transcellular permeability) and carmine red (total transit time) as previously described (Vanhaecke et al., 2017). After 60min, blood samples were taken from the tail vein (heparinized tubes). Plasma was isolated by centrifugation (3200 rpm, 10 min) and plasma FSA fluorescence was determined on a microplate reader (Varioskan, Thermo Fisher Scientific, λ_{exc}: 490 nm, λ_{emi}: 510 nm). Plasma HRP activity was determined by enzymatic assay in presence of Tetramethylbenzidine substrate, using a spectrofluorometer. Results are expressed as fluorescence (or HRP concentration) per mg of plasma. Fecal pellets were monitored at 5 min intervals for the presence of carmine red. Total GI transit time was defined as the interval between the initiation of gavage and the time of first observance of carmine red in feces. Colonic transit (fecal pellet output) was evaluated by quantifying the number of fecal pellets every 15 min during 2 hr after gavage.

Colorectal distension (3.5 months). Mice were individually housed for this experiment. Four days before colorectal distention, 2 electrodes were implanted in the abdominal external oblique musculature of mice previously anesthetized with xylazine and ketamine. Electrodes were exteriorized at the back of the neck and protected by a plastic tube attached to the skin. As previously described (Boué et al., 2014), electrodes were connected to a Bio Amp, which was connected to an electromyogram acquisition system (ADInstruments, Inc, Colorado Springs, CO). A 10.5-mm-diameter balloon catheter was gently inserted into the colon at 5 mm proximal to the rectum. The balloon was inflated in a stepwise fashion. Ten-second distensions were performed at pressures of 15, 30, 45, and 60 mmHg with 5-minute rest intervals. Electromyographic activity of the abdominal muscles was recorded and visceromotor responses were calculated using Chart 5 software (ADInstruments).

Sacrifice, sample collection and *ex vivo* analyses

After behavioral assessment (5.5 months), feces were collected and stored at -80°C (1-2 per animal) before mice were deeply anesthetized with isoflurane and killed by decapitation. Cardiac blood was collected for gut hormones assay in EDTA-coated tubes containing DPP-IV inhibitor (5µl/500 µl blood, Calbiochem) and phenylmethylsulfonyl fluoride (PMSF, 10 mg/ml) and centrifuged at 4°C before plasma was stored at−20°C. Whole brains were collected and stored at -80°C until use. Segments of ileum and distal colon (approximately 1.5 cm) were collected for *ex vivo* intestinal motility experiments. Distal colon segments were finally used for assessment of protein content.

Ex vivo intestinal motility (males only). Ex vivo neuromuscular transmission was evaluated as previously described (Suply et al., 2012). The ileum and distal colon were placed in cold oxygenated (5% CO2-95% O2) Krebs solution containing (in mM) 117.0 NaCl, 4.7 KCl, 1.2 MgCl2, 1.2 NaH2 PO4, 25.0 NaHCO3, 2.5 CaCl2, and 11.0 glucose. Segments were then placed in the longitudinal direction in a 7-ml organ bath containing oxygenated Krebs solution (37°C) and were stretched with a preload of 0.04 to 0.06 mN of tension. Preparations were equilibrated for 60 min. Isometric contractions were recorded with force transducers (no. 7005; Basile, Comerio, Italy) and data acquired onto a PowerMac Performa 7100/80 computer equipped with the MacLab/4s system (ADI, Bremen, Germany). Activation of enteric neurons was performed by electrical field stimulation (EFS) using a stimulator connected to two platinum ring electrodes (10 V, duration of pulse train: 10 s; pulse duration: 300 µs; frequency: 30 Hz). This procedure was repeated three times with 10-min intervals between stimulations. The response of colonic longitudinal muscle to EFS was also measured in the presence of NO synthase (NOS) inhibitor, N-nitro-l-arginine methyl ester (L-NAME, 50 mM, Sigma), and further in presence of atropine (10 µM, Sigma), an antagonist of cholinergic muscarinic receptors. Drugs were applied 15 min before EFS. Tension level, amplitude of spontaneous contractions, and area under the curve (AUC) during each EFS-induced response were measured. Data were normalized to the weight of the tissue.

Microarray transcriptome in the medial prefrontal cortex (mPFC). Total mRNA was extracted from mPFC micropunches (anterioposteriority from bregma: +2.10 to +1.18 mm) using a TRIzol extraction kit (Invitrogen, Life Technologies, Carlsbad, CA, USA) according to the manufacturer's instructions. RNA concentration, purity and integrity were determined using a ND-1000 spectrophotometer (Nanodrop Technologies, Wilmington, DE, USA) and a bioanalyzer (Agilent) (Labrousse et al., 2012). Gene expression profiles were performed at the GeT-TRiX facility (GénoToul, Génopole Toulouse Midi-Pyrénées) using Agilent Sureprint G3 Mouse microarrays (8x60K, design 074809) following the manufacturer's instructions. For each sample, Cyanine-3 (Cy3) labeled cRNA was prepared from 25 ng of total RNA

using the One-Color Quick Amp Labeling kit (Agilent) according to the manufacturer's instructions, followed by Agencourt RNAClean XP (AgencourtBioscience Corporation, Beverly, Massachusetts). Dye incorporation and cRNA yield were checked using Dropsense™ 96 UV/VIS droplet reader (Trinean, Belgium). 600 ng of Cy3-labelled cRNA were hybridized on the microarray slides following the manufacturer's instructions. Immediately after washing, the slides were scanned on Agilent G2505C Microarray Scanner using Agilent Scan Control A.8.5.1 software and fluorescence signal extracted using Agilent Feature Extraction software v10.10.1.1 with default parameters. Microarray data and experimental details are available in NCBI's Gene Expression Omnibus (Edgar et al., 2002) and are accessible through GEO Series accession number GSEXXXXX (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSEXXXXX). Microarray data were analyzed using R (R Development Core Team, 2008) and Bioconductor packages (www.bioconductor.org, v 3.0, (Gentleman et al., 2004) as described in GEO accession GSEXXXXX. Raw data (median signal intensity) were filtered, log2 transformed, corrected for batch effects (microarray washing bath and labeling serials) and normalized using quantile method (Bolstad et al., 2003). A model was fitted using the limma lmFit function (Smyth, 2004). Pair-wise comparisons between biological conditions were applied using specific contrasts. A correction for multiple testing was applied using Benjamini-Hochberg procedure (Benjamini and Hochberg, 1995) for False Discovery Rate (FDR). Probes with FDR ≤ 0.1 were considered to be differentially expressed between conditions.

Fecal Microbiota assessment by high-throughput 16S-sequencing. DNA extraction and sequencing were carried out at the GeT-TRiX facility (GénoToul). Mice stools were aliquoted (150mg) and extracted using both mechanical and chemical lysis (PMID: 20203603). Following DNA concentration estimation by nanodrop, the V3-V4 region of the 16S rRNA gene was amplified (V3F bac339F-TACGGRAGGCAGCAG (modified from Wilson KH, et al. J Clin Microbiol. 1990) and V4R bac806R-GGACTACCAGGGTATCTAAT). Illumina MiSeq sequencing was performed on 46 samples. One sample provided too low sequences number (n=365) and was removed from analysis. Total reads were filtered for length (min length=300bp) and quality (min quality =25). A total of 316,668 reads was obtained (average 7,037 reads/samples). High quality reads were pooled, checked for chimeras, and grouped into operational taxonomic units (OTUs) based on a 97% similarity threshold with uclust software from QIIME. Estimates of phylotypes richness and diversity were calculated using Chao1 index on the rarefied OTU table (n=4,000 reads). Singletons were removed and phylogenetic affiliation of each OTU (average 503 OTUs per sample) was done by using ribosomal database project taxonomy (Cole et al., 2014) and performed from phylum to species level.

The statistical language R was used for data visualization and to perform abundance-based principal component analysis (PCA) and inter-class PCA associated with Monte-Carlo rank testing on the bacterial genera (ade4 library). To decipher the impact of the different set-ups (sex and early adversity) on microbiota composition, principal component analyses with the different clinical factors as instrumental variables were computed based on the abundance of the different bacterial taxa for each individual (one analysis per environmental factor, data not shown). These inter-class PCA are appropriate to represent a typology displaying the diversity between individual's microbiota as it maximizes the variance between populations (here, mice fecal microbiota), instead of the total variance. Hence, inter-class PCA allows highlighting combinations of variables (bacterial phylotypes, or genera etc) that maximize variations observed between qualitative variables (e.g. environmental factors). Based on these inter-class PCA, statistical *p*-values of the link between the different environmental and clinical factors with microbiota profiles was assessed using a Monte-Carlo rank test (1000 replicates).

Statistics

All data were analyzed using Statistica 6.0 (Statsoft). Normality was assessed by using Shapiro–Wilk tests. Student t-tests were used to test the effects of early adversity on pups' body weight, adult offspring behavior and dams' body temperature. Social interaction data were analyzed with non-parametric Mann-Whitney U tests. Two-way ANOVAs with repeated measures followed by Fisher's LSD *post-hoc* tests were used for USVs, marble burying test, locomotor activity, corticosterone response to stress and adult body weight analyses. Pearson correlations were used to examine associations between gut microbiota and behavioral data. Statistical significance was set at $p<0.05$. Graphics were made using GraphPad Prism 5 and Adobe Illustrator CS5.1 was used for artwork. Data are expressed as means ± SEM except for gut microbiota composition (medians).

RESULTS

Effects of early adversity on offspring's body weight

LPS injection on E17 induced significant hypothermia in dams (Student t-test, $t(15)=4.98$, $p<0.001$), indicating that bacterial immune activation was effective **(Supplementary Figure 1)**. There was no significant effect of prenatal LPS on pup body weight on PND2 (males, $t(15)=1.60$, $p=0.1302$; females, $t(15)=0.94$, $p=0.3633$) **(Supplementary Figure 2a)**. However, the combination of prenatal LPS and MS (early adversity) significantly decreased

the body weight of pups at PND15 (males, $t(15)=2.46$, $p=0.0.026$; females, $t(15)=1.62$, $p=0.1269$) **(Supplementary Figure 2b)**. This effect was no longer present at weaning (males, $t(15)=1.67$, $p=0.1159$; females, $t(15)=1.26$, $p=0.2283$) **(Supplementary Figure 2c)**.

In males, early adversity leads to impaired social communication during infancy and altered social interaction at adulthood

In order to assess early deficits in social and communication behavior at PND8, we analyzed the ultrasonic calls emitted by pups when isolated from their dam and littermates. Males showed lower vocalization number when submitted to early adversity relative to controls (two-way ANOVA with repeated measures, early adversity effect: $F(1,14)=4.78$, $p=0.0462$, time x early adversity effect: $F(1,42)=10.74$, $p<0.001$) **(Figure 2a,b)**. This effect was mainly due the marked decrease in USV emission during the first minute (planned comparisons, early adversity *vs* control, $p<0.001$). In addition, the latency to vocalize was increased in early adversity compared with controls (Student *t*-test, $t(14)=3.10$, $p=0.0079$) **(Figure 2c)**.

As adults, males submitted to early adversity spent significantly less time in social interaction with a conspecific (Mann-Whitney *U* test, $U=1$, $p=0.0043$) **(Figure 2d)**. Moreover, social interaction latency was significantly lower relative to controls ($U=3$, $p=0.0152$) **(Figure 2e)**. We further investigated social behavior in males using a resident-intruder paradigm **(Figure 2f,g)**. While the aggressors displayed the same number of attacks in both groups ($U=24$, n.s.) **(Figure 2f)**, early adversity males seemed to spend less time in defensive behavior ($U=13$, $p=0.1135$) **(Figure 2g)**. There was no significant impact of early adversity on the percentages of time spent and distance traveled in the open arms of the EPM (Student *t*-test, all $t(18)<1$, n.s) **(Figure 2h** and data not shown**)**, on the number of buried marbles in the marble burying test (two-way ANOVA with repeated measures, early adversity effect, early adversity x time effect, all $F(1,88)<1$, n.s.) **(Figure 2i)**, or on immobility time in the TST ($t(20)=0.88$, n.s) **(Figure 2j)**, suggesting that anxiety and depressive-like behaviors are unspoiled in males exposed to early adversity. Moreover, locomotor activity was not altered by early adversity (two-way ANOVA with repeated measures, early adversity effect, early adversity x time effect, all $F(1,110)<1$, n.s.; data not shown). Plasma corticosterone levels after restraint stress were not significantly modulated by early adversity (two-way ANOVA with repeated measures, early adversity effect, early adversity x time effect, all $F(1,36)<1$, n.s) **(Figure 2k)**. There was no significant impact of early adversity on male body weight throughout behavioral assessment (data not shown); however, 3 weeks after TST and acute restraint stress, only control males gained significant weight (two-way ANOVA with repeated measures, early adversity effect: $F(1,20)<1$, n.s.; early adversity x time effect, $F(1,20)=5.00$, $p=0.0368$; Fisher LSD's post-hoc test, control pre- *vs* poststress, $p=0.0006$; early adversity

pre- vs poststress, n.s.) **(Figure 2l).** This difference resulted in significantly lower body weight gain in early adversity males relative to controls ($t(20)=2.24$, $p=0.0368$) **(Figure 2m)**, suggesting that they are more sensitive to stress.

In females, early adversity leads to impaired social communication during infancy and exacerbated anxiety at adulthood

In female pups, the number of USV calls in response to isolation was differentially altered by early adversity depending upon time (two-way ANOVA with repeated measures, early adversity effect: $F(1,16)<1$, n.s.; time x early adversity effect: $F(1,48)=2.73$, $p=0.0539$) **(Figure 3a,b)**. The number of USV calls was significantly reduced during the first minute (planned comparisons, early adversity *vs* control, $p=0.0359$). This modest effect was not accompanied by significantly delayed USV latency (Student *t*-test, $t(16)=1.67$, $p=0.1134$) **(Figure 3c)**.

At adulthood, females exposed to early adversity showed similar time spent in social interaction with a conspecific compared with controls (Mann-Whitney *U* test, $U=16$, n.s.) **(Figure 3d)**. Consistently, the latency to first interaction with the conspecific was not different between groups ($U=8$, n.s., data not shown). In the EPM, early adversity females spent significantly less time in the open arms relative to controls (Student *t*-test, $t(22)=2.64$, $p=0.0148$) **(Figure 3e)**. Moreover, the distance traveled in the open arms was also reduced in early adversity *versus* control mice ($t(22)=2.23$, $p=0.0449$) **(Figure 3f)**. Similarly, early adversity females buried significantly more marbles in the marble burying test compared with controls (two-way ANOVA with repeated measures, early adversity effect, $F(1,84)=6.78$, $p=0.0165$) **(Figure 3g)**. Locomotor activity was not altered by early adversity (two-way ANOVA with repeated measures, early adversity effect, early adversity x time effect, all $F(1,110)<1$, n.s., data not shown), suggesting that the above-mentioned behavioral differences are not due to altered locomotion in the early adversity group. Immobility in the TST was not affected by early adversity ($t(21)=0.29$, n.s) **(Figure 3h)**. Plasma corticosterone levels after restraint stress varied depending upon time and the history of early adversity (two-way ANOVA with repeated measures, early adversity effect, $F(1,18)<1$, n.s.; early adversity x time effect, $F(1,36)=3.88$, $p=0.0298$) (**Figure 3i)**. Planned comparisons revealed significantly higher corticosterone levels 60 min after the end of restraint (t90, $p=0.0055$). Further analysis showed that corticosterone recovery to basal levels was lower in the early adversity group ($t(18)=3.31$, $p=0.0039$ **(Figure 3i, inset)**). Finally, as in males, the body weight growth of females exposed to early adversity was stopped after TST and restraint stress exposures, whereas the control group exhibited a normal body weight growth (two-way ANOVA with repeated measures, early adversity effect: $F(1,21)=2.02$, $p=0.1693$, n.s.;

early adversity x time effect, $F(1,21)=5.11$, $p=0.0346$; Fisher LSD's post-hoc test, control pre- *vs* poststress, $p=0.0032$; early adversity pre- vs poststress, n.s.; body weight gain: ($t(21)=2.26$, $p=0.0346$)) **(Figure 3j,k)**.

Early adversity induces gut leakiness in adult males but not females

We next assessed whether the effects of early adversity on behavior would be associated with altered gut physiology. We first measured plasma levels of several metabolic and gut hormones. There was no difference in insulin, leptin, active ghrelin, and total PYY plasma levels between groups either in males (Student *t*-tests, all $t(20)<1.5$, n.s) or in females (all $t(19)<1.5$, n.s) (data not shown). Visceral sensitivity to colorectal distension as well as intestinal permeability were evaluated in both sexes. In males, there was no significant effect of early adversity on visceral sensitivity to colorectal distension (two-way ANOVA with repeated measures, early adversity effect, early adversity x distension pressure effect, all $F(1,39)<1$, n.s.) **(Figure 4a)** or on transcellular permeability to HRP (Student *t*-test, $t(18)=<1$, n.s.). However, paracellular permeability to FSA was significantly increased relative to controls ($t(18)=2.56$, $p=0.0198$) **(Figure 4b).** Total transit time and fecal pellet output were similar between groups (all $t(18)<1.2$; n.s.; data not shown). Intriguingly, early adversity females displayed visceral hyposensitivity, an effect likely driven by a hypo-response to noxious stimuli (two-way ANOVA with repeated measures, early adversity effect: $F(1,13)=4.90$, $p=0.0454$, early adversity x distension pressure effect: $F(1,39)=2.46$, $p=0.0773$; planned comparison for 60 mmHg: $p=0.0636$). **(Supplementary Figure 3a)**. Contrary to males, early adversity had no significant impact on females' intestinal permeability or total transit time (Student *t*-tests, all $t(19)<1$; n.s.) **(Supplementary Figure 3b,c** and data not shown), although it significantly increased fecal pellet output 1 hour after oral gavage ($t(19)=2.60$; $p=0.0176$; data not shown). As early adversity led to gut leakiness in males but not in females, we further assessed intestinal permeability in males at a later age (5.5 months). The increased paracellular permeability was no longer observed in older male animals ($t(21)<1$, n.s.), however, early adversity significantly increased males' transcellular permeability at this age ($t(21)=2.7995$, $p=0.0107$). There was no effect of early adversity on total transit time (all $t(18)<1.4$, n.s.) or fecal pellet output 2 hours after oral gavage (all $t(21)=1.35$, n.s.) but a trend was observed at 1 hour ($t(21)=1.85$, $p=0.0782$). Finally, *ex vivo* motility was studied on the distal colon and on the ileum in the same males (5.5 month-old). Segments of colonic and ileal longitudinal muscle were stimulated by electrical field stimulation (EFS), and EFS-induced contractile responses were then analyzed in the absence or presence of L-NAME or atropine. There was no significant differences in contractile responses to EFS between groups in the ileum or distal colon (all $t(14)<1.5$, n.s.).

In presence of L-NAME, the contractile response to EFS in the ileum and distal colon decreased similarly across groups (all t(14)<1.5, n.s.). In contrast, after atropine application in the bath, the contractile response to EFS was significantly higher in mice subjected to early adversity, but this effect was restricted to the distal colon (t(14)=3.57, p=0.003).
Altogether, our results indicate that multi-hit early adversity leads to different phenotypes in males and females for both behavior and gut physiology. In order to explore potential mechanisms involved in these effects, we analyzed gene expression in the mPFC and gut microbiota composition in the same animals.

Effects of early adversity on gene expression in the medial prefrontal cortex of adult offspring

We examined gene expression in the mPFC, a brain area relevant for the regulation of emotional behaviors, but also highly sensitive to early-life stress. Microarray analysis with the Benjamini-Hochberg correction for multiple testing revealed interactions between sex and early adversity for 30 probes (adjusted *p* value <0.1, among which 8 probes had an adjusted *p* value <0.05) (see gene list and genes full names in **Figure 5** and detailed statistical results in **Supplementary Table 1**), suggesting that the effects of early adversity differed in males *versus* females. Accordingly, the heatmap showed diminished z-scores of relative expression of Klf2, Npas4, Btg2, Gadd45g, Zfp36 and Tob1 genes in males exposed to early adversity, whereas the z-scores of these genes were increased in females exposed to early adversity. Conversely, early stress increased the relative expression of Gm9910 and L3mbtl4 genes in males and reduced it in females. However, Klf2 (Krüppel-like factor 2) was the only gene significantly altered (upregulated) by early adversity specifically in females (*p*adj=0.0157) **(Figure 5b)**.

Early adversity differentially alters gut microbiota composition in adult males and females

Finally, we analyzed gut microbiota composition in both sexes using 16S-based profiling **(Figures 6 and 7 and Supplementary Figure 4)**. As illustrated in **Supplementary Figure 4,** in adult animals, microbiota composition between males and females of the control group strongly differed both qualitatively and quantitatively. Moreover, there were differential effects of early adversity according to sex at several taxonomic levels. In males, PCA based on genus composition revealed a trend towards significantly differential distribution according to the history of early adversity (Monte Carlo simulated *p*-value=0.0779) **(Figure 6a)**. Chao1 index of alpha diversity was not altered by early adversity (Mann-Whitney U test, *U*=65, n.s.) **(Figure 6b)**. Males exposed to early adversity displayed significantly lower proportions of

unclassified Lachnospiraceae (U=27, *p*=0.0156) and *unclassified Porphyromonadaceae (U*=27, *p*=0.0156) genera **(Figure 6c)** and higher proportions of *Bacteroides (U*=34, *p*=0.0512)*, Lactobacillus (U*=29, *p*=0.0225), *Porphyromonas (U*=31, *p*=0.0317)*, Alloprevotella (U*=32, *p*=0.0374) and *unclassified Firmicutes (U*=34, *p*=0.0512) genera **(Figure 6d)** compared with controls. Of note, the effect of early adversity on *unclassified Lachnospiraceae* species (spp.*)*, the most abundant detected genus, was particularly striking (falling from 30% to 20%). Further analysis at the OTU level showed 96 significantly altered OTUs in males with a history of early adversity in comparison with controls: 63 OTUs were depleted (55 from Firmicutes and 8 from Bacteroides phyla) **(Figure 6e)** and 33 OTUs were enriched (18 from Firmicutes, 13 from Bacteroides and 1 from Proteobacteria phyla) **(Figure 6f)**. Notably, an important proportion of the altered OTUs belongs to *Barnesiella* and *Clostridium XIVa* spp. of the Porphyromonadaceae and Lachnospiraceae families, respectively. Although the analysis at the genus level shows overall lower proportions of *unclassified Lachnospiraceae* and *unclassified Porphyromonadaceae,* the OTUs data indicate that different OTUs within the same genus or family vary in opposite directions.

In female offspring, PCA based on genus composition showed no significant dissociation between groups (Monte Carlo simulated *p*-value=0.1968, n.s.) and chao1 index of alpha diversity was not altered by early adversity (*U*=50, n.s.) **(Figure 7a,b)**, suggesting that the gut microbiota of females is less sensitive to early adversity than that of males. Consistently, only two genera were significantly altered by early adversity in females **(Figure 7c)**. Specifically, relative abundance of *Mucispirillum (U*=29, *p*=0.0400) *and Lactobacillus* (*U*=30, *p*=0.0473) genera was significantly decreased in early adversity female mice compared with controls. Interestingly, this depletion of *Lactobacillus* spp. in females contrasts with its enrichment in males submitted to early adversity. Further analysis at the OTU level showed 68 significantly altered OTUs in early adversity females *versus* controls, with 41 depleted (34 from Firmicutes, 5 from Bacteroides phyla, 1 from Actinobacteria and 1 from Deferribacteres phyla) **(Figure 7d)** and 33 enriched (27 from Firmicutes, 15 from Bacteroides and 1 from Proteobacteria phyla) **(Figure 7e)**. As in males, an important proportion of the altered OTUs belongs to *Barnesiella* and *Clostridium XIVa* spp. of the Porphyromonadaceae and Lachnospiraceae families and different OTUs within these families vary in opposite directions. Moreover, a substantial proportion of the depleted OTUs belongs to the *Lactobacillus* genus and may account for the overall effect observed at the genus level.

Overall, the effects of early adversity on gut microbiota composition varied according to sex, with males being the most affected. *Clostridium XIVa* cluster, which represents more than 10% of the total bacteria, and more generally the *Lachnospiraceae* family, accounting for

approximately 40% of total bacteria (Supplementary Figure 4d,e), appear highly sensitive to early adversity in both sexes.

In male animals, correlational analyses conducted on gut microbiota, behavior, gene expression in mPFC and gut function data revealed that *uncl. Lachnospiraceae* was the bacterial genus most often significantly correlated with these parameters. *Uncl. Lachnospiraceae* abundance positively correlated with social interaction (latency for the first interaction: r=0.49, *p*=0.015; time spent in social interaction: r=0.39, *p*=0.06) and it negatively correlated with colonic motility in presence of atropine (r=-0.59, *p*=0.015). The relative abundance of these bacteria in males was also positively correlated with the expression of several genes in the mPFC (Npas4: r=0.46, *p*=0.024; Btg2: r=0.48, *p*=0.02; Gadd45g: r=0.51, *p*=0.012). In female animals, *Lactobacillus* abundance was negatively correlated with the anxiety score in the marble burying test (r=-0.44, *p*=0.03) and Klf2 expression in the mPFC (r=-0.44, *p*=0.042). In contrast, *Lactobacillus* abundance was positively correlated with Gm9910 expression in females' mPFC (r=0.59, *p*=0.005).

DISCUSSION

Several studies have reported sex differences in gut microbiota composition in both humans and animals (Markle et al., 2013; Dominianni et al., 2015). There is a growing number of studies investigating the role of the gut-brain axis, especially gut microbiota, in the regulation of stress-related emotional behaviors in animal models of early-life adversity. However, it is not clear whether early adversity differentially affects the gut microbiota between males and females. Using a mouse model of multifactorial early adversity combining prenatal inflammation (LPS injection), post-natal MS and UCMS in dams, we report that early adversity leads to social deficits in males and hyperanxiety in females. More importantly, we provide clear evidence that multifactorial early adversity differentially alters gut microbiota composition in males and females, an effect accompanied by male-specific gut leakiness.

Consistent with numerous studies, we report that early adversity, including maternal immune activation and MS, induces social deficits in male offspring (Tsuda et al., 2011; Hsiao et al., 2013; Zhu et al., 2014; Rincel et al., 2016; Shin et al., 2016; Kim et al., 2017; Monte et al., 2017; Shin Yim et al., 2017) and increases anxiety in female offspring (Salzberg et al., 2007; Fernández de Cossío et al., 2017). These results are particularly interesting with regards to the high prevalence of autism spectrum disorders in male and anxiety disorders in female subjects. Notably, USVs data suggest early onset social communication deficits in males and show only mild alterations in females, which is consistent with their intact social interaction as adults. However, in contrast to previous findings, we did not find any effect of early adversity

on depressive-like behaviors in the TST in either sex (Varghese et al., 2006; Ghia et al., 2008). In addition, we did not observe overt HPA axis dysfunction, although it is often reported in an early stress context (Huot et al., 2002; Lehmann et al., 2002; Slotten et al., 2006; Cotella et al., 2013; Rincel et al., 2016). These discrepancies could be due to specie differences (mice have been shown to be less susceptible to stress than rats) but also to the strain differences in mice (Millstein and Holmes 2007; Savignac et al., 2011).

Several studies have demonstrated that correction of gut dysbiosis can reverse social deficits (Hsiao et al., 2013; Buffington et al., 2016; Kim et al., 2017; Shin Yim et al., 2017) and anxiety-like behaviors (Bercik et al., 2010; Hsiao et al., 2013; Liang et al., 2015; Emge et al., 2016; Leclercq et al., 2017; Moya-Pérez et al., 2017), highlighting the role of gut microbiota in the regulation of emotional behaviors. Here, we report that early adversity induces sex-differences in gut dysbiosis at adultdhood. In males exposed to early-life adversity, gut dysbiosis was mainly characterized by depleted *unclassified Lachnospiraceae* and *Porphyromonadaceae* spp. and enriched *Bacteroides*, *Lactobacillus*, *Alloprevotella*, *Porphyromonadaceae* and *unclassified Firmicutes* spp., while early adversity female group displayed depleted *Lactobacillus* and *Mucispirillum* spp. These results show that i) the microbiota of females is affected to a lesser extent than that of males and ii) the only genus affected in both sexes is *Lactobacillus*, however the effects of early adversity on this genus are opposed in males and females. We found significant enrichment or depletion of OTUs belonging to *Clostridium cluster XIVa* in the Lachnospiraceae family in both sexes, with different OTUs impacted in males *versus* females. A previous study found increased proportions of Porphyromonadaceae, Prevotellaceae, unclassified Bacteriodales and Lachnospiraceae spp. in the maternal immune activation mouse model of autism (Hsiao et al., 2013). However, males and females were pooled together in this study. Several other studies using MS reported increased *Bacteroides, Lachnospiraceae* and *Clostridium XIVa* in male rats and mice (García-Ródenas et al., 2006; De Palma et al., 2015; Zhou et al., 2016; Murakami et al., 2017). Interestingly, numerous OTUs in the Lachnospiraceae family have been found to be either increased or decreased in stools of depressive patients compared with healthy controls (Zheng et al., 2016), suggesting that the effects of early adversity on these bacteria in animal models are relevant to human psychiatric conditions. In addition, a recent study reveals that the antidepressant effects of probiotics in male mice are associated with an increase of Lachnospiraceae abundance (Guida et al., 2017). Although we do not report altered depressive-like behaviors in the TST, the decrease of social interactions has been reported in numerous preclinical models of depression (Nestler and Hyman, 2010). Interestingly, transfer of intestinal microbiota, including members of Clostridiales and Lachnospiraceae, to microbiota-depleted C57BL/6 recipients was sufficient to induce social

avoidance and change gene expression and myelination in the prefrontal cortex (Gacias et al., 2016). Overall, Lachnospiraceae appear as good candidates for the regulation of emotional behaviors within the microbiota-gut-brain axis in males. Moreover, the effect of early adversity on *Lactobacillus* spp. could also account for the sex-specific behavioral differences, as abundance of these bacteria was increased in males but decreased in females. In females, lower *Lactobacillus* abundance was associated with higher anxiety, but not in males. *Lactobacillus* strains are commonly used as probiotics with beneficial effects on anxiety-like behavior in rodents (Bravo et al., 2011; Liang et al., 2015; Emge et al., 2016; Liu et al., 2016; Leclercq et al., 2017). In humans, randomized, double-blind, placebo controlled trials show that women who received *Lactobacillus rhamnosus* spp. throughout pregnancy had significantly lower depression and anxiety scores in the postpartum period (Slykerman et al., 2017) and that patients with chronic fatigue syndrome displayed reduced anxiety symptoms after 2 months of treatment with *Lactobacillus casei* spp (Rao et al., 2009).

The links between specific gut microbiota alterations and gene expression in the brain remains to be elucidated. In the present study, we report a significant interaction between sex and early-life adversity for eight genes in the mPFC. Among them, Klf2 and Npas4 were of particular interest regarding both behavior and gut microbiota. Klf2 is significantly increased in the mPFC of female mice with a history of early-life adversity and females with the highest expression of Klf2 in the mPFC exhibited the lowest abundance of gut *Lactobacillus* spp*.* KLF2 is a transcription regulator highly expressed in vascular cells. In animal models, Klf2 up-regulation has been reported in several conditions associated with neuroinflammation such as ischemia and cerebral cavernous malformations (Shi et al., 2013; Tang et al., 2017). Remarkably, a recent study demonstrated a link between brain Klf2 signaling and gut microbiota in an animal model of cerebral cavernous malformations (Tang et al., 2017). Gram negative bacteria in the gut constitute a source of LPS that enters circulating blood, activating luminal, brain endothelial toll-like receptor (TLR) 4. Authors proposed that LPS–TLR4 stimulation drives MEKK3–KLF2/4 signaling to induce cerebral malformations. In male mice, *uncl*. *Lachnospiraceae* abundance positively correlated with Npas4 expression in the mPFC. NPAS4 is a transcription factor highly expressed in brain tissues and reported to regulate excitatory and inhibitory synapse balance (Lin et al., 2008; Spiegel et al., 2014). In humans, inhibitory-excitatory unbalance is suspected to underlie the increased risk of neurodevelopmental disorders such as schizophrenia and autism (Maćkowiak et al., 2014; Nelson and Valakh, 2015; Krystal et al., 2017) and it has been shown that such unbalance specifically in the PFC elicited social behavior impairments in mice (Yizhar et al., 2011). It has been recently shown that Npas4 plays a significant role in the development of prefrontal inhibitory circuits (Shepard et al., 2017). Notably, Npas4

deficient mice displayed altered expression of GABAergic markers in the PFC in a sex-specific manner, along with sex-specific emotional impairments when Npas4 deficiency was induced at adolescence, but not adulthood (Shepard et al., 2017). Npas4 is a target gene of HDAC5 (Taniguchi et al., 2017), and this epigenetic enzyme has been shown to be increased in the PFC in a mouse model of social deficit (Aoyama et al., 2014). Interestingly, sodium butyrate treatment (butyrate is a short-chain fatty acid, product of the fermentation of complex carbohydrates by the gut microbiota) attenuates social behavior deficits in this mouse model (Aoyama et al., 2014). Therefore, microbiota-driven alterations in butyrate levels could represent a possible link between decreased prefrontal Npas4 expression and reduced social behavior *via* HDAC5 activity.

In conclusion, our work supports a large literature showing that early adversity has an impact on emotional behavior and gut microbiota later in life. Importantly we demonstrate that offspring's behavior and gut physiology are differentially affected by the multifactorial adversity in males and females. Males seem more prone to develop gut dysfunctions than females, as indicated by the large number of bacteria altered in their microbiota and their gut leakiness. At the behavioral level, early adversity produced social behavior deficit in males and increased anxiety in females. Future studies should target the mechanisms underlying these sex differences, and evaluate potential causality links between gut microbiota alterations and behavioral deficits. Overall, our study highlights the importance of examining both males and females in gut-brain axis research.

FIGURES

F

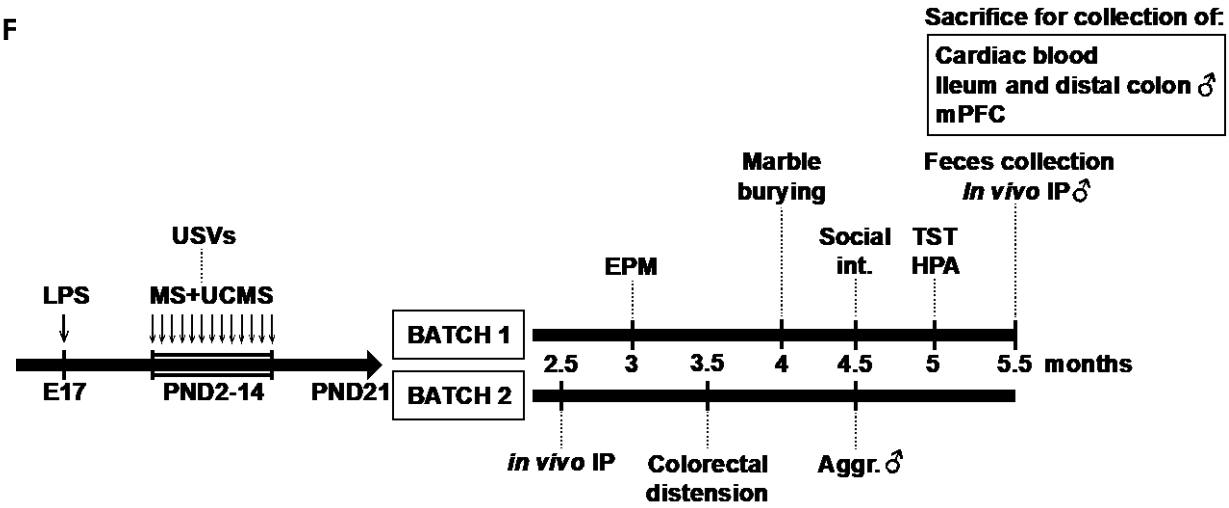

Fig 1. Experimental design. Early adversity consisted in combined maternal immune activation and MS associated with UCMS in dams. On E17, early adversity group received LPS injection (E. Coli O127B8, 120 µg/kg, i.p.), while control group received saline. MS was carried out from PND2 to PND14 (180 min daily). Each day of separation, dams were submitted to UCMS during the 180 min. At PND21, male and female offspring were weaned and separated in two batches. During infancy, ultrasonic vocalizations in response to a short separation were analyzed in PND7 pups. At adulthood (3-5 months), animals underwent a battery of behavioral tests for anxiety (elevated plus maze and marble burying), social behavior and depressive-like behavior (tail suspension test). *In vivo* gut permeability and visceral sensitivity to colorectal distension were evaluated in a subset of animals at 2.5 and 3.5 months, respectively. Finally, HPA axis responsiveness to restraint stress was assessed and the animals were killed 2 weeks later (5.5 months) for fecal microbiota composition analysis by high-throughput 16S RNA sequencing. Gene expression in the medial prefrontal cortex was analyzed by microarrays in the same animals. Unless stated ♂, all the experiments were conducted in both males and females. E, Embryonic day; PND, Post-natal day; LPS, Lipopolysaccharide; USVs, Ultrasonic vocalizations; MS, Maternal separation; UCMS, Unpredictable chronic mild stress; IP, intestinal permeability; EPM, Elevated plus maze; Aggr, interaction with an aggressor; TST, Tail suspension test; HPA, Hypothalamic-pituitary-adrenal axis response to stress; mPFC, medial prefrontal cortex.

Figure 2

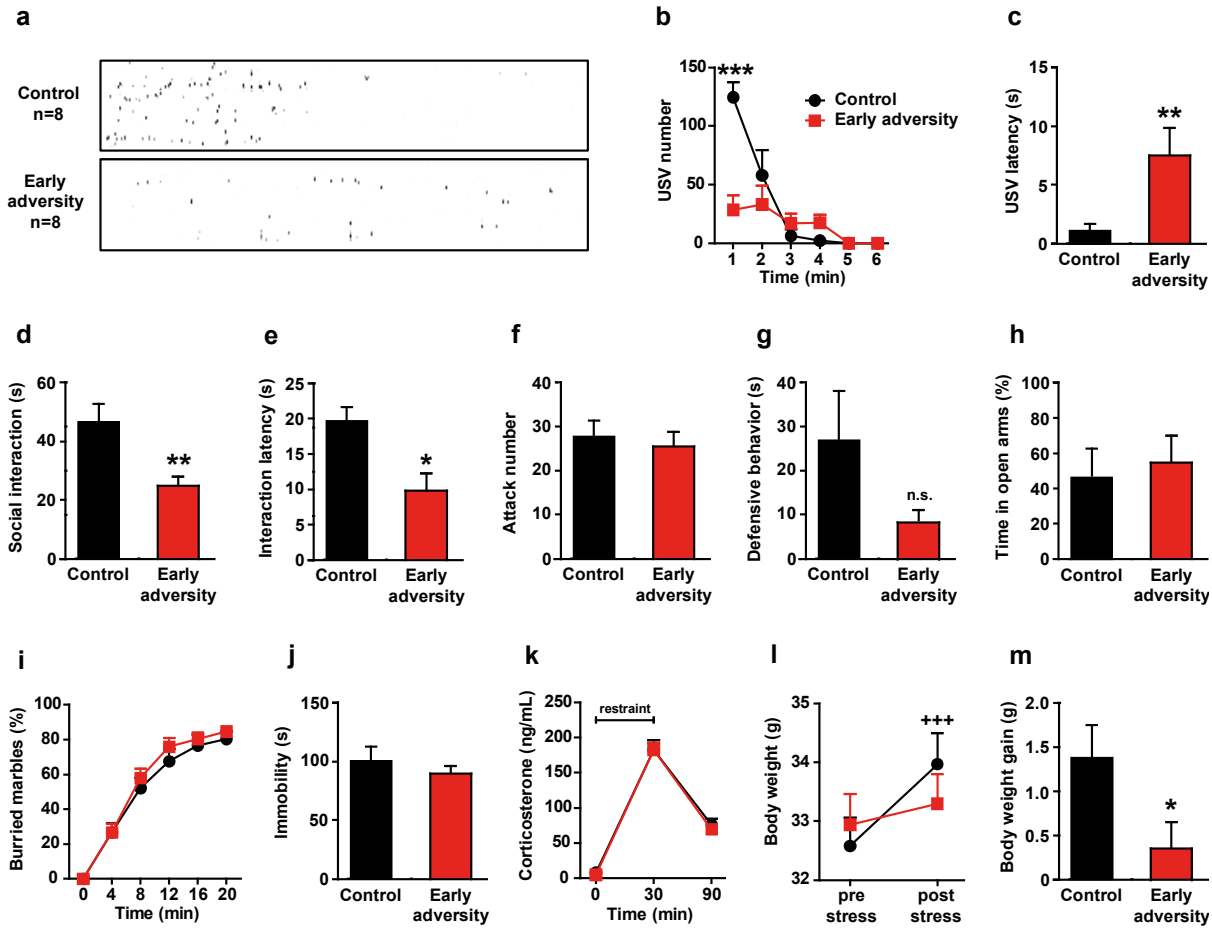

Fig 2. Early adversity leads to social behavior impairment in adult males. (a-c) USV response to acute short separation in PND7 pups (N=9 per group). **(a)** Each line corresponds to one animal and each dash represents one USV. **(b)** USV number across time (min) and **(c)** latency (s) to first USV. **(d-l)** Adult phenotype. **(d)** Time (s) spent in social interaction with a conspecific over 6 min of test and **(e)** latency (s) to first interaction (N=6 pairs per group). **(f)** Number of attacks by the aggressor and **(g)** time (s) spent in defensive behavior over 6 min in the resident-intruder paradigm (N=6-9 per group). **(h)** Time (s) spent in the open arms of the EPM (N=10 per group). **(i)** Number of buried marbles across time (min) in the marble burying test (N=12 per group). **(j)** Time (s) spent immobile over the 6min of test in the TST (N=11 per group). **(k)** Plasma corticosterone (ng/mL) in response to 30-min restraint stress at 0, 30 and 90 min. Inset: recovery to basal levels (%) (N=10 per group). **(l)** Body weight (g) before and 3 weeks after exposure to acute restraint stress (30min) and **(m)** Body weight gain (g) 3 weeks after the TST and acute restraint stress (N=10-12 per group). Data are mean ± SEM. * $p<0.05$, ** $p<0.01$ and *** $p<0.001$ *versus* control group (planned comparison in **b**; Student *t*-tests in **c** and **m**; Mann-Whitney *U* tests in **d, e** and **g**); +++ Fisher LSD's post-hoc test, control pre- *versus* poststress $p<0.001$.

Figure 3

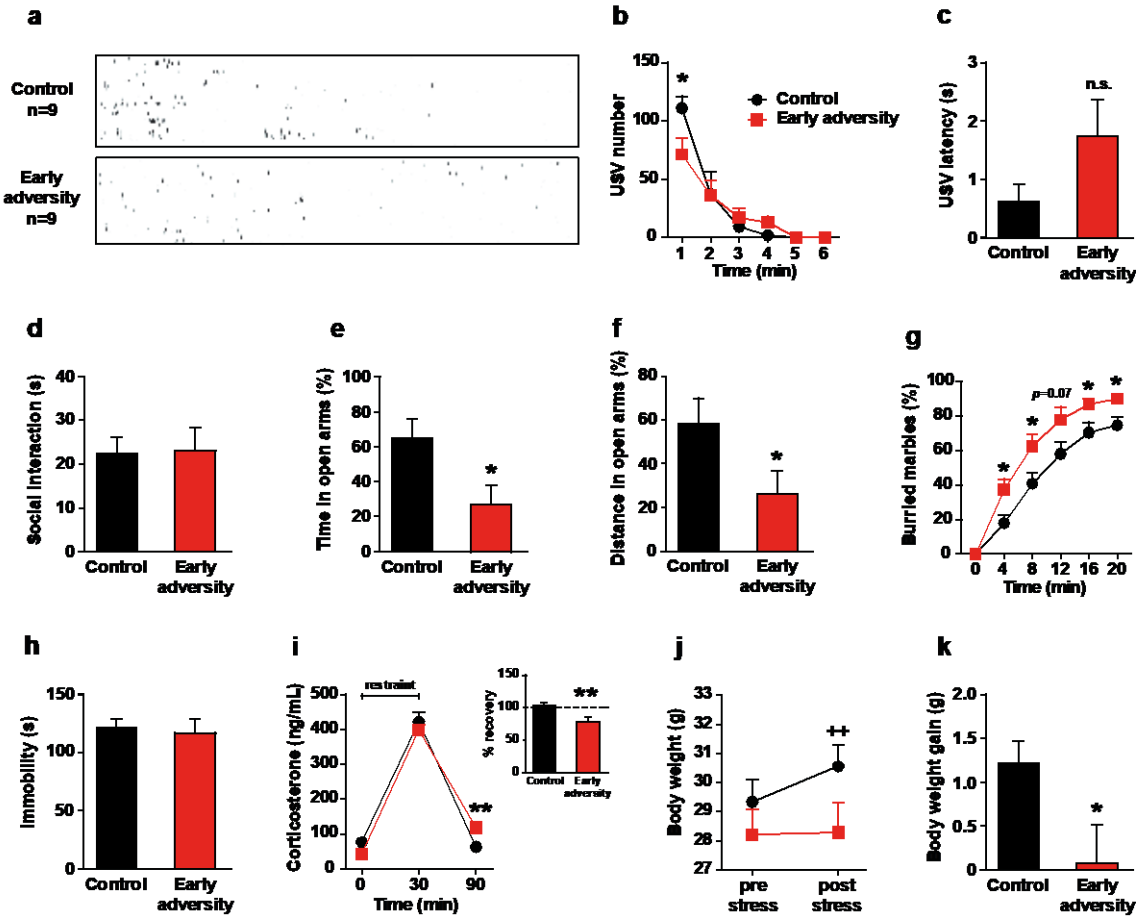

Fig 3. Early adversity leads to hyper-anxiety in adult females. (a-c) USV response to acute short separation in PND7 pups (N=9 per group). **(a)** Each line corresponds to one animal and each dash represents one USV. **(b)** USV number across time (min) and **(c)** latency (s) to first USV. **(d-j)** Adult phenotype. **(d)** Time (s) spent in social interaction with a conspecific over 6 min of test (N=6 pairs per group). **(e)** Time (%) spent and **(f)** distance (%) traveled in the open arms of the EPM (N=12 per group). **(g)** Number of buried marbles across time (min) in the marble burying test (N=11-12 per group). **(h)** Time (s) spent immobile over the 6min of test in the TST (N=12-12 per group). **(i)** Plasma corticosterone (ng/mL) in response to 30-min restraint stress at 0, 30 and 90 min. Inset: recovery to basal levels (%) (N=10 per group). **(j)** Body weight (g) before and 3 weeks after exposure to acute restraint stress (30min) and **(k)** Body weight gain (g) 3 weeks after the TST and acute restraint stress (N=11-12 per group). Data are mean ± SEM. * $p<0.05$ and ** $p<0.01$ *versus* control group (planned comparisons in **b**, **g** and **i**; Student *t*-tests in **c**, **e**, **f**, inset in **I, j** and **k**); ++ Fisher LSD's post-hoc test, control pre- *versus* poststress $p<0.01$.

Figure 4

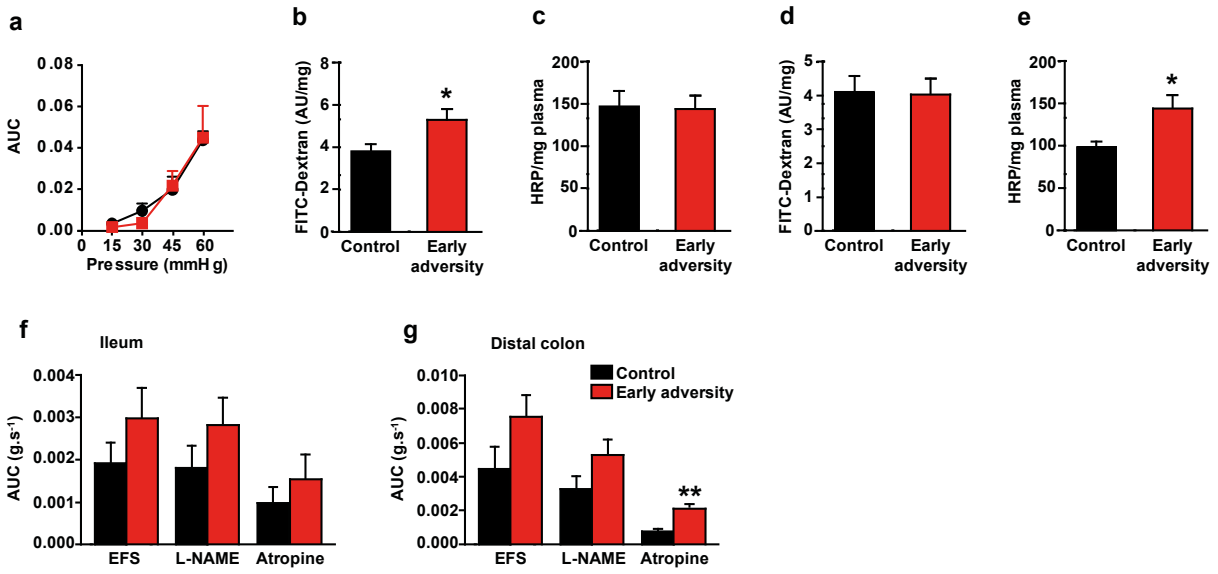

Fig 4. Early adversity increased intestinal permeability and motility in adult males. (a) Visceral sensitivity to colorectal distension (N=7-8 per group). Intestinal permeability to FSA (AU/mg) **(b)** or HRP (HRP/mg plasma) **(c)** at 2.5 months (N=10 per group). Intestinal permeability to FITC-Dextran (AU/mg) **(d)** or HRP (HRP/mg plasma) **(e)** at 5.5 months (N=11-12 per group). Ileal **(f)** and distal colonic **(g)** longitudinal muscle segments were stimulated by electrical field stimulation (EFS) (N=8 per group). The area under the curve (AUC) of EFS-induced contractile response was analyzed in absence or in presence of *N*-nitro-l-arginine methyl ester (l-NAME) or atropine. Data are mean ± SEM. * $p<0.05$ and ** $p<0.01$ *versus* control group (planned comparison in **g**; Student *t*-tests in **b** and **e**).

Figure 5

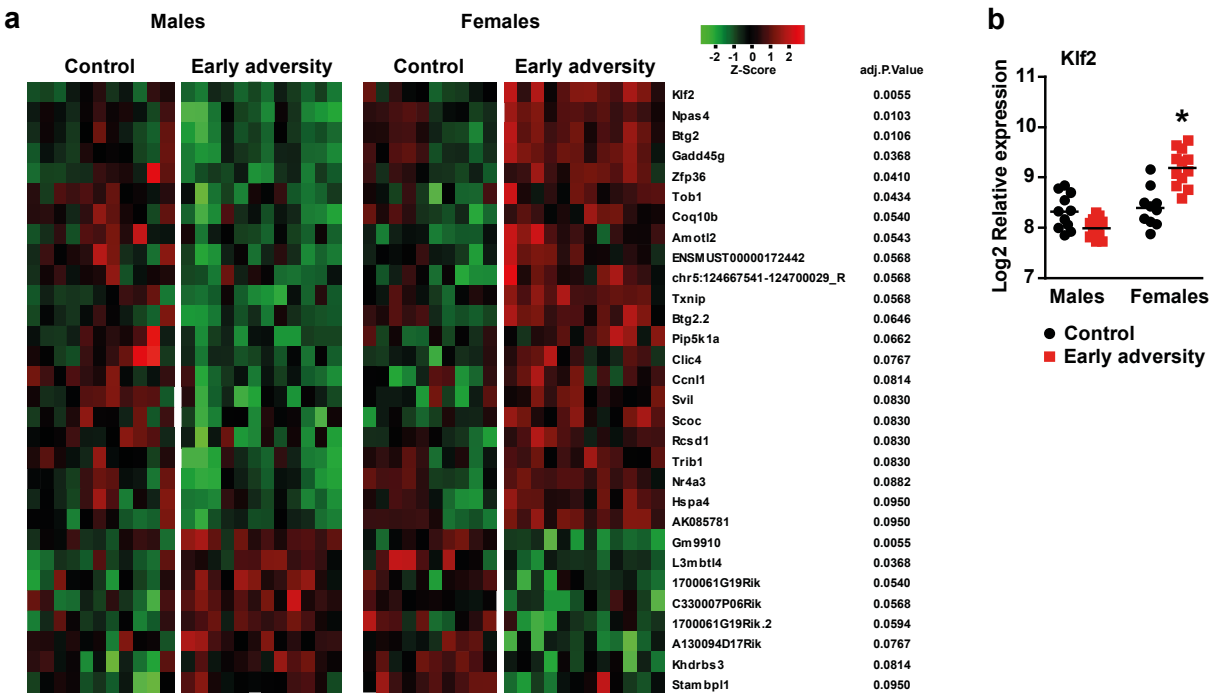

Fig 5. Gene expression in the mPFC. Microarrays revealed a significant early adversity x sex interaction for 30 probes. **(a)** Heatmap shows relative expression (Z-score) of the different probes (rows) in each animal (columns). **(b)** Klf2 mRNA (log2 relative expression) was significantly upregulated in early adversity females relative to controls (bars represent means). N=10-12 per group. * $p<0.05$ *versus* control females. Amotl2, angiomotin-like 2 ; Clic4, chloride intracellular channel 4; Rcsd1, RCSD domain containing 1; Pip5k1a, phosphatidylinositol-4-phosphate 5-kinase, type 1 alpha; Svil, supervillin; Klf2, Kruppel-like factor 2; Zfp36, zinc finger protein 36; Txnip, thioredoxin interacting protein; Tob1, transducer of ErbB-2.1; Scoc, short coiled-coil protein; Ccnl1, cyclin L1; Hspa4, heat shock protein 4; Btg2, B cell translocation gene 2, anti-proliferative; Gadd45g, growth arrest and DNA-damage-inducible 45 gamma; Npas4, neuronal PAS domain protein 4; Coq10b, coenzyme Q10B; Trib1, tribbles pseudokinase 1; L3MBTL4, l(3) mbt-like 4 (Drosophila); C330007P06Rik, RIKEN cDNA C330007P06 gene; Khdrb s3, KH domain containing, RNA binding, signal transduction associated 3; Stamb pl1, STAM binding protein like 1; 1700061G19Rik, RIKEN cDNA 1700061G19 gene; Gm9910, predicted gene 9910; A130094D17Rik, RIKEN cDNA A130094D17 gene.

Figure 6

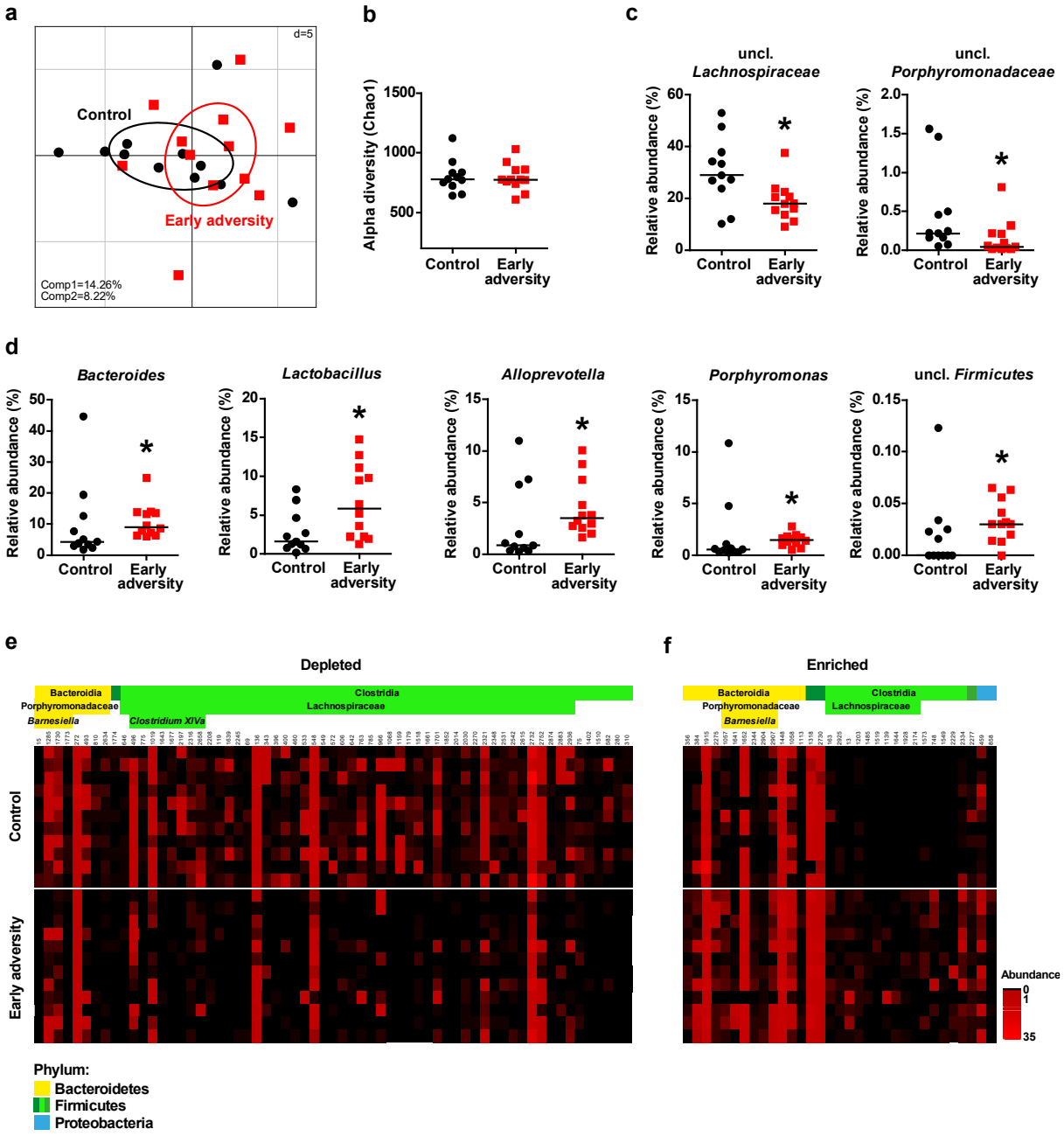

Fig 6. Effects of early adversity on fecal microbiota composition in adult males. (a) PCA based on genus distribution in early adversity and control males. **(b)** Alpha diversity (Chao1 index). Genera with significantly decreased **(c)** or increased **(d)** relative abundance in early adversity *versus* controls. Bars in histograms represent medians. Mann-Whitney U Test, * $p<0.05$. OTUs with significantly decreased **(e)** or increased **(f)** relative abundance in early adversity *versus* controls. Each column represents a single OUT and each row a single animal. Taxa affiliations are color coded. Red hues denote increasing relative abundance. N=11-12 per group.

Figure 7

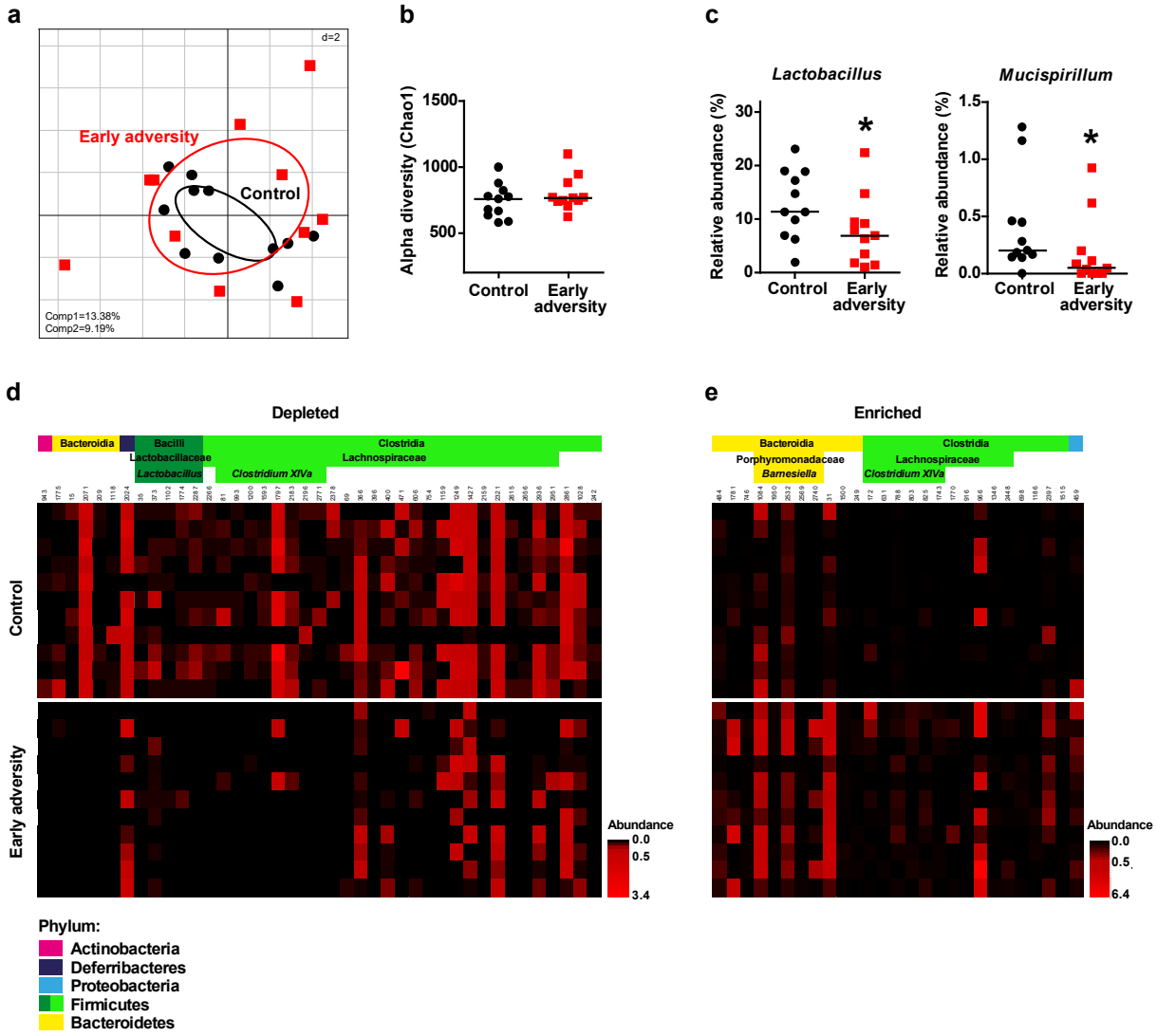

Fig 7. Effects of early adversity on fecal microbiota composition in adult females. (a) PCA based on genus distribution in early adversity and control males. **(b)** Alpha diversity (Chao1 index). **(c)** Genera with significantly decreased relative abundance in early adversity *versus* controls. Bars in histograms represent medians. Mann-Whitney U Test, * $p<0.05$. OTUs with significantly decreased **(e)** or increased **(f)** relative abundance in early adversity *versus* controls. Each column represents a single OUT and each row a single animal. Taxa affiliations are color coded. Red hues denote increasing relative abundance. N=11 per group.

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SUPPLEMENTARY INFORMATION

SUPPLEMENTARY METHODS

Locomotor activity (3.5 months)

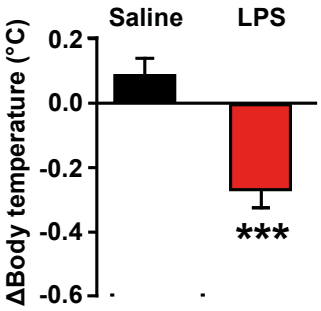
Mice were placed in individual cages (30 x 12 cm) filled with fresh bedding under dim light (light intensity: 30 lux) and videotracked for 60 min using Smart software (Bioseb). Total distance travelled was automatically quantified.

Plasma gut hormones multiplex assay

Plasma leptin, insulin, ghrelin (active), GLP-1 (active) and PYY (total) were measured by multiplex assay (MGTMAG-78K MILLIPLEX MAP Mouse Gut Hormone Magnetic Bead Panel, Millipore, Fontenay sous Bois, France) according to the manufacturer's instructions. Hormones concentrations were determined using the Luminex xMap Technology (Bio-Rad, Marnes-la-Coquette, France). All samples were processed in duplicates. Intra and inter assay coefficients were below 10 and 20%, respectively and crossed reactions were insubstantial (0.01%). Minimum detectable concentrations were 17.80 pg/mL for leptin, 18.89 pg/mL for insulin, 3.79 pg/mL for ghrelin (active), 20.98 pg/mL for GLP-1 (active) and 2.64 pg/mL for PYY (total). Active GLP-1 was not detectable in our samples.

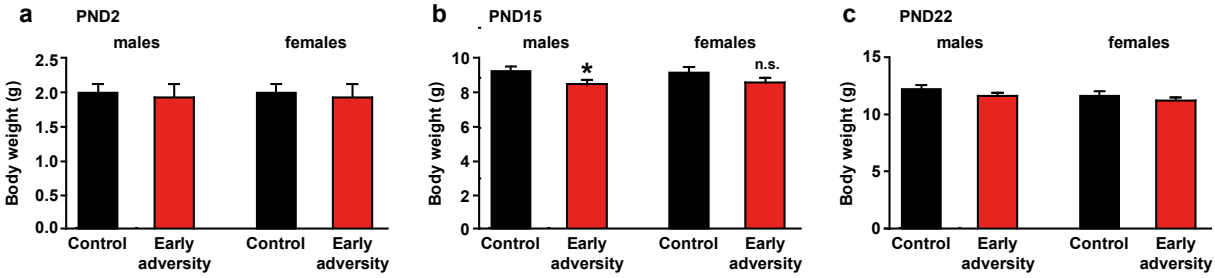
SUPPLEMENTARY FIGURES

Supplementary Figure 1



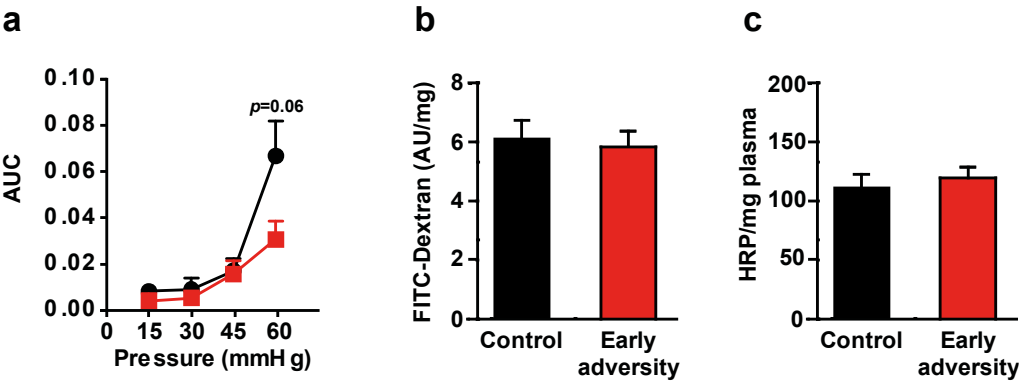
Supplementary Fig 1. LPS injection on gestational day 16 produces hypothermia in dams. Change in body temperature (°C) 3 hours post LPS or saline injection. The significant hypothermia observed in LPS-injected dams provides evidence for LPS response efficacy. N=14 per group. Data are means ± SEM. Student *t*-test: *** $p<0.001$

Supplementary Figure 2



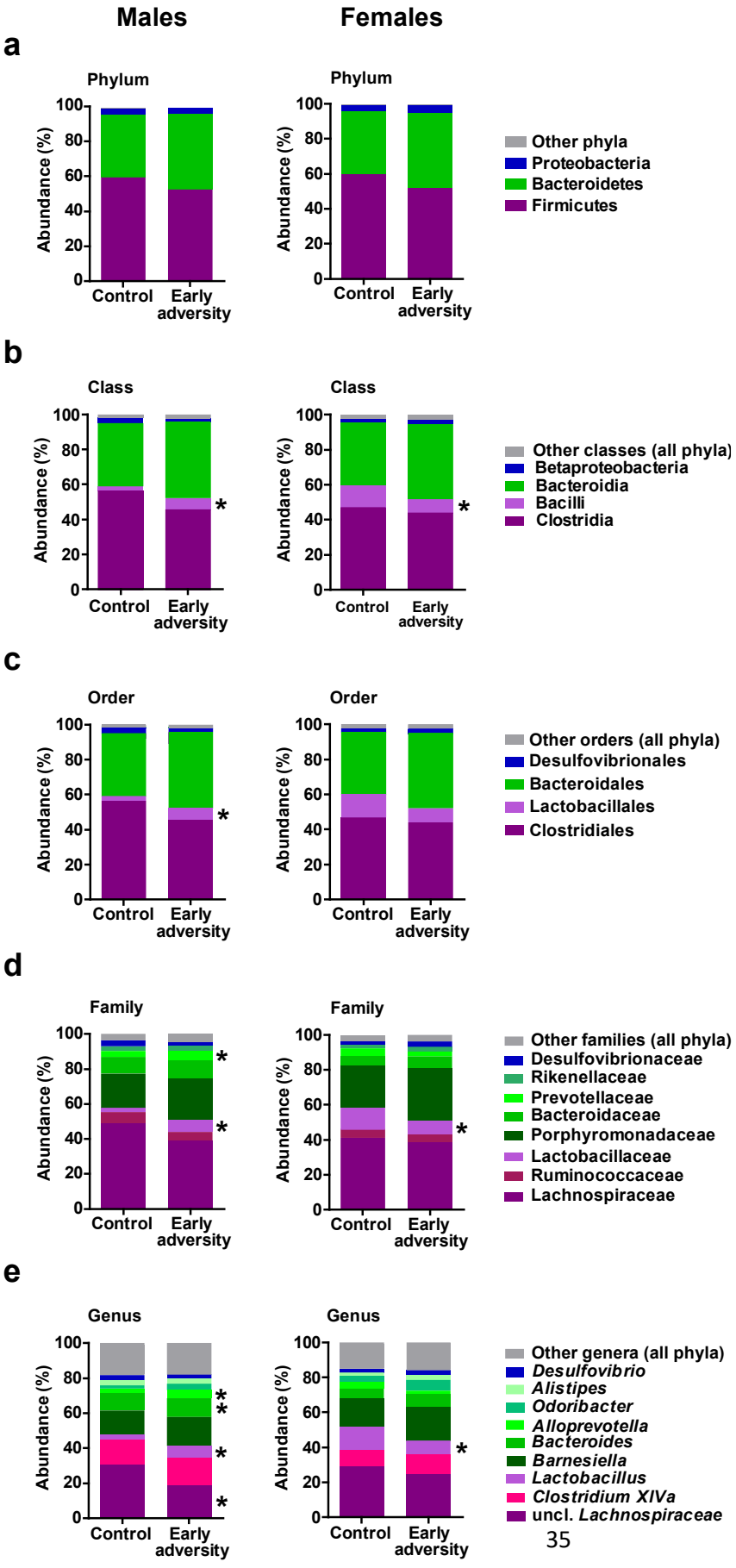
Supplementary Fig 2. Effect of early adversity on male and female offspring body weight. Body weight (g) of male and female pups on PND2 **(a)**, PND15 **(b)** and PND22 **(c)**. N=8-14 per group. Data are means ± SEM. Student *t*-test: * $p<0.05$ *versus* control males.

Supplementary Figure 3



Supplementary Fig 3. Effects of early adversity on visceral sensitivity and permeability in females at 2.5 months. (a) Visceral sensitivity to colorectal distension (N=7-9 per group). Intestinal permeability to FSA (AU/mg) **(b)** or HRP (HRP/mg plasma) **(c)** at 2.5 months (N=11 per group). Data are means ± SEM.

Supplementary Figure 4



Supplementary Fig 4. Effects of early adversity on bacterial taxa distribution in feces of adult males and females. Relative abundance of the different taxa in males (left) and females (right). Only predominant taxa are represented. **(a)** Mean phylum distribution per group. There were 7 detected phyla in males, 6 in females. The Candidatus Saccharibacteria phylum (only represented by *Saccharibacteria incertae sedis* spp.) was found only in two males of the early adversity group. The Deferribacteres phylum was only represented by mucispirillum spp. **(b)** Mean class distribution per group. There were 12 detected classes in males, 11 in females. **(c)** Mean order distribution per group. There were 19 detected orders in males, 18 in females. **(d)** Mean family distribution per group. There were 33 detected families in males, 32 in females. **(e)** Mean genus distribution per group. There were 77 detected genera in males, 73 in females. N=11-12 per group. Mann-Whitney U tests: **p*<0.05 *versus* controls of the same sex.

SUPPLEMENTARY TABLES

Supplementary Table 1. Gene expression in the mPFC. Microarrays revealed a significant early adversity x sex interaction for 30 probes (statistical significance alpha=0.1).

	Sex x early adversity Interaction	Early adversity *vs* Control Males		Early adversity *vs* Control Females	
Gene	**adj.P.Value**	**adj.P.Value**	**logFC**	**adj.P.Value**	**logFC**
Klf2	0.0055	0.5143	-0.33	0.0157	0.79
Npas4	0.0103	0.1464	-0.92	0.8644	0.57
Btg2	0.0106	0.4768	-0.60	0.4183	0.78
Gadd45g	0.0368	0.4901	-0.35	0.4183	0.48
Zfp36	0.0410	0.4768	-0.32	0.6556	0.35
Tob1	0.0434	0.4768	-0.20	0.7526	0.19
Coq10b	0.0540	0.4502	-0.46	0.9663	0.31
Amotl2	0.0543	0.5134	-0.11	0.4183	0.18
ENSMUST00000172442	0.0568	0.4768	-0.29	0.8684	0.24
chr5:124667541-124700029_R	0.0568	0.5184	-0.15	0.4183	0.33
Txnip	0.0568	0.5097	-0.26	0.4183	0.41
Btg2	0.0646	0.5097	-0.47	0.4462	0.74
Pip5k1a	0.0662	0.4768	-0.28	0.9548	0.22
Clic4	0.0767	0.4768	-0.35	0.9560	0.27
Ccnl1	0.0814	0.4768	-0.20	0.9560	0.16
Svil	0.0830	0.4768	-0.24	0.9973	0.18
Scoc	0.0830	0.5143	-0.13	0.6138	0.19
Rcsd1	0.0830	0.4901	-0.20	0.8060	0.21
Trib1	0.0830	0.4502	-0.75	0.9998	0.41
Nr4a3	0.0882	0.4768	-0.86	0.9998	0.52
Hspa4	0.0950	0.4985	-0.24	0.8060	0.28
AK085781	0.0950	0.5158	-0.35	0.4462	0.64
Gm9910	0.0055	0.4768	0.21	0.3264	-0.28
L3mbtl4	0.0368	0.4901	0.21	0.4462	-0.25
1700061G19Rik	0.0540	0.4502	0.36	0.9642	-0.24
C330007P06Rik	0.0568	0.4768	0.22	0.9560	-0.16
1700061G19Rik	0.0594	0.4901	0.21	0.7258	-0.23
A130094D17Rik	0.0767	0.5143	0.17	0.4183	-0.33
Khdrbs3	0.0814	0.5076	0.18	0.6900	-0.22
Stambpl1	0.0950	0.4905	0.14	0.8060	-0.15

DISCUSSION

DISCUSSION

In the last decade, there has been a huge interest in the gut-brain axis, especially as regards stress-related emotional behaviors. The gut microbiota emerges as a key node in this axis. The developmental period constitutes a critical window of sensitivity to stress. Indeed, early-life adversity increases the risk to develop psychiatric diseases, but also GI disorders such as IBS. Animal models including models of prenatal and post-natal adversity demonstrate lasting deleterious effects on both the gut and the brain. One of the most used models is maternal separation. Several studies suggest that alterations of gut microbiota composition in animals exposed to MS underlie some of its effects on brain and behavior (Desbonnet et al., 2010; De Palma et al., 2015; Moya-Pérez et al., 2017). Moreover, intestinal hyper-permeability has been consistently reported in MS animals (Söderholm et al., 2002)(Barreau et al., 2004a, 2004b; García-Ródenas et al., 2006; Gareau et al., 2007b; Moussaoui et al., 2014). However, although gut microbiota-directed interventions have been found to modulate gut permeability (Ait-Belgnaoui et al., 2012; Hsiao et al., 2013), the potential role of gut barrier function in mediating the long-term behavioral effects of MS has not been investigated. Importantly, sex differences as regards emotional vulnerability and gut microbiota composition exist in both humans and animals. However, a vast majority of the studies are conducted in males only. In this regard, we aimed to determine the role of gut permeability in the long-term neurobehavioral effects of early-life stress and to test whether early adversity differentially impacts gut microbiota and permeability according to sex.

In a first study, we showed that previously reported protective effects of maternal HFD on MS-induced neurobehavioral alterations are associated with prevention of gut leakiness in rat pups. This observation led us to ask whether gut permeability could contribute to the emergence of the behavioral alterations associated with early stress. We hypothesized a causal relationship between gut leakiness during early-life and MS-induced endophenotypes including behavior, stress response and gut dysbiosis. Using two complementary strategies, we demonstrated that pharmacological inhibition of gut leakiness during early-life prevents some of the long-term effects of MS on behavior and endocrine response to stress, and conversely, genetically-driven gut leakiness in transgenic mice that were not submitted to MS recapitulated some of its behavioral and endocrine phenotypes. These results are the first proof of concept that gut permeability can affect brain and behavior and support the hypothesis that maternal HFD may protect the developing brain notably *via* the attenuation of gut leakiness in MS pups. Moreover, we showed that inhibition of gut leakiness during development lastingly modulated the effects of MS on gut microbiota composition. The study

in transgenic mice also provided new insight into the sex differences in behavior and neurobiological correlates and importantly showed that gut leakiness differentially affects males and females. Finally, we report further sex differences in gut and brain vulnerability to early-life adversity in a multiple-hit model.

Here, we will first compare the results obtained in our different studies and discuss the findings on the role of gut leakiness and dysbiosis in the psychoneuroendocrine effects of early adversity. In a second part, we will discuss possible mechanisms of gut-brain communication downstream gut permeability and microbiota, as well as potential biological bases for the observed sex differences in our models.

I – Commonalities and disparities in our studies

Effects of early adversity and gut permeability manipulations on brain, HPA axis and behavior

In line with the literature and our previous results (Rincel et al., 2016, **ANNEXE 1)**, we found that MS produces hyperanxiety in male rats **(ARTICLE 2)**. It appears that male C3H mice are resistant to the effect of multifactorial early adversity on anxiety-like behavior **(ARTICLE 4)**. However, female C3H mice do develop hyperanxiety after early adversity. These findings can be compared with the CA-MLCK study **(ARTICLE 3)**, in which females, but not males, display increased anxiety levels.

Surprisingly, we did not find social deficits in male rats submitted to MS in our ML-7 study **(ARTICLE 2)**, contrasting to our previous findings (Rincel et al., 2016, **ANNEXE 1)** and other reports. A possible explanation is that the MS protocol was slightly different due to daily vehicle injections in both control and MS groups, which might constitute a mild stressor for control rats which were undisturbed in our previous work. However, male C3H submitted to multifactorial early adversity show decreased social interaction whereas females do not **(ARTICLE 4)**, suggesting that males are more prone to social behavior impairment than females.

Another, striking discrepancy between our studies is the increased number of isolation-induced ultrasound calls in MS male rats **(ARTICLE 2)** *versus* its decrease in male C3H mice exposed to early adversity **(ARTICLE 4)**. Although in both cases, the altered pattern of vocalization can be interpreted as a disturbance in communication with the dam, it suggests that this behavior is different across species. Ultrasonic vocalizations (USVs) are generally

recognized as a sensitive measure of altered social communication in mouse models of autism (Scattoni et al., 2009), and/or anxiety-like behavior in rat pups (Hofer, 1996). MS in rats is widely used as a model of anxiety and/or depressive disorders and we report, in line with previous studies (Litvin et al., 2016), that MS increases USV in developing pups. Interestingly, the increased USV number in male MS rats is not associated with later social behavioral impairment in adulthood, but rather with adult hyperanxiety **(ARTICLE 2)**. It has been shown that rats selectively bred for their high rates of isolation-elicited USVs at PND10, for 20 generations, display higher anxiety levels compared with rats selectively bred for low rates of USVs (Zimmerberg et al., 2005). In contrast, in C3H mice exposed to multifactorial early adversity, USVs are reduced in both sexes during development, but adult male offspring exhibit altered social behavior while adult females show hyperanxiety **(ARTICLE 4)**. Importantly, the multifactorial model involves prenatal inflammation, which is used to model autism in mice, and leads to reduced social behavior (Knuesel et al., 2014). Moreover, the MS protocol used in C3H mice also include unpredictable chronic mild stress in dams and thus is different from MS in rats. A limitation in the latter study is that we did not dissociate the respective effects of prenatal inflammation and chronic post-natal stress on offspring's behavior **(ARTICLE 4)**. Altogether, our results and other findings in the literature indicate that the relationship between early stress and USVs in neonatal rodents is complex and may vary according to several factors such as the species and the type of early stress considered.

Overall, it seems that emotional vulnerability differs between male rats and mice. The literature suggests that female rats are more resistant to perinatal stress than males (Barna et al., 2003; Dimatelis et al., 2016; Prusator and Greenwood-Van Meerveld, 2016), which does not seem to be the case in mice. A major limitation is that our studies conducted in rats involved only males (Rincel et al., 2016, ANNEXE **1; ARTICLES 1 and 2)**.

We showed in male rats that prevention of gut leakiness during development protects against the long-term sexual reward seeking impairment induced by MS **(ARTICLE 2)**. In CA-MLCK male mice that display gut leakiness, we observed decreased sexual reward seeking and HPA axis hyper-response to stress, that resemble MS phenotype in rats **(ARTICLE 3)**. On the other hand, CA-MLCK males do not exhibit increased anxiety or reduced sucrose preference, and such alterations were not restored by ML-7 in MS rats. Together, these results consistently suggest that developmental gut permeability in males plays a role in the maturation of brain areas involved in sexual reward seeking behavior and HPA axis function. However, further studies using transgenic mice with overexpression of the CA-MLCK in the gut specifically during the early post-natal life, for instance using the lactase promoter (Lee et

al., 2002), may help to unravel the differential impact of gut leakiness during development or at adulthood.

Regarding the effects of early adversity and gut permeability manipulations on the brain, comparisons are difficult because we did not use the same neurobiological readouts across studies, which constitutes a main limitation. Nevertheless, we found significant alterations in the PFC of rats and mice (neuronal morphology, stress-related gene expression and transcriptome, in **ARTICLE 1, 3 and 4** respectively), highlighting the key role of this brain area in a context of early stress but also in gut-brain communication. A recent report in Nature demonstrated that maternal immune activation produces abnormalities in the primary somatosensory cortex of adult offspring, and that activation of pyramidal neurons in this cortical area is sufficient to induce the long-term autistic-like behavioral effects of maternal immune activation (Shin Yim et al., 2017). Therefore, it would be interesting to examine whether animals exposed to early adversity or chronic gut leakiness display similar cortical abnormalities. Moreover, we have not investigated the neurobiological impact of gut permeability manipulations during development, in particular on dendritic spine density, that we reported to be altered by MS in PND21 juvenile rats **(ARTICLE 1)**. Further studies will be required to fill this gap.

Effects of early adversity and gut permeability manipulation on visceral sensitivity

MS is a widely used model of IBS and MS offspring are hypersensitive to colorectal distension in adulthood. We previously showed that visceral hypersensitivity in adult male rats exposed to MS was prevented by perinatal exposure to HFD. The results obtained in our first study **(ARTICLE 1)** suggest that this effect of HFD on visceral sensitivity could be underlied by the protective effect of HFD on gut permeability in MS pups. However, we have not assessed visceral sensitivity in rats treated with the ML-7 or in CA-MLCK mice **(ARTICLES 2 and 3)**. Unpublished data from our collaborators show that female CA-MLCK mice are hyposensitive to colorectal distension under basal conditions, whereas they display hypersensitivity following acute water avoidance stress (Ferrier et al., unpublished data). Although there are no data available in male CA-MLCK mice, these findings are particularly interesting with respect to our results in C3H mice exposed to multifactorial adversity **(ARTICLE 4)**. Indeed, in this study, female mice showed a trend towards decreased visceral sensitivity under basal conditions. It would have been interesting to expose them to acute stress and re-perform colorectal distension. These data also suggest that visceral sensitivity is differentially impacted in males *versus* females according to the species. Finally, it seems that altered visceral sensitivity goes with hyperanxiety in our models. Indeed, male

MS rats, female CA-MLCK mice, as well as female C3H exposed to early adversity all display hyperanxiety and altered visceral sensitivity, whereas male C3H exposed to early adversity are not hyperanxious and do not display altered visceral sensitivity (Rincel et al., 2016, **ANNEXE 1** and **ARTICLES 3 and 4)**. It will be necessary to analyze visceral sensitivity in female MS rats and male CA-MLCK to confirm this relationship. Other studies reported concomitant anxiety and visceral sensitivity alterations in MS rodents (Schwetz et al., 2005; Moloney et al., 2015b). Moreover, a study comparing different mouse strains has described similar relationships, with CBA/J and C3H/HeN mice being the most anxious and viscerally hypersensitive (Moloney et al., 2015c). However, whether visceral sensitivity and anxiety cause each other remains to be demonstrated.

Effects of early adversity and gut permeability manipulation on the gut microbiota

In male rats, the preventive effects of ML-7 on MS-associated endocrine and behavioral phenotypes were accompanied by attenuation of gut dysbiosis at adulthood **(ARTICLE 2)**. Unpublished data from our collaborators reveal that CA-MLCK mice also exhibit dysbiosis, including increased *Clostridium XIVa*, *Clostridium IV* and *Desulfovibrio* spp. and decreased *Enterococcus*, *Prevotella*, *Bacteroides* and *Bifidobacterium* spp. (Ferrier et al., unpublished data). The enrichment in *Desulfovibrio* and depletion in *Bifidobacterium* spp. are in line with our results in MS rats (same alterations but restored by ML-7 treatment). However, these observations were made in female CA-MLCK mice. Further investigation is needed in both sexes to better understand the effects of gut barrier dysfunction on gut microbiota composition. In addition, such data could provide new insight into the behavioral sex differences observed in the transgenic mice.

Using a different model of multifactorial early adversity in C3H mice, we have also analyzed the effects of early-life stress on gut microbiota composition in both sexes **(ARTICLE 4)**. Comparison with gut microbiota data in rats submitted to MS did not highlight robust similarities at the genus level in male animals. At the OTU (operational taxonomic unit) level (data not shown in MS rats), a significant proportion of the altered bacterial populations belongs to Clostridiales or Bacteroidales orders in both studies; however, the corresponding families within these orders are different in rats *versus* mice. Nonetheless, the greatest number of altered OTU in each experiment are Lachnospiraceae spp. Previous studies also reported altered abundance of these bacteria in animal models of early adversity including MS (De Palma et al., 2015; Zhou et al., 2016; Murakami et al., 2017). More interestingly, numerous OTUs in the Lachnospiraceae family have been found to be either increased or decreased in stools of depressive patients compared with healthy controls (Naseribafrouei et al., 2014;

Zheng et al., 2016), suggesting that the effects of early adversity on these bacteria in animal models are relevant to human psychiatric conditions.

It is worth mentioning that these comparisons between species (humans, rats and mice) and between mouse strains may not make so much sense, as it appears that variations in gut microbiota composition are observed even among mice of the same strain. For instance, several studies reported that gut microbiota composition of C57BL/6 mice differs according to the animal provenance (Jackson Laboratories versus Taconic Biosciences) (Ivanov et al., 2009; Kim et al., 2017; Rosshart et al., 2017). In addition, the DNA extraction protocols, analytic pipelines (identification of taxa) and data processing used differ between our studies. It has been shown that these parameters influence microbiota compositional data both quantitatively and qualitatively (Fouhy et al., 2016; Salonen et al., 2010). For DNA extraction, we used mechanical lysis (beads) in the rat study **(ARTICLE 2)** and both mechanical and chemical lysis in the C3H mice study **(ARTICLE 4)**. In addition, OTU grouping was carried out using FROGS pipeline in rats and uclust software (QIIME) in C3H mice. Finally, a negative binomial fit model with Benjamini-Hochberg method to control the False Discovery Rate was applied in the rat study (logarithmic transformation), whereas row relative abundance (%) data were used in the C3H mice study. Key protocol issues including the 16S RNA targeted regions (V3-V4 regions) and sequencing method (Illumina MiSeq) were however conserved across the studies. These methodological considerations obviously also apply to comparisons with other works in the literature. In conclusion, despite the methodological differences found in our studies, populations among the Lachnospiraceae family seem to be fundamentally affected by both early-life adversity and gut permeability manipulation.

II –Mechanisms underlying the effects of early adversity on emotionality and stress response

Role of gut leakiness in stress-induced dysbiosis

The mechanisms by which MS leads to changes in microbiota composition are not yet understood. It has been shown that stress decreases gastric acid and increases bicarbonate secretion in rats, thereby leading to changes in luminal pH and possibly favoring certain bacterial communities. Especially, it has been shown that some *Clostridium XIVa* spp. (Lachnospiraceae) survive well in an acidic environment compared with other bacterial strains from the Bacteroidetes phylum (Duncan et al., 2009). Moreover, these bacteria are important

SCFA producers and SCFAs are suspected to lower the colonic pH (Macfarlane et al., 1992), possibly resulting in a self-perpetuating loop. In addition, MS effects on gut mucosa and mucus layer as well as mucosal immune and enteric nervous systems likely play a role. Indeed, decreased mucus layer thickness has been reported in MS animals (O'Malley et al., 2010). Since different bacterial strains reside preferentially in the mucus layer, it is conceivable that a change in mucus thickness would alter the composition of gut bacteria. Moreover, our present results suggest that gut leakiness *per se* can, at least in part, contribute to the MS-induced gut dysbiosis **(ARTICLE 2)**. Bacterial translocation has been shown to occur in MS animals (Barreau et al., 2004a; Moussaoui et al., 2014). We can speculate that increased occurrence of bacterial invasion in the lamina propria due to gut leakiness stimulates the immune system in a way that it would increase the production of IgA and antimicrobial peptides to negatively regulate microbial density. Such changes in the cross-talk between host immune cells and microorganisms could lead to modifications of gut microbiota composition.

Role of gut leakiness and dysbiosis in the regulation of emotional behavior

Gut permeability and gut microbiota are tightly connected, making it hard to isolate their separate effects on brain and behavior. Indeed, our results together with the literature show that gut leakiness is often concomitant with gut dysbiosis **(ARTICLES 2 and 4)**. Although the causal role of gut bacteria has been robustly demonstrated by the use of GF animals, fecal transplantation and probiotics, it is not clear whether these effects are independent of gut permeability. In our models, only adult C3H females submitted to multifactorial early adversity seem to display gut dysbiosis despite intact gut barrier function, even though we cannot exclude that gut leakiness occurs earlier in life in these animals **(ARTICLE 4)**. An interesting study published in 2015 demonstrated that MS fails to induce emotional behaviors in GF mice, but leads to increased anxiety and depressive-like behaviors upon colonization with the gut microbiota of a conventional control mouse (De Palma et al., 2015). However, colonization with the microbiota of a maternally separated animal did not transfer the MS-associated behavioral phenotype in naive GF mice. These findings suggest that gut bacteria are necessary but not sufficient to mediate the behavioral effects of MS. A hypothesis could be that GF mice display gut leakiness, conferring latent vulnerability only unmasked by microbial colonization. Conversely, control animals not exposed to MS would not display gut leakiness, which would explain that altered gut microbiota following fecal transplantation from a MS donor has no deleterious effects on emotional behaviors. Together with these

previous findings, our results suggest that both gut dysbiosis and gut leakiness are required for the long-term behavioral effects of MS.

As previously underlined, the protective effect of ML-7 treatment in early-life is restricted to some MS-induced alterations (i.e. decreased sexual reward sensitivity and sustained corticosterone response to stress) **(ARTICLE 2)**. Since the gut dysbiosis in MS rats was not fully prevented by the ML-7 treatment, a hypothesis could be that the remaining alterations in certain bacterial communities account for the lack of preventive effect of ML-7 on specific behaviors. For instance, hyperanxiety and reduced sucrose preference, which are unaffected by ML-7, could be underlied by enriched *Acetitomaculum*, *Escherichia*, *Intestinibacter* or *Parabacteroides* genera, or depleted *Turicibacter* or *Adlercreutzia* genera, that are also unaffected by ML-7. Indeed, a study showed that supplementation with *Escherichia coli* produces increased anxiety levels in male Wistar rats (Tennoune et al., 2015). In this regard, it would be interesting to test the effects of fecal transplantation with the microbiota of control animals (C Veh) in both MS Veh and MS ML-7 groups. Furthermore, some studies have shown that early colonization of GF mice could normalize their neuroendocrine and behavioral phenotypes, whereas adult colonization failed (Sudo et al., 2004; Heijtz et al., 2011). We have seen that early ML-7 treatment restored gut permeability in PND14 pups, but no longer in PND21 or PND49 rats. Another hypothesis could be that intact gut barrier function is not only critical during early post-natal life, but also during adolescence and/or adulthood for behaviors not restored by early ML-7 (anxiety and sucrose preference). Therefore, testing the effects of ML-7 treatment during adulthood would provide deeper insight in the existence of potential windows of sensitivity.

The behavioral impact of gut leakiness in CA-MLCK males seems to be restricted to decreased sexual reward sensitivity and spatial memory impairment **(ARTICLE 3)**. Since CA-MLCK mice display altered HPA axis responsivity to stress, it would be interesting to expose them to chronic stress such as MS or chronic restraint stress in adulthood. Moreover, an important consideration is that CA-MLCK mice display only mild increases in gut permeability. For instance, gut leakiness in CA-MLCK mice is not associated with bacterial translocation and mucosal damage (Su et al., 2009; Edelblum et al., 2017), that are found in maternally separated animals (Barreau et al., 2004a; Moussaoui et al., 2014). The group of Turner reported that CA-MLCK mice are more sensitive to experimental colitis but exhibit only subclinical systemic inflammation (Su et al., 2009). Therefore, we can hypothesize that an immune challenge such as LPS injection may aggravate their behavioral alterations and/or produce other alterations, possibly through neuroinflammatory processes. As a matter of fact,

only heterozygous CA-MLCK mice can be used because breedings involving CA-MCLK females fail (Ferrier personal communication). It would be interesting to develop new tools to produce larger increases in gut permeability. Moreover, the use of WT dams precludes the investigation of the effects of *in utero* exposure to maternal gut leakiness, that might be also deleterious for offspring's brain development.

Finally, it is worth noting that our MLCK-directed strategies to manipulate gut permeability are restricted to paracellular, tight-junction-dependent transport. Because of the different nature of the trafficking molecules, we can imagine that para and transcellular hyperpermeability do not produce the same effects locally in the gut and thus could be associated with different behavioral outcomes. For instance, certain bacterial metabolites may be transported *via* transcellular pathways and others may travel between cells (Ménard et al., 2010). Moreover, para and transcellularly transported molecules may differentially affect the mucosal immune system, which responds to both intra and extra-cellular antigens (Eberl, 2016).

Role of other gut microorganisms in the gut-brain axis

Although it also applies to most of the current research on gut microbiota, especially in the gut-brain axis, a limitation is that our characterizations of gut microbiota composition are restricted to bacteria. Gut bacteria provide a wide array of microbial antigens and metabolites that have been demonstrated to affect mucosal immunity, but also CNS functions. However, gut bacteria are not the only inhabitants of the gut and the contribution of archaebacteria, viruses and fungi in our effects on brain and behavior remains to be determined. These microorganisms also tightly interact with the mucosal immune system and likely play an important role in regulating the nature, number, and function of bacterial communities. We do not rule out that some of these microbial populations could be primarily impacted by early-life adversity or gut permeability manipulation and participate in broader effects within the gut-brain axis. For instance, a recent study reported that the mycobiome (fungi) is responsible for visceral hypersensitivity in maternally separated rats (Botschuijver et al., 2017). The authors elegantly showed that visceral hypersensitivity was abolished in fungicide-treated MS rats, and further, only fecal transplantation with non-fungicide-treated caecal content was able to reinstate visceral hypersensitivity in these rats. To date, the role of the gut mycobiome in the regulation of emotional behaviors is unknown.

Role of the gut immune system

Our results underscore remaining big questions in the gut-brain axis field such as how can gut dysbiosis and leakiness exert their long-term effects in the CNS. A recent study published in

Nature has demonstrated that segmented filamentous bacteria (SFB), members of the Clostridiaceae, play a critical role in the effects of poly(I:C)-induced maternal immune activation (MIA) on autistic-like behaviors in the offspring (Kim et al., 2017), *via* the induction of T helper 17 (Th17) cells in gestant dams. Intriguingly, they found out that offspring of dams from Jackson Laboratories – which lack SFB in their gut flora – were resistant to maternal immune activation, as they did not show any of its associated behavioral disturbances, contrary to offspring of dams from Taconic Biosciences – that are known to carry SFB. Strikingly, Jackson dams' fecal transplantation with the microbiota of mice mono-colonized (or mono-associated) with SFB was sufficient to reinstate MIA susceptibility. Interestingly, a recent study showed that mucosal immune responses to intestinal pathogen infection are different in CA-MLCK mice compared with WT (Edelblum et al., 2017). Indeed, CA-MLCK mice show increased numbers of $CD4^+$ T cells in the lamina propria. The authors reported that this phenotype was absent in mice from Jackson Laboratories and depended upon IL-17 production in the presence of specific bacteria found in mice from Taconic Biosciences, most likely SFB, known as potent Th17 cell inducers (Gaboriau-Routhiau et al., 2009; Ivanov et al., 2009). Together, these findings suggest that altered abundance of gut commensal bacteria with a propensity to induce Th17 cells may increase the neuropsychiatric vulnerability in CA-MLCK mice, but also in our other models. For instance, it would be interesting to compare the behaviors of GF CA-MLCK mice, ex-GF CA-MLCK mice colonized with the microbiota of a conventional mouse, or monocolonized with SFB or lachnospiraceae spp.

SFB is a group of spore-forming gram-positive bacteria, defined on the basis of their morphology and their characteristic adhesion to epithelial cells (Davis and Savage, 1974). They are widely distributed in vertebrates, although their 16S rRNA gene sequences differ among host species (Snel et al., 1995; Yin et al., 2013). Thus, SFB do not constitute a phylogenetic group, but they belong to the order of Clostridiales within the phylum Firmicutes. Although we did not observe significant stress-induced differences in rodent SFB (Clostridiaceae) in our models, it is plausible that other bacteria including Lachnospiraceae spp. have similar immunomodulatory effects. Indeed, Lachnospiraceae spp. have been shown to reside in close proximity to the intestinal epithelium (Van den Abbeele et al., 2013) and SFB in arthropods belong to the Lachnospiraceae family (Thompson et al., 2012). In addition, it has been shown that, apart from rodent SFB, other bacterial strains with epithelial-cell-adhesive properties also cause a robust induction of Th17 cells in the mouse colon (Atarashi et al., 2015). Furthermore, a recent study conducted in C3H mice, the same mouse strain that

we used in the multifactorial early adversity model, showed that mono-association with *Roseburia hominis,* a Lachnospiraceae spp., increases the number of $Foxp3^+$ regulatory T cells (Tregs) in the lamina propria (Patterson et al., 2017). $Foxp3^+$ Tregs regulate the number and polarization of effector T cells and play a pivotal role in suppressing excessive immune responses deleterious to the host (Sakaguchi et al., 2008); (Wang et al., 2014b). It has been shown that transient systemic Treg depletion affects the brain's choroid plexus, a selective gateway for immune cell trafficking to the CNS, and is associated with subsequent recruitment of monocyte-derived macrophages and Tregs to cerebral sites in an animal model of Alzheimer's Disease (Baruch et al., 2015).

In rat pups submitted to MS, we did not observe evident increases in proinflammatory cytokines mRNA expression in the hippocampus or PFC **(ARTICLE 2)**. However, deeper analyses such as cytokines protein level determination, but also quantification of $CD4^+$ T cells, especially CD45high/CD11bhigh cells that represent infiltrating macrophages, or microglial cells, could reveal potential neuroinflammatory processes, that may be affected by the ML-7 treatment. For instance, it has been shown that MS pups exhibit increased numbers of activated microglia in the PFC and hippocampus (Gracia-Rubio et al., 2016). Microglia play a critical role in post-natal brain wiring notably by regulating the number and quality of dendritic spines, a process known as synaptic pruning (Thion and Garel, 2017). As seen in the introduction, the gut microbiota has been shown to regulate the properties and maturation of microglia including during development (Erny et al., 2015; Matcovitch-Natan et al., 2016). Notably, microglia of GF mice show a downregulation of genes associated with inflammation (Matcovitch-Natan et al., 2016). Interestingly, sex differences exist in microglia density and phenotype during development (Schwarz and Bilbo, 2012; Hanamsagar and Bilbo, 2016). For instance, males have overall more microglia early in postnatal development. Moreover, gene expression of numerous cytokines, chemokines and their receptors is highly dependent upon sex (Schwarz and Bilbo, 2012). Microglia have estrogen receptors, and it has been shown that microglial activation during embryonic development is influenced by sex hormones (Lenz et al., 2013). Our work shows that the impact of early adversity on brain, HPA axis function and behavior, but also gut permeability, visceral sensitivity and gut microbiota is different in males *versus* females **(ARTICLE 4).** It is widely accepted that there are differences in immune function between sexes. Males are generally more susceptible to infections, whereas prevalence of autoimmune disorders is much higher in females (Klein, 2000; Ruggieri et al., 2016). Moreover, gut microbiota composition also differs between males and females (Markle et al., 2013). Recent evidence suggests that sex differences in gut microbiota composition

could underlie the aforementioned immune sex differences (Fransen et al., 2017). In light of our results, it could be hypothesized that both differences in gut microbiota and differences in immunity, possibly in interaction with gut permeability, contribute to the differential effects of early adversity on brain and behavior in males and females. Future work exploring the effects of gut permeability manipulation on neuroimmune processes will shade new light on the molecular and cellular mechanisms underlying its effects on brain and behavior.

Role of short-chain fatty acids

Gut bacteria produce numerous metabolites including SCFAs, which have been reported to exert a variety of biological effects in the host (Shenderov, 2012). For instance, SCFAs can activate different G-protein coupled receptors, including in the brain (Stilling et al., 2016). Butyrate receptors are found in neutrophils, monocytes and Tregs and there is accumulating evidence that butyrate has anti-inflammatory potential (Bollrath and Powrie, 2013), suggesting that butyrate could play a role in neuropsychiatric conditions associated with chronic inflammation such as depression (Raison et al., 2006). In addition, SCFAs have been identified as potent HDAC inhibitors (especially butyrate) (Candido et al., 1978; Licciardi et al., 2011; Stilling et al., 2014b). For instance, SCFAs have been shown to strengthen the integrity of the gut epithelial barrier by the upregulation and reorganization of tight junction proteins, notably *via* HDAC inhibition (Suzuki et al., 2008; Peng et al., 2009; Wang et al., 2012). In addition, gut microbiota has been shown to modulate gut homeostasis and inflammatory response in a HDAC3-dependent manner (Alenghat et al., 2013). Interestingly, epigenetic regulations have been proposed to underlie some aspects of microbiota-gut-brain bottom-up communication (Stilling et al., 2014a, 2014b). In this respect, Stilling and colleagues suggested that the concept of hologenome could be extended to 'holo-epigenome'. A study by Braniste et al. suggests that SCFAs regulate BBB function, although the exact underlying mechanisms are not clear (Braniste et al., 2014). Remarkably, Erny and coworkers demonstrated a role for SCFAs in the maintenance of microglia homeostasis (Erny et al., 2015). In addition, numerous studies have reported that peripheral administration of sodium butyrate or 4-phenylbutyrate in adult animals modulates brain physiology and function including anxiety, depressive and autistic-like behaviors (see Stilling et al., 2016 for review). For instance, adult sodium butyrate administration has been shown to exert antidepressant-like effects both in naive mice and in chronically stressed mice (Schroeder et al., 2007; Tsankova et al., 2006) and to attenuate social deficits in a genetic mouse model of autism (Kratsman et al., 2016). De Vadder et al. have shown that propionate-enriched diet increased the number of C-Fos$^+$ cells in the NTS of rats, suggesting an involvement of the vagus nerve

in the effects of SCFAs on behaviors (De Vadder et al., 2014). Considering the potential role of the vagus nerve in gut-brain communication, Stilling et al. highlighted the interest of exploring epigenetic modifications in the brainstem (Stilling et al., 2016). Although to our knowledge, no such investigation has been published to date, recent findings suggest that stress-induced visceral hyperalgesia is driven by epigenetic mechanisms in the spinal cord (Hong et al., 2015). Indeed, rats submitted to chronic water avoidance stress exhibited epigenetic marks in dorsal root ganglia of the lombosacral spinal cord, at several genes including Nr3c1 (GC receptor). Specific knockdown of DNMT1 and HAT P300 in lombosacral dorsal root ganglia neurons reduced DNA methylation and histone acetylation and prevented stress-induced increases in visceral pain (Hong et al., 2015). Consistently, another study reported similar beneficial effects of peripheral HDAC inhibition on visceral pain in the MS model (Moloney et al., 2015b).

As seen in the introduction, a variety of epigenetic marks have been reported in the brain of MS offspring, notably at the promoter of genes encoding GC receptors. Moreover, similar marks have been found in the brains of suicide victims with a history of childhood maltreatment (McGowan et al., 2009, 2013). In addition, recent insights in animals support neuroimmune and neuroepigenetic bases for the sex differences observed in the brain (McCarthy et al., 2017). Altogether, these findings support a role of SCFAs in the long-term alterations of gut-brain communication induced by early adversity. Interestingly, lachnospiraceae (especially *Clostridium cluster XIVa*) members are important butyrate producers (Flint et al., 2008; Louis and Flint, 2009), suggesting that altered abundance of some lachnospiraceae spp. could account for the psychoneuroendocrine alterations observed in our models, possibly *via* a change in SCFA quantity and quality. Accordingly, it would be pivotal to quantify SCFAs in the gut and plasma in our models.

Conclusion

In conclusion, our work shows that early adversity leads to sex differences in emotional behavior, gut permeability and gut microbiota composition. We provide new insight into the mechanisms of gut-brain communication and highlight intestinal permeability as a key actor. Furthermore, our results point to a potential role of Lachnospiraceae members in these effects. Our findings have relevance for psychiatric conditions associated with early-life adversity, including autism, anxiety and mood disorders, and might contribute, ultimately, to develop alternative preventive and therapeutic strategies for these disorders.

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ANNEXES

ANNEXE 1

OPEN

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ORIGINAL ARTICLE

Maternal high-fat diet prevents developmental programming by early-life stress

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Anxiety disorders and depression are well-documented in subjects exposed to adverse childhood events. Recently, maternal obesity and/or maternal consumption of high-fat diets (HFD) have been also proposed as risk factors for offspring mental health. Here using an animal model in rats, we explored the combinatorial effects of a maternal HFD (40% of energy from fat without impact on maternal weight; during gestation and lactation) and maternal separation (MS) in offspring. In the prefrontal cortex (PFC) of pups, MS led to changes in the expression of several genes such as *Bdnf* (brain derived neurotrophic factor), *5HT-r1a* (serotonin receptor 1a) and *Rest4* (neuron-restrictive silencer element, repressor element 1, silencing transcription factor (*Rest*), splicing variant 4). Surprisingly, perinatal HFD strongly attenuated the developmental alterations induced by MS. Furthermore, maternal HFD totally prevented the endophenotypes (anxiety, spatial memory, social behavior, hypothalamic–pituitary–adrenal (HPA) axis response to stress, hippocampal neurogenesis and visceral pain) associated with MS at adulthood. Finally, we also demonstrated that HFD intake reduced anxiety and enhanced maternal care in stressed dams. Overall, our data suggest that a HFD restricted to gestation and lactation, which did not lead to overweight in dams, had limited effects in unstressed offspring, highlighting the role of maternal obesity, rather than fat exposure *per se,* on brain vulnerability during development.

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INTRODUCTION

The etiology of the majority of psychiatric disorders remains unknown. It is, however, well-accepted that psychosocial adversity in childhood can contribute to an increased risk of depressive and anxiety disorders later in life.[1–6] In modern societies, a considerable amount of the population including childbearing women and children is exposed to low-cost energy-dense food with a high content in fat. Yet, it has been recently proposed that maternal obesity and/or maternal consumption of fat-rich diets could also constitute risk factors for offspring's mental health.[7] It is therefore crucial to unravel the possible combinatorial effects of perinatal exposure to fat-rich diets and early-life stress on the developing brain.

Early disruption of the mother–infant relationship in rats leads to a wide range of abnormalities[8,9] that are also found in depressive and anxious patients with an history of early-life stress.[6] These include altered hypothalamic–pituitary–adrenal (HPA) axis response to stress,[10,11] reduced hippocampal neurogenesis,[12] altered emotionality,[11,13] increased visceral pain[14] and cognitive impairments.[12,15] Similarly, beside the well-known effects on offspring metabolism,[16,17] maternal obesity and/or maternal high-fat diet (HFD) consumption can also affect behavior and brain function in offspring.[18] Indeed, altered hippocampal neurogenesis,[19] spatial learning deficits[20] and hyperanxiety,[21–23] have been reported, suggesting that maternal stress and maternal HFD may produce similar effects on the brain during development.

In humans, early-life adversity has marked impact on child brain and particularly on the prefrontal cortex (PFC).[24,25] In rodents, ontogenic molecular changes within the PFC (between post natal day (PND) 7 and PND14) have been described in pups submitted to maternal separation (MS) and are suggested to participate to the programming effects of early-life stress.[26,27] Indeed, the increase of the neuronal transcription factor *Rest4* (neuron-restrictive silencer element, repressor element 1, silencing transcription factor (*Rest*), splicing variant 4) in pups' medial prefrontal cortex (mPFC) is responsible for the molecular signature of MS, characterized by upregulation of genes such as *5HT-r1a* (serotonin receptor 1A) and *Bdnf* (brain derived neurotrophic factor).[27] Moreover, *Rest4* overexpression in the mPFC specifically during early post-natal development, but not in adulthood, is sufficient to produce MS-associated adult endophenotypes, especially hyperanxiety. Here we aim to determine whether exposure to maternal HFD in rats can mimic MS and potentiate the MS-induced developmental alterations in the PFC. To dissociate HFD effect from maternal obesity effects, we used a protocol of maternal HFD exposure (40% from fat, restricted to gestation and lactation periods), which does not produce maternal obesity.[28] We further aim to evaluate the long-lasting impact of maternal HFD exposure on MS-induced alterations of emotional and cognitive behaviours, as well as some typical neuroendocrine and neurobiological changes affected by MS. Since MS is widely used as an animal model of irritable bowel syndrome (IBS),[14] we also examined the effects of these early

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manipulations on visceral pain in adulthood. Finally, given that the dams were directly exposed to the HFD, and that previous reports suggest that HFD could modulate stress response, we also examined the behavioral effects of HFD in stressed dams.

MATERIALS AND METHODS

Experimental procedures

All experiments were approved by the Bioethical committee of our University (N° 50120186-A) and région Aquitaine Veterinary Services (Direction Départementale de la Protection des Animaux, approval ID: A33-063-920) according to the European (Directive 2010/63/EU, 22 September 2010) legislation.

Animals were maintained in a 12-h light/12-h dark cycle (lights on at 0800 hours) in a temperature-controlled room (22 °C) with free access to food and water. Seventy six pregnant female Wistar rats (11-week old, Janvier, Le Genest, Saint-Isle, France) were randomly assigned to either standard diet (SD) or HFD. A mixture of vegetable oils was used as the source of fat (SD, 12% and HF, 40% of energy from fat, Supplementary Table 1).[28] Dams were maintained under these diets from the first day of gestation to postpartum day (PP) 21. At birth, litters were culled to 8–10 pups with balanced sex-ratios and randomly assigned to control group or MS group. From PND2 to PND14, stressed pups underwent daily MS for 180 min as previously described.[29] During the separation sessions, dams were placed in new cages with free access to food (according to their respective diet) and water, whereas pups were placed in individual containers in another room under controlled temperature (28 ± 2 °C). Control pups remained undisturbed with the dams. At PND21, male pups were weaned onto laboratory chow and housed four per cage (from different litters) until the testing age (Supplementary Figure S1). A maximum of two pups per litter was used for each measure to prevent from any litter effect.[30] To investigate the effects of maternal diet and MS on brain during development, the expression of genes (*Rest4*, *Rest*, *5HT-r1a*, *Bdnf*, *Adcy5*, *Camk2a* and *Crh*) known to be affected by early stress[26,27] was assessed in the PFC at PND11 (cohort 1, see gene list in Supplementary Table 2). For that purpose, male pups of the four groups (SD–control, HFD–control, SD–MS and HFD–MS) were killed on PND11, with stressed pups killed either before or after the 180 min-period of MS. PND11 time point was chosen based on previous studies showing ontogenic changes in gene expression between PND7 and PND14 in MS pups.[26,27] Plasma levels of metabolic hormones were also assessed after 180 min of separation at PND11. In adulthood (cohort 2), rats underwent a battery of behavioral tests. First, they were tested for anxiety-like behaviour (4 months), then for spatial learning, spatial memory (5 months), anhedonia (6 months) and social interaction (7 months). At 8 months, they were killed and a random subset of each group was used for biochemical or immunohistochemistry analysis. Plasma corticosterone, *Crh* (corticotropin-releasing hormone) mRNA expression in the hypothalamus (HT) and C-FOS expression in the paraventricular nucleus of the HT (PVN) in response to an acute stress (10 min of open-field) were examined, as well as hippocampal neurogenesis. Visceral sensitivity was evaluated in a separate set of animals at adulthood (2 months, cohort 3).

The following experiments were conducted in stressed dams fed a SD or HFD (PP2-14, cohort 1). Food intake during the 180 min separation and out of the stress-sessions in the home cage was measured. Maternal care was analyzed on PP2 and PP10 in a subset of stressed dams. At PP11, blood samples were withdrawn at the end of the 180 min of separation for corticosterone determination. Dams' anxiety-like behavior was tested in the light–dark test on the last day of the MS procedure (PP14). Dams were killed 2 weeks after weaning and fat tissue (mesenteric and perigonadic) was collected and weighed.

Behavioral assessments in adult offspring

Open field. Rats were placed in the corner of the open field (100 × 100 cm) and exploration of the center (40 × 40 cm) was recorded for 10 min using videotracking (Bioseb, Vitrolles, France). Distance traveled and number of visits in the center were automatically quantified.

Sucrose preference. Rats were individually housed and presented two bottles of tap water to measure basal water consumption. After 48 h of habituation, animals were presented one bottle filled with 1% sucrose solution and one bottle of water. Both intakes were measured after 24 h of test and sucrose preference was calculated as percentage of the volume of sucrose intake over the total volume of fluid intake. Bottle side was randomized to control for any side bias.

Social interaction. Pairs of weight-matched rats from the same experimental group were placed in a new cage, under dim light (30 lux) for 8 min. Social behavior (sniffing, allogrooming and crawling over) were recorded and scored using an ethological software (The observer, Noldus Information Technology, Wageningen, The Netherlands).[31]

Morris water maze. Spatial learning and memory were assessed as previously described.[28] Learning consisted of six sessions (four daily trials each) during which distance traveled to reach the hidden platform was recorded (Bioseb, Vitrolles, France). After the last session, animals were given 48 h of retention time and were tested for reference memory during a 90 s probe trial without the platform. Time spent in each quadrant was analyzed.

Colorectal distension. Visceral sensitivity was evaluated using electromyography recordings in response to progressive colorectal distension as previously described.[32] For more information see Supplementary Methods.

Behavioral assessments in stressed dams

Food consumption. From PP2 to PP14, 24 h and 180 min stress food intakes were measured. Data were expressed as a percentage of food intake during separation on MS day 2.

Light-dark box. On the last day of separation (PP14), dams' anxiety was assessed in the light–dark box paradigm during separation. The total time spent in the light compartment was recorded for 10 min as previously described.[31]

Maternal behavior. On PP2 and PP10, five 60-min periods in the light (1300 and 1900 hours) and dark (2200, 0100 and 0500 hours) phases were video-recorded. Dams' behaviors were scored every 5 min (12 observations per hour) and classified into either 'maternal behavior' (arched back posture, licking/grooming and passive nursing, including nesting and pup retrieving) or 'non-maternal behavior' (off nest, including eating/drinking, self-grooming).[33]

Molecular and biochemical analysis

Real-time quantitative PCR (pups and adult offspring). Total mRNA was extracted from PFC of pups and HT of adult offspring using a TRIzol extraction kit (Invitrogen, Life Technologies, Carlsbad, CA, USA) according to the manufacturer's instructions. The RNA concentration and purity were determined using a ND-1000 spectrophotometer (Nanodrop Technologies, Wilmington, DE, USA). cDNA was synthetized from 1 µg of RNA using Superscript III reverse transcriptase (Invitrogen, Life Technologies) as previously described.[34] Quantitative PCR was performed using SYBR assays (Supplementary Table 2). See Supplementary Methods for further details.

Plasma metabolic hormones multiplex assay (pups). Trunk blood was collected from PND11 pups after 180 min of MS and centrifuged at 4 °C before plasma was stored at − 20 °C. Plasma leptin, insulin, total glucagon-like peptide 1 (GLP-1) and peptide YY (PYY) of PND11 pups were measured by multiplex assay (MILLIPLEX MAP Rat Metabolic Hormone Magnetic Bead, Millipore, Fontenay sous Bois, France) according to the manufacturer's instructions. Hormone concentrations were determined using the Luminex xMap Technology (Bio-Rad, Marnes-la-Coquette, France). All samples were processed in duplicates. Intra and inter assay coefficients were below 15% and crossed reactions were insubstantial (0.01%).

Corticosterone radioimmunoassay (dams and adult offspring). Blood samples were collected from the tail vein (between 0900 and 1200 hours), centrifuged at 4 °C and plasma was stored at − 20 °C until use. Total plasma corticosterone was measured with an in-house radio immunoassay, by competition between cold corticosterone (B) and 3H-B (B*) for a specific anticorticosterone antibody, as previously described.[35] The sensitivity of this assay is around 5 ng ml^{-1}. Intra- and interassay variations were $< 15\%$.

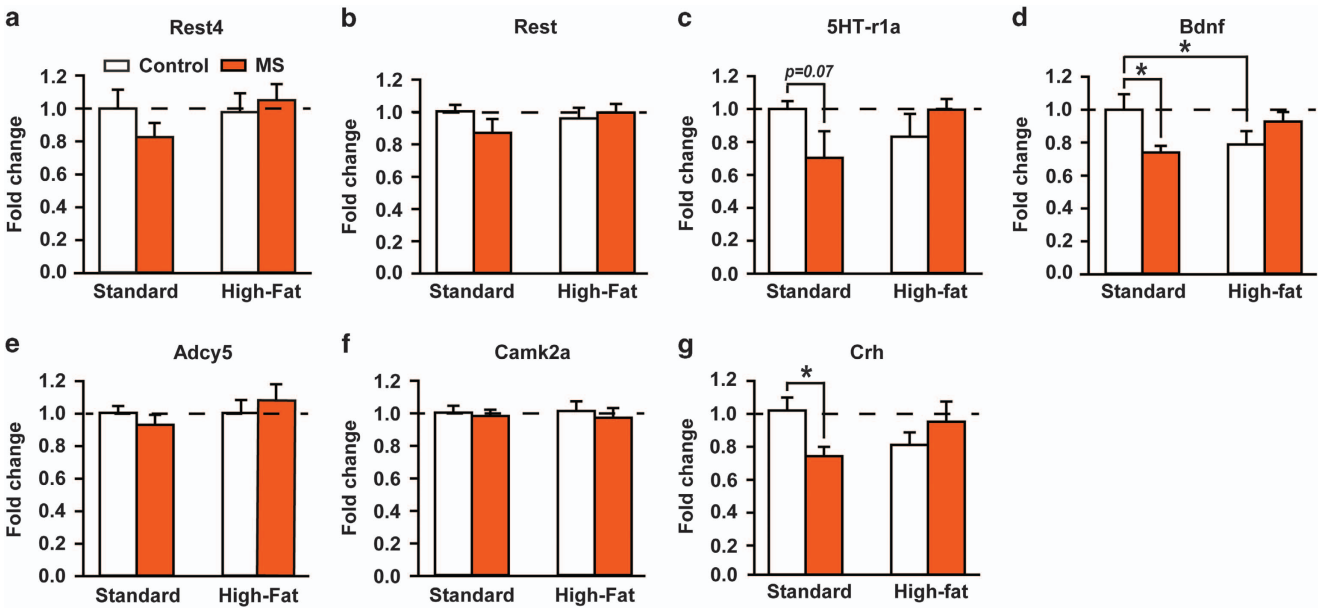

Figure 1. Independent and combined effects of maternal high-fat diet and maternal separation on pups' prefrontal cortex gene expression. Gene expression of (**a**) *Rest4*, (**b**) *Rest*, (**c**) *5HT-r1a*, (**d**) *Bdnf*, (**e**) *Adcy5*, (**f**) *Camk2a* and (**g**) *Crh* in the PFC of 11-day-old pups ($n = 10$ for SD–control and HFD–control, $n = 7$ for SD–MS and $n = 9$ for HFD–MS; except for *Camk2a:* $n = 5$ for SD–MS, $n = 9$ for HFD–control and $n = 8$ HFD–MS). All data are expressed relative to the housekeeping gene *β2m* (fold change). Pups of SD-fed dams exposed to chronic MS exhibited a slight decrease in *5HT-r1a* mRNA levels, and a significant down-regulation of *Bdnf* and *Crh* mRNA. Expression of these markers was restored by maternal HFD. *Adcy5*, Adenylate cyclase5; *Bdnf*, Brain-derived neurotrophic factor; *Camk2a*, Calcium/calmodulin-dependent protein kinase 2 α; *Crh*, Corticotropin-releasing hormone; HFD, high-fat diet; MS, maternal separation; *Rest*, Neural-restrictive silencer element, repressor element 1 (RE1), silencing transcription factor; *Rest4*, *Rest* splicing variant 4; SD, standard diet; *5HT-r1a*, Serotonin receptor 1A. *$P < 0.05$.

Immunohistochemistry in adult offspring

Neuronal activation in PVN. Anesthetized rats (Pentobarbital, 50 mg kg^{-1}) were intracardially perfused with Phosphate Buffer Solution followed by 4% Paraformaldehyde. Brains were post-fixed in the same fixative for 24 h, cryoprotected in 30% sucrose, and stored at − 80 °C until use. Immunostaining for C-FOS was used to measure neuronal activation 1 h post stress. Free-floating sections (40 µm) containing PVN (−1.80 to − 2.12 mm posterior to Bregma)[36] were treated as previously described.[37] C-FOS immunoreactive (IR) cells were counted with the optical fractionator method using a microscope (Olympus, Hamburg, Germany, BX51) equipped with an objective (×100), a video camera (Nikon digital camera DMX 1200, Champigny sur Marne, France), and a stereological software (Mercator, ExploraNova, La Rochelle, France). Quantification of C-FOS-IR cells was carried out in two PVN sections per animal. The fields of view were systematically sampled using a step size of 50 µm along the *x* and *y* axes. The dissector counting frames were 150 × 150 µm. Results are expressed as C-FOS-IR cells in the total PVN.

Hippocampal neurogenesis. Hippocampal sections (from bregma 2.30 to 5.20 mm) were treated for doublecortin (DCX) immunoreactivity using a goat polyclonal antibody (1:1000, Santa Cruz Biotechnology, Santa Cruz, CA, USA) and a biotinylated donkey anti-goat secondary antibody (1:200, Amersham, Chicago, IL, USA) as previously described.[38] Adult neurogenesis in the dentate gyrus (DG) was evaluated in eight coronal slices of hippocampus. For each rat ($n = 4$ per group), four matched-sections for dorsal (1.06 to − 2.06 mm) and ventral (−3.08 to − 3.80 mm) hippocampus were selected.[39] DCX-IR cells were counted within the granular cell layer. The number of DCX-IR cells was then expressed per mm^2.

Statistical analysis

Three different cohorts were used to evaluate the independent and combined effects of maternal HFD and MS. Sample sizes were determined based on power analysis and common practice in behavioral (~10 animals per group) and molecular biology (~5 animals per group) experiments. The exact number of animals tested in each group is specified in the figure legends. All data were analyzed using Statistica 6.0 (StatSoft, Tulsa, OK, USA). Graphs showing the means ± s.e.m. were graphed using Prism 5.0 (GraphPad Software, San Diego, CA, USA). Normality was assessed by Shapiro–Wilk tests. Statistical outliers were detected with the Grubb's test and highly significant outliers ($P < 0.01$) were removed from analyses. Data were analyzed using two or three-way analysis of variance (ANOVA) with repeated measures when appropriate, followed by Fisher's LSD *post hoc* tests or planned comparisons (% dams' food intake throughout the maternal separation sessions). Unpaired Student *t*-tests were used to test the effects of maternal diet in stressed animals. Pearson correlation was used to examine the link between anxiety in the open-field and memory performance in the water maze. Neurogenesis and maternal behavior were analyzed by Mann-Whitney *U*-tests. Data quantifications that potentially include subjective bias (social interaction, maternal care, C-FOS and DCX quantification) were conducted by observers blind to the experimental group. Statistical significance was set at $P < 0.05$.

RESULTS

Maternal high-fat diet prevents the molecular signature of maternal separation in pup's prefrontal cortex

To examine the respective and combined effects of maternal HFD and MS on the developing brain, we assessed mRNA expression of *Rest4* and related genes in the PFC of PND11 pups, with stressed pups killed before the stress session (Figure 1a–g). Expression of the housekeeping *B2m* gene did not significantly vary across groups in any of condition (data not shown). *Rest4*, *Rest*, *Adcy5* (adenylate cyclase 5) and *Camk2a* (calcium/calmodulin-dependent protein kinase 2 α) mRNA levels were not significantly altered by maternal HFD nor MS (Figure 1a,b,e,f). However, there was a significant interaction between maternal diet and early stress for *5HT-r1a* (two-way ANOVA, $F_{(1,31)} = 4.3221$, $P = 0.0460$), *Bdnf* ($F_{(1,32)} = 6.6418$, $P = 0.0148$) and *Crh* ($F_{(1,32)} = 5.3553$, $P = 0.0272$) mRNA levels (Figure 1c–g). Pups of SD dams exposed to chronic MS exhibited a trend toward a decrease of *5HT-r1a* mRNA (Fisher's LSD *post hoc,* SD–MS versus SD–control, $P = 0.0726$), and a significant downregulation of *Bdnf* ($P = 0.0282$) and *Crh* ($P = 0.0443$) mRNA expression. The effect of maternal HFD alone was restricted to a decrease of *Bdnf* expression (HF-control versus

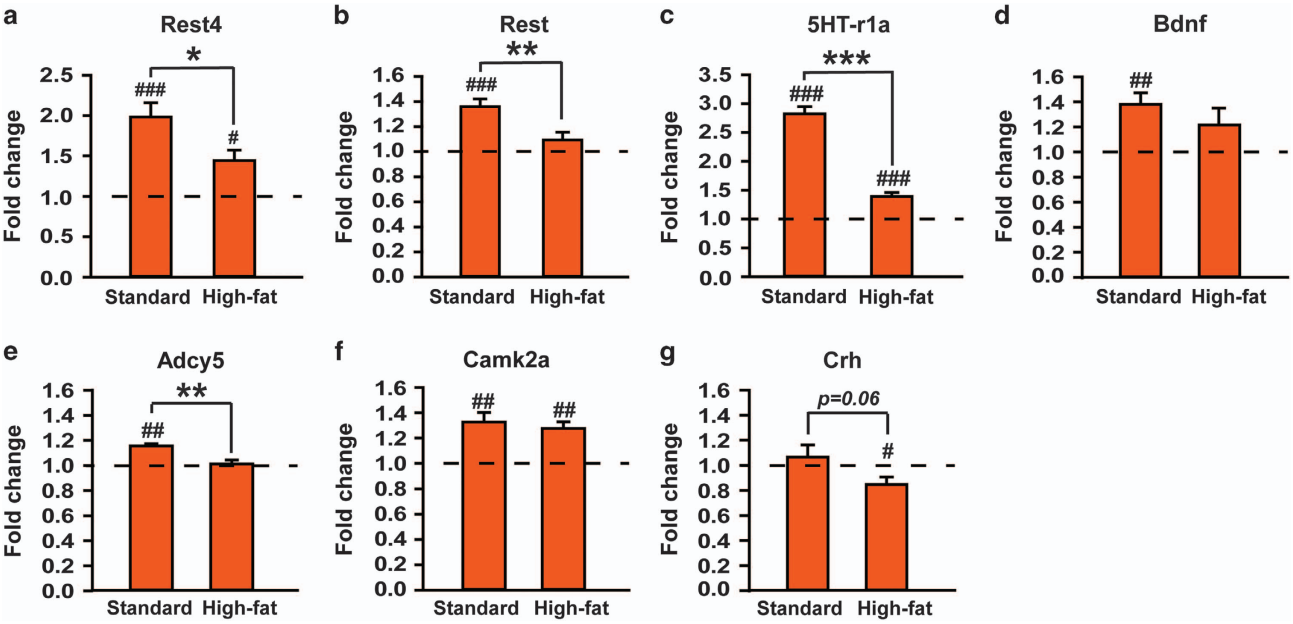

Figure 2. Maternal high-fat diet prevents gene expression changes in the prefrontal cortex of pups following 180- min of maternal separation. mRNA levels of (**a**) *Rest4*, (**b**) *Rest*, (**c**) *5HT-r1a*, (**d**) *Bdnf*, (**e**) *Adcy5*, (**f**) *Camk2a* and (**g**) *Crh* in the PFC of 11-day-old pups after 180-min of separation ($n = 8$ per group). All data are expressed relative to gene expression in pups killed before separation. In pups of SD-fed dams, expression of all these markers, except *Crh*, was increased after the acute separation, compared with baseline. However, the acute separation-induced rises of *Rest*, *Rest4*, *5HT-r1a* and *Adcy5* were blunted in pups born to high-fat-fed dams. *Adcy5*, Adenylate cyclase5; *Bdnf*, Brain-derived neurotrophic factor; *Camk2a*, Calcium/calmodulin-dependent protein kinase 2 α; *Crh*, Corticotropin-releasing hormone; PFC, prefrontal cortex; *Rest*, Neural-restrictive silencer element, repressor element 1 (RE1), silencing transcription factor; *Rest4*, *Rest* splicing variant 4; SD, standard diet; *5HT-r1a*, Serotonin receptor 1A. $^{*}P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ and $^{\#\#\#}P < 0.001$ compared with the standard value of 1 representing mRNA levels in pups killed before the separation.

SD–control, $P = 0.0482$). Unexpectedly, the combination of maternal HFD and MS led to *5HT-r1a*, *Bdnf* and *Crh* mRNA levels similar to control levels (HFD–MS versus SD–control, $P = 0.0980$, $P = 0.4979$, $P = 0.5887$, respectively), suggesting a preventive effect of maternal HFD on the developing PFC in MS pups.

On PND11, we further examined PFC gene expression at the end of the stress session (that is, 180-min) in chronically stressed pups, as compared with expression in stressed animals (SD or HFD) killed before stress (Figure 2a-g). There was a significant rise in mRNA expression of *Rest4* (one sample *t*-test, $t_{(7)} = 5.5237$, $P = 0.0009$), *Rest* ($t_{(7)} = 6.2155$, $P = 0.0004$), *5HT-r1a* ($t_{(7)} = 14.4684$, $P < 0.0001$), *Bdnf* ($t_{(7)} = 4.2398$, $P = 0.0038$), *Adcy5* ($t_{(7)} = 4.7668$, $P = 0.0020$) and *CamK2a* ($t_{(7)} = 4.3180$, $P = 0.0035$) in stressed pups of SD-fed dams (Figure 2a-f). Maternal HFD strongly blunted MS-induced upregulation of *Rest4* (unpaired Student *t*-test, $t_{(14)} = 2.4514$, $P = 0.0280$), *Rest* ($t_{(14)} = 3.4289$, $P = 0.0041$), *5HT-r1a* ($t_{(14)} = 9.9536$, $P < 0.0001$) and *Adcy5* ($t_{(14)} = 3.0611$, $P = 0.0085$), but not *Bdnf* nor *Camk2a* ($t_{(14)} = 1.0520$, $P = 0.3106$ and $t_{(14)} = 0.5439$, $P = 0.5951$, respectively). Finally, *Crh* mRNA levels were decreased in MS pups from dams fed with HFD (one sample *t*-test, $t_{(7)} = 2.4416$, $P = 0.0581$; Figure 2g). Overall, our data highlight an unexpected, protective effect of maternal HFD on the molecular changes associated with MS during brain development.

Maternal high-fat diet prevents adult endophenotypes associated with early-life stress

Since *Rest4* overexpression in the mPFC during development leads to long-lasting deleterious effects resembling the MS phenotype,[27] we hypothesized that the restoration of *Rest4* expression in the PFC at PND11 would alleviate MS-associated behavioral endophenotypes in adult offspring of HFD-fed dams. Therefore, we next examined anxiety, anhedonia, social behavior and spatial learning and memory, which have been extensively reported as affected in adult MS offspring[10,11,13,40] (Figure 3a-f). In the open-field test, the effects of MS on the distance traveled in the center area differed with respect to the maternal diet (two-way ANOVA, maternal diet × early stress effect: $F_{(1,54)} = 4.8826$, $P = 0.03138$; Figure 3a). In offspring of SD dams, MS tended to decrease the distance in center compared with the control group (Fisher's LSD *post hoc,* SD–MS versus SD–control, $P = 0.0676$), suggesting a higher anxiety. This effect was attenuated in MS offspring exposed to maternal HFD (HFD–MS versus SD–control, $P = 0.0001$). Maternal HFD exposure had no impact on anxiety-like behavior in non stressed animals (HFD–control versus SD–control, $P = 0.23791$). A similar profile was found for the number of visits in the center (two-way ANOVA, maternal diet × early stress effect: $F_{(1,54)} = 4.3119$, $P = 0.0426$; Figure 3b). In contrast, anhedonia, assessed by the sucrose preference test was not significantly altered by MS or maternal HFD (maternal diet × early stress effect: $F_{(1,54)} = 0.5020$, $P = 0.4818$; Figure 3c). In the social interaction test, MS rats spent significantly less time in interaction over the 8-min of the test compared with controls, independently of maternal diet (three-way ANOVA with repeated measures, early stress effect: $F_{(1,24)} = 6.9739$, $P = 0.0143$; data not shown). However, the analysis of the first minute, which can be considered the most anxiogenic, revealed a significant interaction between early stress and maternal diet (two-way ANOVA, $F_{(1,24)} = 6.1909$, $P = 0.0202$; Figure 3d). MS rats exposed to a maternal SD displayed reduced social interaction time compared with their control counterparts (Fisher's LSD *post hoc*, SD–MS versus SD–control, $P = 0.0007$). In contrast, in offspring of HFD dams, MS had no effect on social behavior (HFD–MS versus HFD–control, $P = 0.7355$). Again, maternal HFD alone had no significant impact on social behavior (HFD–control versus SD–control, $P = 0.1139$). As early-life stress is also associated with cognitive dysfunctions,[12,15] we examined the impact of maternal HFD on spatial memory performance in the water maze task. All groups performed equally over the spatial

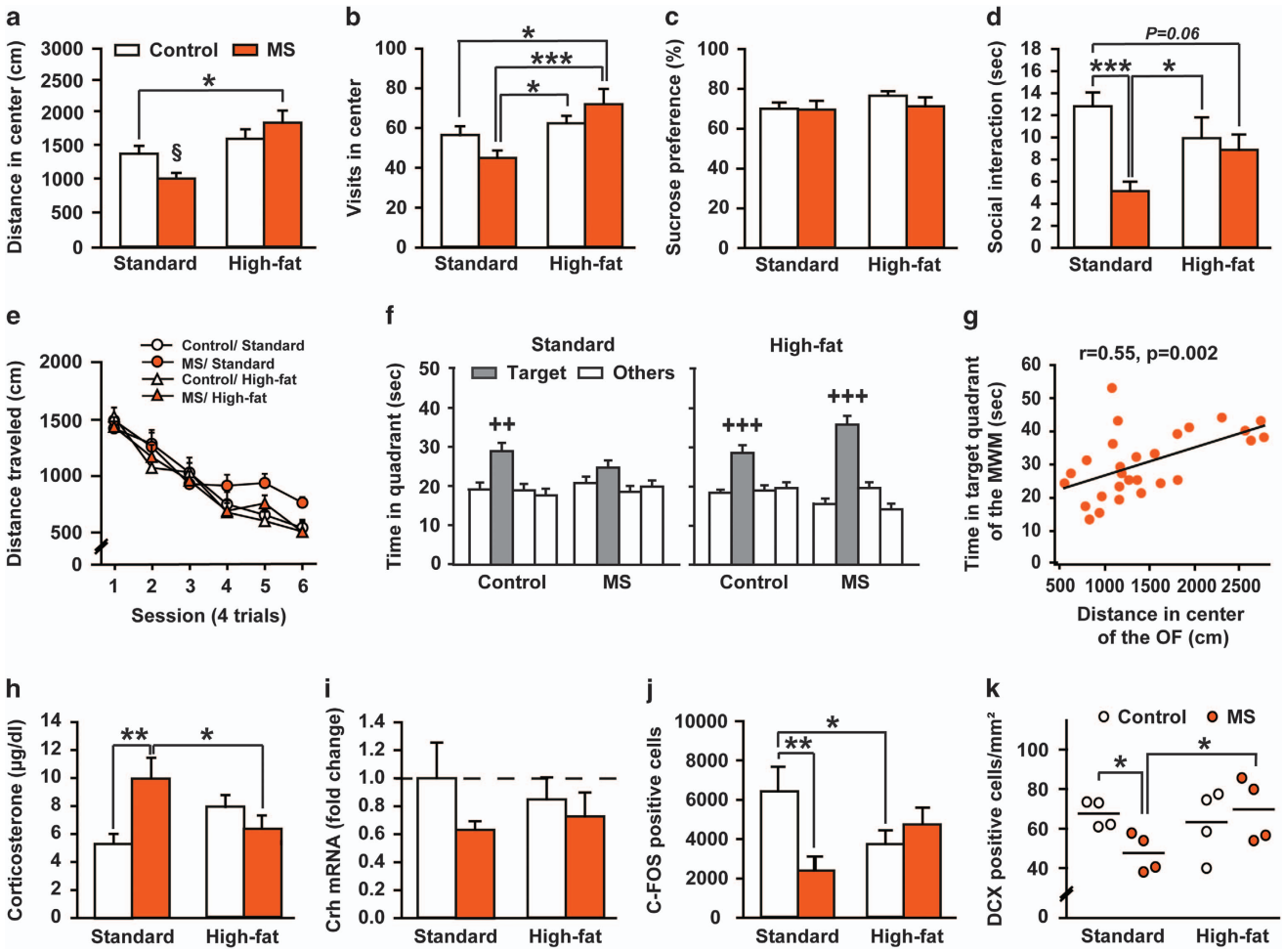

Figure 3. Maternal high-fat diet alleviates offspring endophenotypes induced by maternal separation. (**a**) Distance traveled (cm) and (**b**) number of visits in the center of the open field ($n = 15$ for SD-control and HFD–control, $n = 14$ for SD–MS and HFD–MS). (**c**) Sucrose preference (percentage of sucrose solution consumption relative to total fluid intake) over 24 h ($n = 15$ for SD–control and HFD–control, $n = 14$ for SD–MS and HFD–MS). (**d**) Time (sec) spent in social interaction ($n = 7$ *per* group). (**e**) Distance traveled (cm) to reach the hidden platform during learning and (**f**) time (sec) spent in the target quadrant during the probe test, 48 h after the last training session ($n = 15$ for SD–control and HFD–control, $n = 14$ for SD–MS and HFD–MS). (**g**) Significant positive correlation between distance traveled in the center of the open field and time spent in target quadrant in the water maze in adult offspring exposed to MS ($n = 28$). (**h**) Plasma corticosterone levels (µg dl^{-1}) ($n = 15$ for SD–control and HFD–control, $n = 14$ for SD–MS and HFD–MS), (**i**) *Crh* mRNA expression in the hypothalamus (fold change) ($n = 6$ for SD–control, SD–MS and HFD–MS; $n = 7$ for HFD–control) and (**j**) Number of C-FOS-IR cells in the PVN 1 h after 10 min open-field exposure ($n = 8$ for SD–control, SD–MS and HFD–control; $n = 7$ for HFD–MS). (**k**) Number of DCX-IR cells in the DG of the hippocampus (cells per mm^2; $n = 4$ *per* group). *Crh*, Corticotropin-releasing hormone; DCX, Doublecortin; HFD, high-fat diet; MS, maternal separation; SD, standard diet. $^{*}P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$; $^{\S}P = 0.07$ compared with SD–control and $P < 0.05$ compared with HFD–control and HFD–MS; ++ at least $P < 0.01$ and +++ at least $P < 0.001$ compared with all other quadrants.

learning sessions (three-way ANOVA with repeated measures, maternal diet × early stress × session effect: $F_{(5,270)} = 0.6710$, $P = 0.6459$ (Figure 3e). In the probe test 48 h later (Figure 3f), control offspring of SD and HFD dams spent significantly more time in the target quadrant compared with other quadrants (ANOVA with repeated measures, quadrant effect: SD–control, $F_{(3,42)} = 6.1010$, $P = 0.0015$; HFD–control $F_{(3,42)} = 8.1473$, $P = 0.0002$; Figure 3f, left panel). In contrast, offspring of SD-fed dams submitted to MS did not discriminate the target quadrant (quadrant effect: $F_{(3,39)} = 1.4501$, $P = 0.2431$). This memory impairment was suppressed by maternal HFD (quadrant effect: $F_{(3,39)} = 25.1083$, $P < 0.0001$; Figure 3f, right panel). Given that the water maze task is aversive, we tested a possible link between memory performance and anxiety levels. We found a significant positive correlation between distance traveled in the center of the open-field and time spent in target quadrant of the water maze during the probe test in the MS groups (Pearson correlation: $r = 0.55$, $P = 0.002$; Figure 3g). Indeed, animals that are the most anxious also exhibit the lowest spatial memory performance, suggesting that exacerbated anxiety may be involved in the memory deficit reported in MS animals.

To better characterize the effect of maternal HFD on MS-associated endophenotypes, we next explored HPA axis response to stress (including plasma corticosterone, *Crh* mRNA expression in the HT, and C-FOS expression in the PVN) and hippocampal neurogenesis, both widely reported as affected by early-life stress.[10–13,40] At the end of the behavioral characterization, animals were killed following an acute stress (open-field exposure; Figure 3h-k). The effect of MS on plasma corticosterone levels differed with respect to the dam's diet (two-way ANOVA, maternal diet × early stress effect: $F_{(1,54)} = 8.7133$, $P = 0.0047$). MS offspring of SD dams exhibited significantly higher plasma corticosterone levels compared with controls (Fisher's LSD *post hoc,* SD–MS versus SD–control, $P = 0.0030$). Maternal HFD *per se* tended to

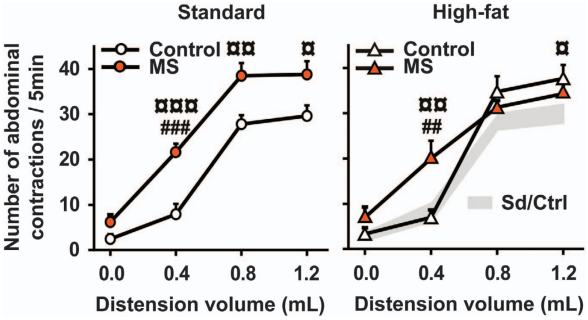

Figure 4. Impact of maternal high-fat diet and maternal separation on offspring visceral sensitivity at adulthood. Number of abdominal contractions (number/5 min) in response to gradual colorectal distension volumes ($n = 11$ for SD–control; $n = 10$ for SD–MS; $n = 9$ for HFD–control and HFD–MS). $^{¤}P < 0.05$, $^{¤¤}P < 0.01$ and $^{¤¤¤}P < 0.001$ compared with control SD; $^{\#\#}P < 0.01$ and $^{\#\#\#}P < 0.001$ compared with control HFD. HFD, high-fat diet; MS, maternal separation; SD, standard diet.

produce similar effects to MS (HFD–control versus SD–control, $P = 0.0824$). In contrast, MS offspring of HFD dams displayed normalized corticosterone levels (HFD–MS versus SD–control, $P = 0.5072$) (Figure 3h). Whatever the maternal diet, there was a non-significant decrease of *Crh* gene expression in the hypothalamus of early stressed animals (two-way ANOVA, early stress effect: $F_{(1,21)} = 1.9295$, $P = 0.1794$; Figure 3i). In addition, we stereologically counted C-FOS IR cells in the PVN of the hypothalamus (Figure 3j). Again, there were differential effects of MS depending on the maternal diet (two-way ANOVA, maternal diet × early stress effect: $F_{(1,27)} = 7.9363$, $P = 0.0090$). In comparison with control SD animals, MS or maternal HFD similarly decreased the number of C-FOS-positive cells in the PVN (Fisher's LSD *post hoc,* SD–MS versus SD–control, $P = 0.0034$; HFD–control versus SD–control, $P = 0.0397$). In contrast, MS offspring of HFD dams showed normalized C-FOS expression (HFD–MS versus SD–control, $P = 0.2148$). Finally, neurogenesis was examined by counting the total number of DCX-positive cells in the DG of the hippocampus. In offspring of SD dams, the total number of DCX-positive cells was significantly lowered by MS (Mann-Whitney *U*-test, SD–MS versus SD–control, $U = 0.0000$, $P = 0.0286$) (Figure 3k). This MS-induced decrease of newborn neurons was not observed in offspring of HFD dams (HFD–MS versus SD–control, $U = 8.0000$, $P = 1.0000$). Maternal HFD alone did not affect hippocampal neurogenesis in control animals ($U = 8.0000$, $P = 1.0000$).

In humans, anxiety disorders are highly co-morbid with the IBS, which is characterized by chronic visceral pain.[41] Since MS is widely used as an animal model of IBS,[14] we next evaluated visceral sensitivity to pain using colorectal distension.[42] Gradual colorectal distension increased the number of abdominal contractions in a volume-dependent manner (three-way ANOVA with repeated measures, volume effect: $F_{(3,105)} = 176.9673$, $P < 0.0001$), and this effect was modulated by both maternal diet and early stress (maternal diet × early stress × volume effect: $F_{(3,105)} = 2.8488$, $P = 0.0410$; Figure 4). Specifically, in offspring of SD-fed dams, MS significantly increased abdominal contractions compared with the control group (Fisher's LSD *post hoc,* SD–MS versus SD–control, $P = 0.0011$, $P = 0.0089$ and $P = 0.0230$ for 0.4, 0.8 and 1.2 ml, respectively; Figure 4, left panel). Maternal HFD alone significantly increased the number of abdominal contractions for the distension volume of 1.2 ml (HFD–control versus SD–control, $P = 0.0505$). In contrast, maternal HFD suppressed the effect of MS for the highest volumes of 0.8 and 1.2 ml (HFD–MS versus SD–control, $P = 0.3813$ and $P = 0.2441$, respectively; Figure 4, right panel), without any protective effect on pain threshold (0.4 ml distension volume, $P = 0.0043$).

High-fat diet consumption dampens anxiety and increases maternal care in stressed dams

Maternal HFD had no impact on dams' body weight at the end of gestation (SD 430.8 ± 11.8, $n = 8$; HFD 415.2 ± 11.2 g, $n = 9$; $t_{(15)} = 0.9555$, $P = 0.3545$), nor on fat mass at death (2 weeks after weaning; fat mass expressed in % of dams' body weight: SD–MS, 4.11 ± 0.27, $n = 13$; HFD–MS, 3.69 ± 0.32, $n = 12$; $t_{(23)} = 1.0322$, $P = 0.3127$). Pups' body weight at birth (SD 7.19 ± 0.15, $n = 24$; HFD 6.94 ± 0.21 g, $n = 24$; $t_{(46)} = 0.9532$, $P = 0.3455$) or after 180-min of MS (Supplementary Table 3) was not affected by maternal HFD either. Moreover, plasma levels of leptin, insulin, GLP-1 and PYY in stressed animals were not significantly different between SD and HFD pups (Supplementary Table 3), suggesting that the protective effect of maternal HFD could not be explained by metabolic adaptations. Considering that separation from pups constitutes a potent stress for dams[43] and that HFD consumption could exert an anti-stress effect in MS dams, we thus explored the effects of HFD consumption in stressed dams (Figure 5). Consistently, HFD dams increased their food intake during the 180-min MS sessions over the 2 weeks compared with SD dams (two-way ANOVA with repeated measures, maternal diet × separation day effect: $F_{(10,230)} = 8.8712$, $P < 0.0001$; planned comparisons, SD–MS versus HFD–MS, at least $P < 0.05$ for sessions 11, 12 and 13; Figure 5a). However, mean daily energy intake during the 2 weeks of MS was similar between SD and HFD dams (unpaired Student *t*-test, $t_{(23)} = 0.7684$, $P = 0.4501$) (Figure 5b), suggesting that HFD dams efficiently adapted their intake with respect to the calories provided by the HFD. We also found a significant reduction of anxiety levels in stressed dams fed a HFD (Figure 5c). Indeed, stressed HFD dams spent more time in the light compartment of the light-dark box compared with stressed SD dams (unpaired Student *t*-test, $t_{(23)} = 2.5346$, $P = 0.0185$). However, changes in anxiety were not associated with significant differences in plasma corticosterone levels after stress (PP11, SD 14.4 ± 2.1, $n = 14$; HFD 12.9 ± 1.4 μg dl^{-1}, $n = 11$; $t_{(23)} = 0.5826$, $P = 0.5659$). We finally explored the impact of HFD on maternal care, which is determinant for later vulnerability to stress[44] and has been recently demonstrated to be increased in HFD-fed dams.[45] Stressed dams under HFD displayed increased global maternal care (that is, arched back posture, licking–grooming, and passing nursing) during the dark phase at PP2 (Mann–Whitney *U*-test, $U = 0.0000$, $P = 0.0357$; Figure 5d). This effect was no longer present on PP10 (data not shown). Of note, maternal care during the light phase was not significantly affected by maternal diet (data not shown).

DISCUSSION

Early-life stress is associated with increased vulnerability to neuropsychiatric diseases later in life.[1,2,5,6] Similarly, obesity, excessive weight gain, metabolic disorders and unhealthy HFD during pregnancy have been recently hypothesized to increase the incidence of mental health disorders.[7] Here we examine whether maternal HFD can have similar effects to MS and/or can exacerbate the effects of MS in the offspring. Contrary to our hypotheses, maternal HFD alone has only small impact on gene expression in pups' PFC and on behavior in adulthood. More importantly, maternal HFD alleviates MS-induced endophenotypes (anxiety, spatial memory, social behavior, HPA axis response to stress and visceral pain) in adulthood and reduces maternal anxiety in stressed dams.

HFD exposure has been recently proposed to act as a stressful challenge during pregnancy.[46] Herein, we report that maternal HFD (40% from fat) alone has no major consequence in offspring, neither on PFC gene expression in pups nor on behavior in adults. Indeed, as we previously reported,[28] HFD (40% fat) exposure restricted to gestation and lactation did not lead to maternal

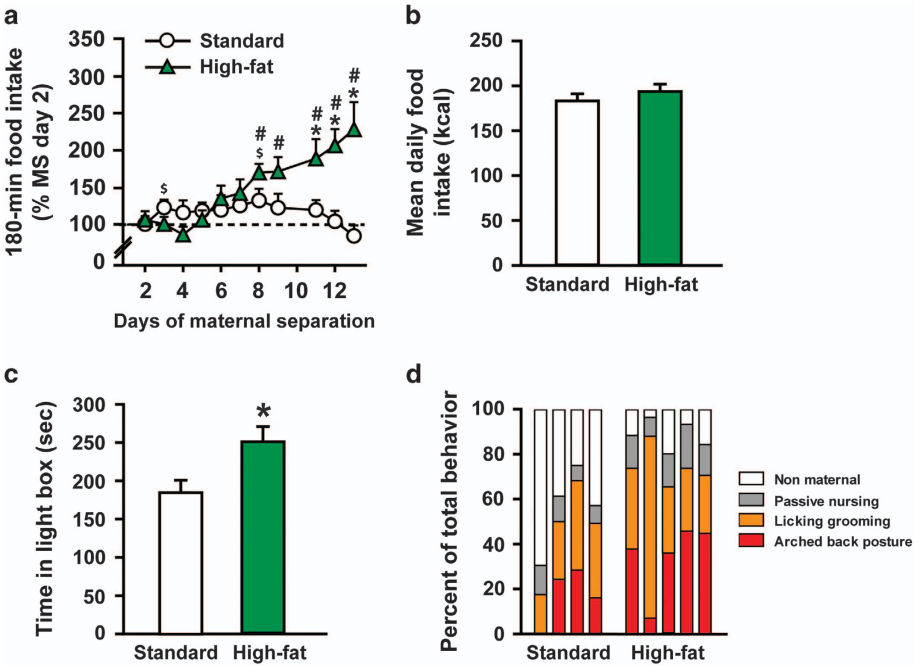

Figure 5. Impact of high-fat diet consumption on stressed dams' behavior. (**a**) Food intake (% of intake on MS-day 2) during the 180 min separations and (**b**) mean daily food intake (kcal) over the two weeks of the MS procedure ($n = 13$ for SD–MS and $n = 12$ for HFD–MS). Stressed dams fed a HFD increased their food intake during the 180 min of separation over the 2 weeks of MS and consumed significantly more food compared with standard diet (SD)-fed stressed dams. However, daily food intake (homecage) was not different between standard and high fat-fed dams. (**c**) Time (s) in light compartment in the dark/light box on PP14 ($n = 13$ for SD–MS and $n = 12$ for HFD–MS). HFD dams showed a reduction of their anxiety-like behavior. (**d**) Dams' behavior (percent of total behaviors measured) during the dark phase at PP2 ($n = 4$ for SD–MS and $n = 5$ for HFD–MS). HFD dams displayed higher global maternal care toward their progeny (arched back posture, licking–grooming and passive nursing together). * at least $P < 0.05$ compared with standard diet; $ at least $P < 0.05$ compared with MS-day 2 in SD–MS; # at least $P < 0.05$ compared with MS day 2 in HFD–MS. HFD, high-fat diet; MS, maternal separation.

overweight. In contrast, in studies where the dams are overweight or obese (60% fat or longer HFD exposure), maternal HFD could impact the developing brain[19,47] and lead to exacerbated anxiety[21–23] impaired memory[20] and decreased neurogenesis[19] in adult offspring. Taken together, these results suggest that maternal obesity, rather than maternal HFD consumption, is critical for the detrimental effects of HFD in offspring.

A major finding of the present study is that early exposure to HFD unexpectedly prevents neurodevelopmental gene expression alterations in the PFC of stressed pups. Indeed, chronic MS led to downregulated *5HT-r1a*, *Crh* and *Bdnf* baseline mRNA levels in PND11 pups, which is prevented by maternal HFD exposure. Interestingly, BDNF and serotonin, notably through the 5HT-R1A receptors, are necessary for proper wiring of neural circuits during development,[48–52] shaping normal anxiety in adulthood.[53] An ontogenic upregulation of *Rest4* and associated markers has been previously reported in the mPFC of MS pups, but not in the amygdala or in the hippocampus.[27] Here we demonstrate that maternal HFD reversed the MS-induced upregulation of *Rest4* and related genes following separation. As *Rest4* overexpression in the mPFC during development leads to hyperanxiety in adulthood,[27] our data suggest that the protective effects of maternal HFD exposure on behavior in adulthood might in part result from the normalization of *Rest4* mRNA levels in pups' PFC. We do not rule out that MS-induced changes in gene expression during development also take place in other brain structures. Furthermore, alterations occurring in the PFC during the perinatal period may lead to altered connectivity with other brain areas such as the amygdala or the hippocampus, which have been shown to play a role in anxiety and cognitive functions.

Although the molecular mechanisms underlying the protective effects of maternal HFD remain to be elucidated, our results demonstrate that HFD exposure early in life attenuates MS-induced hyperanxiety, but also MS-related impairments in spatial memory, social behavior and visceral pain. Moreover, hypercorticosteronemia and altered hippocampal neurogenesis, which are associated with hyperanxiety and spatial memory disturbances,[54,55] were ameliorated by maternal HFD exposure in MS animals. Previous studies have shown that palatable food consumption in adulthood can attenuate the deleterious effects of early stress on emotional behaviours.[11,56–58] However, to our knowledge, our work is the first to demonstrate that an exposure to HFD restricted to the developmental period can protect against the long lasting disturbances induced by early stress.

The protective effects of maternal HFD on the offspring might depend upon several mechanisms acting synergistically. In particular, it could affect pups' metabolism through feeding or pups' stimulation through the level of maternal care.[59] In stressed pups, neither body weight nor plasma metabolic markers after the 180 min separation differed according to the maternal diet. Thus, maternal HFD does not lead to a better metabolic adaptation to the 3 h fasting occurring during the separation sessions. A large body of evidence highlights the importance of maternal behavior in later offspring emotional behavior and HPA response to stress.[60] Moreover, previous work reports that dams maintained on HFD during lactation spent more time nursing their pups.[45] Consistently, we showed that stressed dams fed a HFD increased care toward their pups compared with stressed SD dams. In humans, it has been demonstrated that food choices are modified under stress with a shift in preference toward more palatable, energy-dense snacks.[61] Dallman proposed that overconsumption of palatable food dampens negative emotions associated with stress.[62] MS acts as a potent stressor for dams.[43,63] We demonstrate that stressed dams fed a HFD increased their food

intake specifically during the 180-min stress session of separation, an effect associated with a reduction of their anxiety. These results extend previous findings showing that high-fat intake modulates stress response in adult animals[11,64,65] and alleviates postpartum anxiety and depressive-like behavior in mother rats subjected to MS.[66] Maternal stress is detrimental for maternal care quality,[67] thus it could be hypothesized that the increase of HFD intake during lactation exerts an anti-stress effect on dams, which could promote maternal behavior allowing optimal brain maturation in pups. Further experiments are needed to confirm the comfort food effect of HFD in stressed dams. As stress increases C-FOS expression, future studies should be conducted to examine whether maternal HFD would blunt dams' C-FOS response to the separation stress. Epigenetic regulations are likely candidate for persistent changes in brain function as a consequence of perinatal environment. Indeed, some of the effects of parental obesity persist across multiple generations.[68] Therefore, it would be interesting to examine whether the protective effect of maternal HFD could be epigenetically transmitted across generations in our model.

Contrary to the prevailing belief that HFD exposure is detrimental for the developing brain, our results suggest that obesity, rather than fat consumption *per se*, is critical for brain vulnerability. Furthermore, to our knowledge, we report for the first time a protective effect of maternal HFD in a context of early-life stress. Further work is needed to better document and understand this phenomenon. Although maternal HFD prevents stress-induced emotional alterations in our study, it is important to consider other health outcomes, such as effects on metabolic or cardiovascular diseases vulnerability that might be exacerbated by HFD. Overall, our findings highlight the importance of taking nutrition into account in clinical studies on early-life adversity and mental health.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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Supplementary Information accompanies the paper on the *Translational Psychiatry* website (http://www.nature.com/tp)

ANNEXE 2

ANNEXE 3

ANNEXE 4

médecine/sciences 2016 ; 32 :93-9

Environnement précoce et vulnérabilité neuropsychiatrique

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› Du fait de son immaturité à la naissance, le nouveau-né est totalement dépendant des soins parentaux pour sa survie et pour son développement cérébral. Des altérations de la qualité de l'environnement précoce, non seulement lors de l'enfance, mais également en période prénatale, ont un impact, à long terme, sur la santé. Récemment, chez l'animal, des travaux ont mis en évidence des modulations épigénétiques après des stress précoces qui pourraient se transmettre sur plusieurs générations, *via* des modifications dans les cellules germinales. ‹

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Adversité durant l'enfance et vulnérabilité neuropsychiatrique : données cliniques et modèles animaux de stress psycho-social

Il existe des périodes critiques durant lesquelles l'organisme est fortement influencé par son environnement *(Figure 1)*. L'adversité précoce est associée à de nombreux problèmes de santé à l'âge adulte et augmenterait le risque de développer des troubles neuropsychiatriques tels que la dépression ou l'état de stress post-traumatique, mais également une obésité ou des maladies cardiovasculaires [1]. L'adversité précoce est généralement définie comme l'exposition à des maltraitances dans l'enfance, telles que des négligences parentales, des abus sexuels, physiques ou psychologiques. Avant et après la naissance, d'autres facteurs comme la maladie, la malnutrition ou l'exposition à des drogues constituent aussi des stress précoces importants. À ce titre, l'environnement socio-économique pendant le développement, par la pluralité des aspects qu'il intègre, est un facteur déterminant pour l'état de santé psychique et physique de l'enfant devenu adulte [2]. Dans cet article nous présenterons les recherches montrant les conséquences de stress précoces (pendant l'enfance et *in utero*) sur la santé mentale chez l'homme et sur les comportements, dans les modèles animaux. Nous illustrerons également des mécanismes épigénétiques associés au stress précoce.

Chez beaucoup de mammifères, les petits naissent immatures et totalement dépendants des soins parentaux. L'immaturité du cerveau les rend très sensibles à leur environnement. Depuis les recherches menées par James Harlow, René Spitz et John Bowlby, père de la théorie de l'attachement, il est admis que le développement cognitif et affectif de l'enfant requiert la mise en place d'un lien affectif privilégié avec une figure d'attachement (le parent ou son substitut). Des enfants exposés à des stress sévères tels que des carences affectives précoces ou des maltraitances parentales présentent des atteintes neurodéveloppementales de structures clefs dans la régulation des processus cognitifs et émotionnels (atrophie du cortex préfrontal et de l'hippocampe, atrophie ou hypertrophie de l'amygdale, selon les études). À l'âge adulte, ces effets peuvent perdurer et s'accompagner d'altérations de

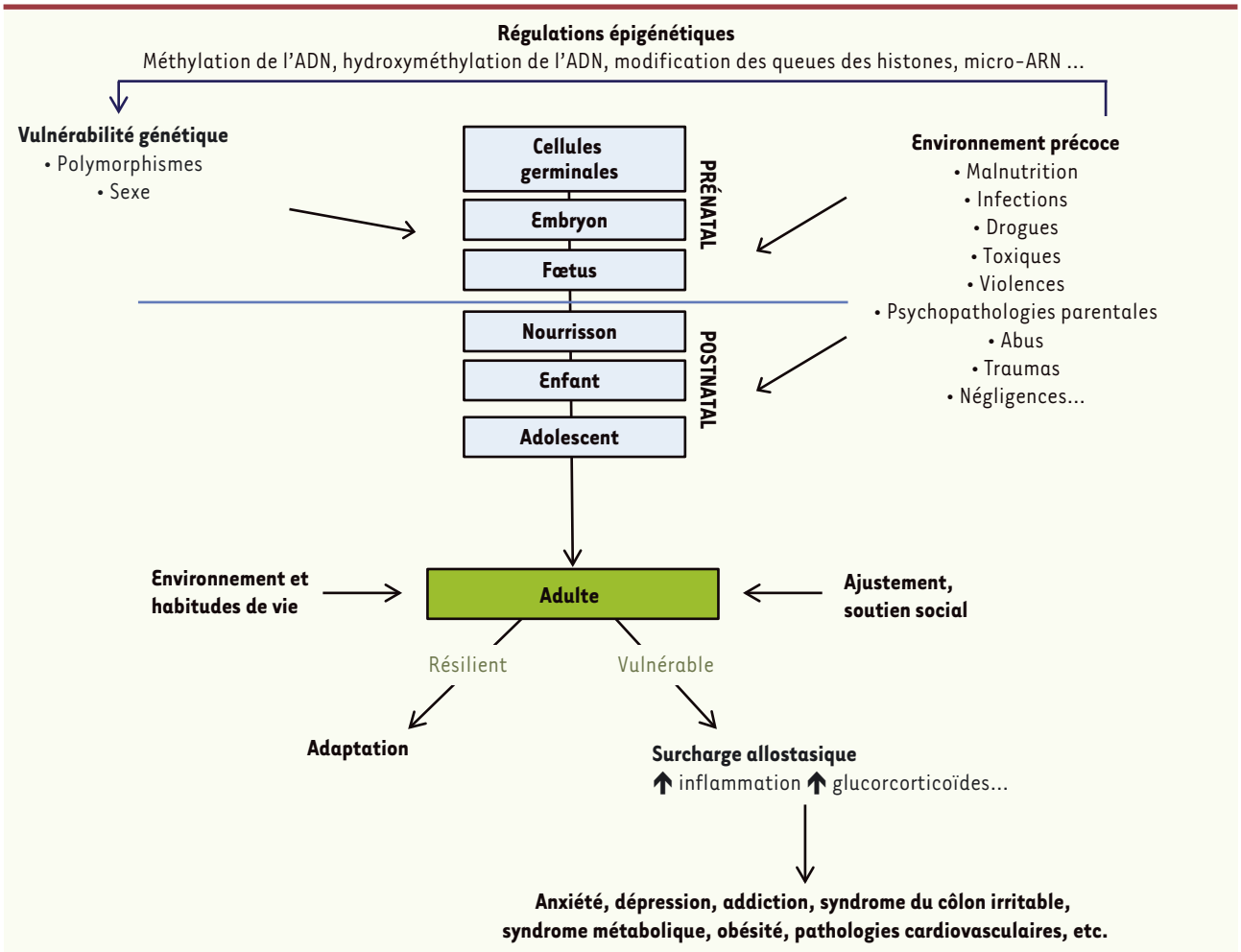

Figure 1. ***Stress précoces, surcharge allostasique et vulnérabilité à l'âge adulte.*** Les premières phases de vie constituent une période critique lors de laquelle les facteurs environnementaux laissent une empreinte durable, suggérant ainsi un phénomène de « programmation précoce » du phénotype adulte. Ainsi, l'adversité précoce, se présentant sous de multiples formes telles que la malnutrition ou encore des violences parentales, augmente la vulnérabilité aux troubles neuropsychiatriques et métaboliques à l'âge adulte. Les régulations épigénétiques représentent un mécanisme majeur pour expliquer l'effet de l'environnement sur l'individu en développement. De plus, la vulnérabilité de l'individu adulte dépend également de son contexte génétique propre. Un contexte génétique inadapté et une pression environnementale trop forte aboutiront à un état de rupture de l'homéostasie physiologique ou surcharge allostasique, lequel est caractérisé par une incapacité d'adaptation à de nouveaux stress.

l'axe corticotrope (système neuroendocrinien du stress) et d'un risque plus élevé de troubles neuropsychiatriques *(Figure 2)* [3, 4].

Chez le rongeur, les travaux princeps menés par Seymour Levine ont mis en évidence des périodes critiques durant lesquelles l'environnement inscrit une empreinte durable sur le cerveau [5]. La femelle rat « materne » de façon intense ses petits (allaitement actif en position arc-boutée, léchage ano-génital) ; ce soin maternel est déterminant pour la maturation cérébrale et les futures capacités adaptatives de la progéniture. Il a été montré qu'à l'âge adulte les descendants des mères à faible maternage présentent des altérations comportementales, une réponse exacerbée de l'axe corticotrope face à un stress et une diminution des récepteurs des glucocorticoïdes dans l'hippocampe *(Figure 3)* [2]. En outre, l'adoption de ratons issus de mères biologiques à fort maternage, par des mères à faible maternage, produit un phénotype semblable à celui observé chez la descendance de mères à faible maternage. Enfin, après des adoptions croisées, la descendance femelle présente, à l'âge adulte, le même comportement maternel que celui de la mère adoptive [6]. Chez le rat, la séparation répétée mères-petits pendant de longues périodes produit, chez la descendance, un phénotype proche de celui induit par le faible maternage : altérations de l'axe corticotrope, perturbations émotionnelles (hyperanxiété, anhédonie) et mnésiques *(Figure 3)* [7]. Ces changements sont associés, entre autres, à des altérations morphofonctionnelles dans le cortex préfrontal médian et l'hippocampe (diminution de l'arborisation et du nombre d'épines dendritiques

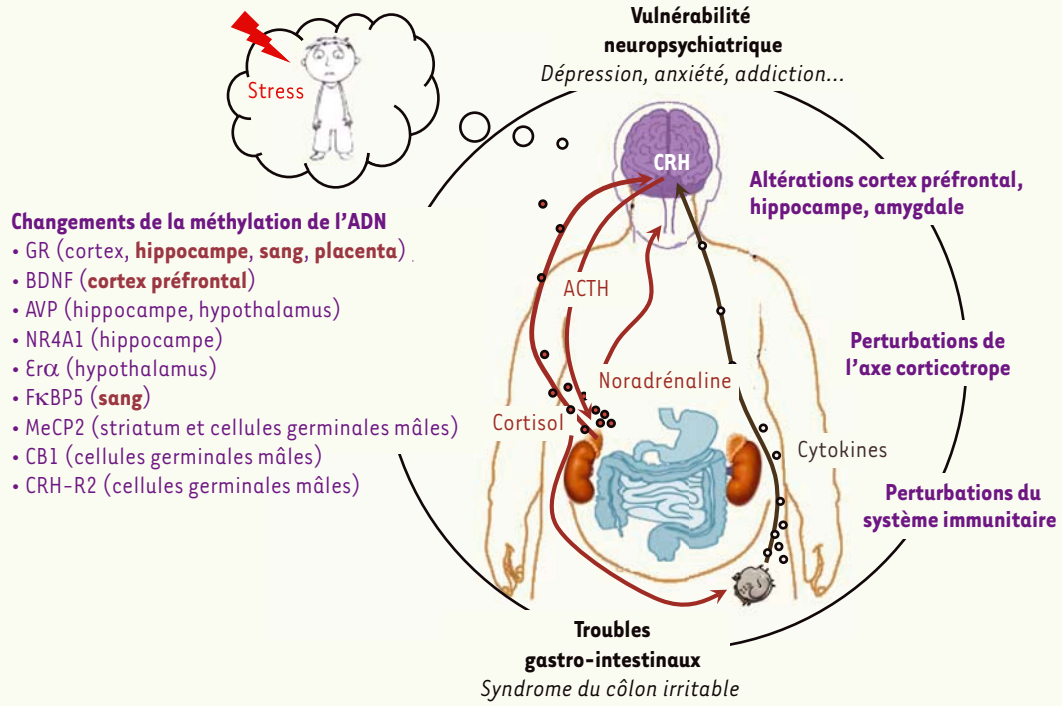

Figure 2. ***Changements physiologiques à l'âge adulte associés à l'adversité dans l'enfance et mécanismes épigénétiques potentiellement impliqués.*** L'adversité précoce est associée à des altérations de divers systèmes physiologiques tels que le système nerveux central et périphérique, le système immunitaire ou encore le système gastrointestinal. Ces perturbations physiologiques sont accompagnées de modifications épigénétiques comme la méthylation ou la déméthylation de l'ADN dans les régions promotrices de nombreux gènes (les changements observés chez l'homme sont en rouge gras, les autres changements ont été observés chez l'animal). L'ensemble de ces altérations pourrait participer à l'émergence de pathologies neuropsychiatriques. CRH : *corticotropin releasing hormone* ; ACTH : *adrenocorticotropic hormone* ; GR : *glucocorticoid receptors* ; BDNF : *brain derived neurotrophic factor* ; AVP : *arginin vasopressin* ; NR4A1 : *nuclear receptor subfamily 4, group A, member 1* ; Erα : *estrogen receptor alpha* ; FκBP5 : *Fκ506 binding protein 5* ; MeCP2 : *methyl CpG binding protein 2* ; CB1 : *cannabinoid receptor type 1* ; CRH-R2 : *corticotropin releasing hormone receptor 2.*

des neurones pyramidaux, perturbations de la potentialisation à long terme[1]). La carence maternelle produit, dans l'hippocampe adulte, une diminution du facteur neurotrophique BDNF (*brain derived neurotrophic factor*) et des altérations des récepteurs du glutamate, NMDA (*N-methyl-D-aspartate receptor*). Elle induit par ailleurs une hypersensibilité aux effets récompensant des drogues d'abus et une hypoactivité du système enképhalinergique[2] dans les noyaux caudé-putamen et accumbens [8]. Les systèmes opioïdes sont très impliqués dans la mise en place des relations entre la mère et le petit, et leur dysfonctionnement pourrait être responsable des altérations comportementales des rats stressés. D'autres perturbations de l'environnement postnatal précoce, comme la réduction de la disponibilité du matériel de nidification, ont également des effets délétères à long terme sur la descendance. En revanche, des séparations mères-petits de courtes durées (15 min par jour) ont des effets opposés, caractérisés par un phénotype résilient attribué à la stimulation du comportement maternel [9].

Par ailleurs, de nombreuses études ont montré que l'adversité précoce constitue un facteur de risque à l'émergence de troubles gastro-intestinaux, en particulier du syndrome du côlon irritable[3] *(Figure 2)*. En effet, il existe une communication bidirectionnelle cerveau-intestin, fortement influencée par le stress. La période néonatale est caractérisée par une grande plasticité neuronale des circuits sensitifs somatiques viscéraux. Chez le rongeur, la séparation maternelle affecte de façon durable l'homéostasie intestinale. Les conséquences majeures sont : (1) des modifications de l'immunité de la muqueuse intestinale, (2) l'apparition d'une hypersensibilité viscérale, et (3) l'altération de la fonction de la barrière de l'intestin. Ces animaux

[1] La potentialisation à long terme est caractérisée par une amélioration durable de l'efficacité synaptique. Ce phénomène est généralement associé à la notion de plasticité neuronale et de mémoire.

[2] Les enképhalines sont des endorphines (morphine endogène) qui se fixent sur des récepteurs opioïdes. Elles jouent un rôle en particulier dans l'inhibition des messages de douleur et la régulation des réponses de stress.

[3] Appelé aussi syndrome de l'intestin irritable, il est caractérisé par une douleur abdominale chronique et des désordres de la fonction intestinale (diarrhée ou / et constipation).

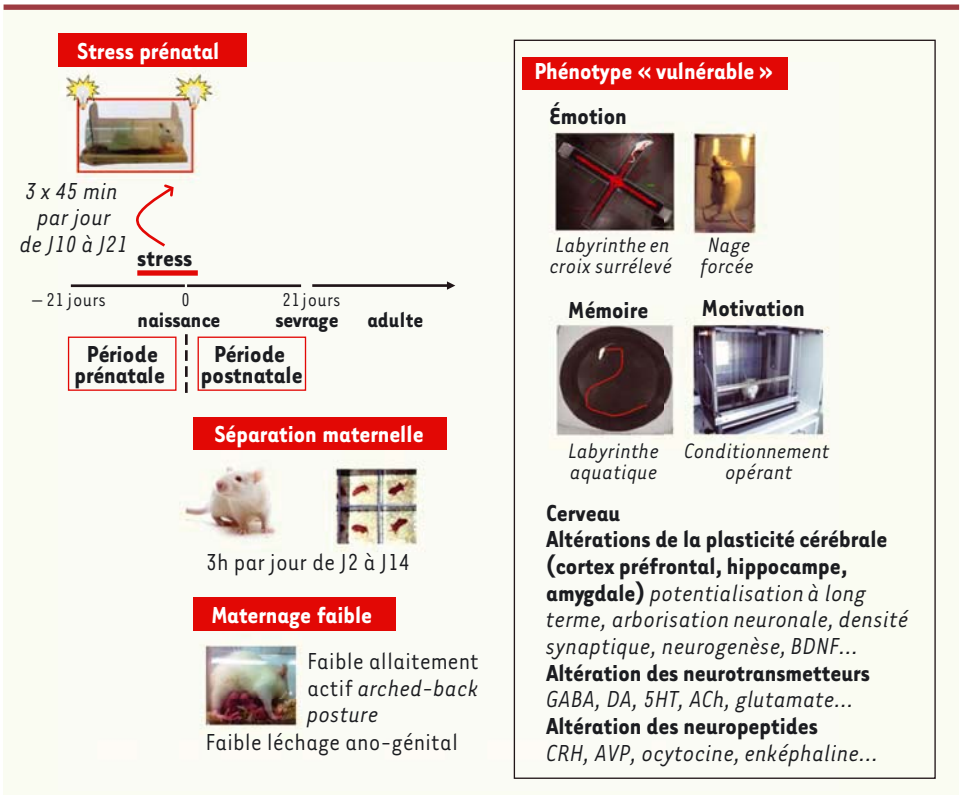

Figure 3. ***Exemples de modèles de stress précoces chez l'animal produisant un phénotype vulnérable à l'âge adulte.*** Dans des tests précliniques, les animaux exposés aux stress précoces présentent des altérations émotionnelles (caractérisées, en autres, par une faible exploration des bras ouverts dans le labyrinthe en croix surélevé et une forte immobilité dans le test de la nage forcée). Ces effets sont atténués par un traitement avec des antidépresseurs. Les animaux exposés aux stress précoces présentent également des altérations cognitives, en particulier une diminution des capacités d'apprentissage et de mémoire dans des tâches spatiales dépendantes de l'hippocampe. Enfin les stress précoces exacerbent la motivation pour les drogues d'abus. Sur le plan neurobiologique, le stress précoce affecte la plasticité cérébrale, les systèmes de neurotransmetteurs et de nombreux neuropeptides. BDNF : *brain derived neurotrophic factor* ; GABA : *gamma-aminobutyric acid* ; DA : dopamine ; 5HT : 5-hydroxytryptamine (sérotonine) ; ACh : acétylcholine ; CRH : *corticotropin releasing hormone* ; AVP : *arginin-vasopressin.*

présentent à l'âge adulte une augmentation du nombre des mastocytes et des polynucléaires neutrophiles muqueux, ainsi qu'une réponse locale cytokinique lymphocytaire de type Th1 (T *helper*) et Th2 témoignant d'un tonus inflammatoire intestinal à bas bruit[4] [10]. Plus récemment il a été montré que la susceptibilité à une hypersensibilité viscérale des animaux soumis à la séparation maternelle peut être transmise à la génération suivante.

Qu'en est-il du stress avant la naissance ?

Pendant la grossesse, l'environnement utérin constitue à la fois une protection contre les agressions externes, et, *via* le placenta, un système de « conduction » qui relie le fœtus à son futur environnement. L'état physique de la mère détermine fortement le développement du fœtus qu'elle porte (➜).

(➜) Voir les Synthèses de B. Le Magueresse *et al.*, et de P. Chavatte-Palmer *et al.*, pages 51 et 57 de ce numéro

L'exposition à certains médicaments, drogues d'abus, virus, toxines microbiennes peut affecter l'embryon *via* leur passage placentaire et avoir des effets irrémédiables. De plus, les procédures d'assistance médicale à la procréation, comme la fécondation *in vitro* suivie du transfert d'embryons, ou les traitements hormonaux aboutissant à une superovulation, ont des conséquences potentielles à long terme sur la descendance, qui restent encore mal connues. Enfin, la question du rôle de l'état émotionnel de la mère sur le développement du fœtus a aussi été posée. Cependant, la démonstration d'un lien causal demeure difficile à établir, du fait de la lourdeur des études prospectives et des biais associés aux études rétrospectives. Depuis de nombreuses années, Vivette Glover étudie l'incidence du stress maternel lors de la grossesse sur le devenir de l'enfant [11]. Certains travaux rapportent une augmentation du risque de troubles émotionnels et cognitifs chez l'enfant, et des retards de langage. Ces effets sont en partie indépendants de causes pouvant survenir après la naissance de l'enfant, comme une dépression de la mère.

Chez l'animal, dès les années 1960, des études ont suggéré que le comportement de la descendance pouvait être modulé par des changements hormonaux de l'environnement intra-utérin ou par les expériences vécues par la femelle lors de la gestation, voire même avant la conception [12]. Depuis, une littérature abondante a porté sur le stress gestationnel chez le rongeur et son incidence sur la descendance [13, 14]. Chez le rat, un stress prénatal (contention maternelle lors de la

[4] Activation chronique des processus immunitaires mais à un niveau relativement faible.

gestation, *Figure 3*) provoque, chez la progéniture, des changements biochimiques et comportementaux durables, qui reflètent l'induction d'une programmation pathologique précoce. À l'âge adulte, lors d'un nouveau stress, les mâles ayant subi un stress prénatal présentent une sécrétion prolongée de corticostérone, caractéristique d'un déficit de rétrocontrôle négatif de l'axe corticotrope [15]. Sur le plan comportemental, l'exposition de la mère à un stress pendant la gestation produit chez la progéniture (1) une augmentation des comportements de types anxieux et dépressifs, (2) une augmentation de la motivation et/ou de la sensibilité aux drogues d'abus [13] et (3) des atteintes cognitives (retard d'apprentissage spatial, déficit de consolidation de la mémoire de peur, déficits de mémoire de travail). Une partie des déficits comportementaux associés au stress prénatal pourrait impliquer l'hippocampe. En effet, sur le plan neurobiologique, le stress prénatal entraîne des atteintes de la plasticité hippocampique, telles que la suppression de la potentialisation à long terme, l'atrophie des neurones granulaires et pyramidaux ou encore une réduction de la neurogenèse adulte [16], un effet certainement lié à l'hyperactivité chronique de l'axe corticotrope. Ces altérations sont accompagnées d'une baisse de l'expression des récepteurs métabotropiques [17] et ionotropiques (NMDA [acide N-méthyl-D-aspartique] et AMPA [α-amino-3-hydroxy-5-méthylisoazol-4-propionate]) du glutamate dans l'hippocampe [18]. De façon intéressante, un bon nombre des altérations comportementales et neurobiologiques que l'on observe chez l'animal adulte sont atténuées par les antidépresseurs [19, 20].

Étant donnés les liens existant entre axe corticotrope et stress [15], la programmation à long terme des effets du stress prénatal sur la descendance pourrait impliquer les glucocorticoïdes. Le stress de contention provoque une activation de l'axe corticotrope et une augmentation des taux circulants de corticostérone chez la femelle gestante *(Figure 3)*. En condition normale, le fœtus est protégé par la 11βHSD2 (11β-hydroxystéroïde déhydrogénase de type 2), une enzyme qui inactive la corticostérone. Cependant, le stress maternel altère l'expression placentaire et l'activité de cette enzyme [21]. De plus, la surrénalectomie, réalisée chez les mères stressées, atténue certains effets sur la descendance [22].

Le stress gestationnel est vraisemblablement associé à un stress postnatal. En effet, l'exposition à des stress pendant la gestation perturbe le comportement de maternage, ce qui, comme on l'a vu précédemment, détermine les capacités d'adaptation au stress de la descendance. Par ailleurs, des manipulations postnatales précoces (adoption, stimulations tactiles, etc.) réduisent les altérations provoquées par le stress prénatal [23].

Stress précoces et épigénome

L'étude princeps concernant la programmation épigénétique et le stress précoce est parue en 2004. Elle montrait que les effets du faible maternage sur la descendance mettaient en jeu des régulations épigénétiques de la région promotrice du gène codant le récepteur des glucocorticoïdes (Nr3c1 [*nuclear receptor subfamily 3, group C, member 1*]) dans l'hippocampe *(Figure 2)*. En effet, le faible maternage produit dans cette structure une augmentation de la méthylation de l'ADN et une diminution de l'acétylation des histones, lesquelles induisent une diminution de la transcription pouvant aboutir à des altérations comportementales et endocriniennes [24]. Ces altérations sont atténuées par des infusions intracérébroventriculaires d'un inhibiteur d'histone déacétylases (HDAC) à l'âge adulte. Les régulations épigénétiques associées à l'adversité précoce concernent aussi d'autres gènes tels que ceux codant le BDNF (*brain derived neurotrophic factor*), la vasopressine, le CRH (*corticotropin releasing hormone*) ou le récepteur CRH-R2 (*corticotropin releasing hormone receptor 2*) [4] *(Figure 2)*. La plus grande sensibilité aux opiacés des rats exposés à une séparation maternelle implique aussi des processus épigénétiques. En effet, la séparation maternelle produit une augmentation de la MeCP2 (*methyl-CpG-binding protein*) et de l'histone déacétylase HDAC2, ainsi qu'une diminution de l'acétylation des histones H3 et H4 dans les noyaux caudé-putamen et accumbens. De manière intéressante, le comportement de dépendance et l'hypoactivité du système enképhalinergique sont supprimés par le valproate de sodium, un inhibiteur d'histone déacétylases [25]. Certaines des altérations épigénétiques décrites chez l'animal sont présentes chez l'homme *(Figure 2)*. Ainsi, une augmentation de la méthylation de la région promotrice du gène *Nr3c1* a été observée dans l'hippocampe de suicidés (souffrant de dépression) ayant subi des maltraitances dans l'enfance [3]. Par ailleurs, chez l'animal comme chez l'homme, des études révèlent des changements de méthylation de l'ADN dans de nombreuses régions du génome après des stress postnataux précoces, que ce soit dans le système nerveux central (hippocampe, cortex préfrontal) ou dans les tissus périphériques (cellules sanguines, salive, épithélium buccal) [4, 25]. Le stress *in utero* est également associé à des changements épigénétiques : (1) une baisse de l'expression placentaire de la 11βHSD2 (qui inactive la corticostérone), accompagnée d'une augmentation de la méthylation de l'ADN dans les sites 3CpG du promoteur du gène codant cette enzyme et (2) des changements de méthylation de l'ADN dans les régions promotrices des gènes codant le CRH et le récepteur des glucocorticoïdes [27]. Cependant, des régulations épigénétiques opposées sont rapportées en fonction de l'intensité du stress (stress léger : augmentation de la méthylation de l'ADN ; stress fort : diminution de la méthylation) et du sexe (HDAC4 augmentée dans l'hippocampe des mâles ; diminuée dans le cortex préfrontal des femelles).

Les effets délétères de l'adversité précoce semblent transmis à travers les générations [28] (➜).

(➜) Voir la Synthèse de C. Junien *et al.*, page 35 de ce numéro

Les processus potentiellement impliqués dans la transmission du stress parental à la descendance sont multiples, mais récemment il a été proposé que le stress puisse agir sur les cellules germinales. Chez la souris, plusieurs études ont rapporté une transmission intergénérationnelle par le père. Ainsi, un stress chronique chez des souris mâles produit des changements neurocomportementaux qui se maintiennent chez sa descendance (F1) et à la 2e génération (F2). Ces changements pourraient être transmis *via* des marques épigénétiques dans le sperme. En effet, une diminution de la méthylation de l'ADN dans la région promotrice du gène *CRH-R2* et une hyperméthylation des sites CpG du promoteur du gène *MeCP2* ont été observées dans le sperme des F1 et F2.

Conclusion-perspectives : promouvoir le bien-être précoce pour favoriser la résilience ?

Depuis plus de 50 ans, les recherches sur l'adversité précoce soulignent ses conséquences sur la santé mentale et la santé en général. Des travaux récents rapportent des changements épigénétiques que l'on observe à l'âge adulte après une exposition à des stress lors de la période de développement. Il faut cependant souligner qu'une part non négligeable des études n'observe aucun effet, voire des effets bénéfiques sur l'adaptation au stress, y compris chez l'homme [9]. Vraisemblablement, le type de stress, son intensité et sa durée jouent un rôle dans les effets observés, de même que le sexe et les caractéristiques génétiques de l'individu qui est exposé au stress.

L'hypothèse selon laquelle l'environnement préconceptionnel et périnatal crée une empreinte sur l'individu est une problématique clé en psychobiologie du développement. Elle met en jeu des questions éthiques importantes [29] (➜). Comment les résultats des recherches sur la transmission intergénérationnelle du stress vont-ils permettre d'améliorer la santé humaine ? Une publication récente parue dans *Science* [30] montre que la stimulation cognitive et sociale précoce (entre 0 et 5 ans) chez des enfants exposés à un environnement socioéconomique défavorisé améliore significativement l'état de santé (marqueurs métaboliques, tension artérielle, poids) 30 ans plus tard. Ces résultats offrent une perspective intéressante en terme de politique publique**.** Comme le souligne le prix Nobel d'économie James Heckman, le retour sur investissement est maximal pour des prises en charges très précoces, entre 0 et 3 ans, puis, plus on s'éloigne de cette période critique, plus la balance coût-bénéfice se déséquilibre [31]. ◊

(➜) Voir la Synthèse de E. Rial-Sebbag *et al.*, page 100 de ce numéro

SUMMARY

Early life stressful experiences and neuropsychiatric vulnerability: evidences from human and animal models

The human newborn is highly dependent on parental care for its survival but also for the healthy development of its brain. A large body of literature demonstrates the impact of early life adversity, even during the prenatal period, on the adult's health. The susceptibility to neuropsychiatric diseases is often potentiated by early stress. If there is an agreement that a critical developmental period exists, the mechanisms underlying the long term effects of early life adversity are still poorly understood. Recent studies in animals highlight the involvement of epigenetic processes in the transmission of such vulnerabilities, notably *via* modifications in germ cells, which can be transmitted in the next generations. ◊

LIENS D'INTÉRÊT

Les auteurs déclarent n'avoir aucun lien d'intérêt concernant les données publiées dans cet article.

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TIRÉS À PART
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