Sex-specific effects of chronic stress on intestinal health and depression-like behaviours

Mémoire

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Résumé

La dépression majeure est devenue la cause principale d'incapacité dans le monde. Pourtant, les antidépresseurs les plus courants sont inefficaces chez 30-50% des patients traités. La dépression présente une comorbidité élevée avec les troubles gastro-intestinaux, avec une pathologie commune comprenant le dysbiose du microbiote un profil périphérique hautement inflammatoire, ce qui suggère une perméabilité intestinale accrue chez ces patients. Il est proposé que le stress chronique soit lié à la détérioration de la barrière intestinale et à la dérégulation de la signalisation intestin-cerveau, cependant, les mécanismes biologiques restent à identifier. Nous avons utilisé les modèles de la dépression pour étudier les effets du stress chronique sur la perméabilité intestinale chez les souris mâles et femelles. Le séquençage du microbiote a montré une modification des populations microbiennes après le stress. De plus, l'expression génétique des jonctions serrées intestinales a été altérée avec des effets spécifiques au sexe, en fonction du type et de la durée du stress. Certaines modifications des jonctions serrées sont associées à la résilience ou à la susceptibilité à l'exposition au stress, déterminée par des tests comportementaux. Nous avons également identifié la protéine de liaison au lipopolysaccharide (LBP) comme un biomarqueur potentiel lié à la susceptibilité au stress chronique.

En étudiant les différences entre individus et selon les sexes, nos résultats contribueront à la connaissance des mécanismes moléculaires qui déterminent la vulnérabilité ou la résilience au stress chronique. Les femmes ayant un risque environ deux fois plus élevé de développer une dépression, l'identification des différences entre les sexes est particulièrement pertinente. Ces études contribueront à l'élaboration de nouvelles stratégies thérapeutiques et diagnostiques pour le traitement de la dépression. Des traitements ciblant l'intégrité de la barrière intestinale pourraient avoir des effets positifs sur les voies inflammatoires périphériques et centrales impliquées dans la dépression.

Abstract

Major depressive disorder (MDD) is the leading cause of disability worldwide. Still, common antidepressants are ineffective in 30-50% of treated patients, highlighting that biological mechanisms remain to be elucidated. MDD has high comorbidity with gastrointestinal disorders including patterns of microbiota dysbiosis and inflammatory peripheral markers, suggesting enhanced intestinal permeability in these patients. Chronic stress, the main environmental risk factor for MDD is linked to intestinal barrier deterioration and dysregulated gut-brain signalling. Therefore, we investigate effects of chronic stress on manifestations of intestinal permeability in both male and female mouse models of depression. Sequencing showed altered microbial populations post-stress. Furthermore, gene expression of intestinal tight junctions was altered with sex-specific effects, dependent on stress type and duration. Certain tight junction changes associated with resilience or susceptibility to the stress exposure, as determined by behavioural tests. We also identified Lipopolysaccharide binding protein (LBP) as a potential biomarker related to susceptibility to chronic stress.

By investigating individual and sex differences, our results will be contributing to the knowledge of molecular mechanisms underlying vulnerability or resilience to chronic stress. As women have roughly a twofold higher risk of developing depression, identifying sex differences is particularly relevant. These studies will help to develop more effective and appropriate therapeutic strategies for the treatment of depression and possibly identify biomarkers which are greatly needed in the field. Targeting the intestinal barrier and potentially promoting barrier integrity, future treatments could have positive downstream effects on peripheral and central inflammatory pathways implicated in depression.

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List of abbreviations

ACC, Anterior cingulate cortex
AGG, Aggressor
AHR, Aryl hydrocarbon receptor
AMY, Amygdala
ANOVA, Analysis of Variance
ASCA, Anti- Saccharomyces cerevisiae antibody
BBB, Blood Brain Barrier
BDNF, Brain-derived neurotrophic factor
CDNA, complementary DNA
CD14, Cluster of differentiation 14
CT, Cholera toxin
CLDN, Claudin
CNS, Central nervous system
CRP, C-reactive protein
CSDS, Chronic social defeat stress
CVS, Chronic variable stress
DCE-MRI, dynamic contrast-enhanced magnetic resonance imaging
DSM-5, Diagnostic and Statistical Manual of Mental Disorders, 5th Edition
ELISA, Enzyme-linked immunosorbent assay
E. coli, Escherichia coli
F-actin, Actin filaments
FABP2, Intestinal fatty acid binding protein
fMRI, Functional magnetic resonance imaging
GBA, Gut-brain axis
GI, Gastrointestinal
HDRS24, Hamilton Depression Rating Scale-24 item (HDRS24)
HIPP, Hippocampus
HPA, Hypothalamic-pituitary-adrenal
IBD, Inflammatory bowel disease
ICAM-1, Intercellular adhesion molecule-1
IDO-1, Indoleamine 2,3-Dioxygenase 1
IFN, Interferon
Ig, Immunoglobulin
IL, interleukin
JEJ, Jejunum
KYN, Kynurenine
LPS, Lipopolysaccharide
LBP, Lipopolysaccharide binding protein
MARVELD2, MARVEL domain-containing protein
MCP-1, Monocyte chemoattractant protein-1
MDD, Major depressive disorder
MMP, Matrix metalloproteinases
mPFC, Medial prefrontal cortex
MRI, Magnetic resonance imaging
mRNA, Messenger ribonucleic acid

MUC2, Mucin-2
NAC, Nucleus accumbens
NFκB, Nuclear factor-κB
NOD2, Nucleotide-binding oligomerization domain 2 protein
OCLN, Occludin
OFC, Orbital frontal cortex
PBS, Phosphate-buffered saline
PCoA, Principle Coordinate Analysis
PERMANOVA, Permutational multivariate analyses of variance
PET, Positron Emission Tomography
PFC, Prefrontal cortex
PRR, pattern recognition receptor
qPCR, Quantitative polymerase chain reaction
RES, Resilient
SCFA, Short-chain fatty acids
SCVS, Subchronic variable stress
sIL-6R, soluble interleukin 6 receptor
SI, Social interaction
SP1, Specificity Protein 1
SS, Susceptible
SSRI, Selective serotonin reuptake inhibitors
STAT3, Signal transducer and activator of transcription 3
S. aureus, Staphylococcus aureus
S100B, S100 calcium-binding protein B
TEER, Transepithelial/transendothelial electrical resistance
TFF3, Trefoil factor 3.
TJP, Tight junction protein
TJP1, Tight junction protein 1 /Zonula occludens-1
TLR, Toll-like receptor
TNF-α, Tumor necrosis factor alpha
TRP, Tryptophan
TSPO, Translocator protein
VCAM-1, Vascular cell adhesion protein 1
VTA, Ventral tegmental area
V. cholerae, Vibrio cholerae
ZOT, Zonula occludens toxin
5-HT, 5-hydroxytryptamine/ Serotonin

List of acronyms

KEGG (Kyoto Encyclopedia of Genes and Genomes)

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Introduction

General overview

This thesis examines the effects of chronic stress on behaviours, gene expression in the brain and the gut and peripheral biomarkers in animal models of depression. In the introductory sections an overview of MDD characteristics and main hypothesis related to its pathogenesis will be presented followed by animal models of depression relevant for this thesis. Finally, a layout of gut-related basic knowledge and a highlight of preclinical and clinical evidence supporting its potential role in MDD precedes my thesis main hypothesis and objectives.

1 Major depressive disorder

1.1 Current state and mechanistic insights

Major depressive disorder (MDD) is currently the most prevalent mood disorder and the leading cause of disability worldwide [4]. Depressive disorders fall along a range of severities from a milder, persistent dysthymia to severe MDD. Core symptoms include low mood, irritability, anhedonia, apathy, difficulty concentrating, disrupted appetite and sleep (**Table 1**) [5]. Still, this disorder is highly heterogeneous and the experience, symptoms and response to treatment varies highly across individuals. Furthermore, comorbidity between MDD and other stress and affective disorders is common [6–8]. Many biological factors have been suggested as risk factors for MDD, however, it is most plausible that MDD has a multifactorial etiology. Chronic stress is the leading environmental risk factor for MDD [9]. However, not every individual exposed to chronic stressors develops an affective disorder, and therefore individual differences in reactivity to stress exposure could bring insight for novel therapeutic strategies.

	Prevalence		Symptomatology	Treatment resistance
	Overall	**Males vs. Females**		
Global	2.87%	♂2.22%/ ♀3.52%	Decreased energy/Fatigue Difficulty concentrating Weight fluctuations Gastrointestinal problems Anhedonia Sadness Anxiety Helplessness Thoughts of death or suicide	~50% do not respond to current antidepressant treatments [1]. > 1/3 of patients are resistant to conventional pharmacologic, psychologic, or somatic treatments [1,2].
Canada	3.39%	♂ 2.50% /♀4.22%		
US	4.95%	♂ 3.58% /♀6.28%		
Europe	3.26%	♂ 2.38% /♀4.12%		

Table 1. Prevalence and symptomatology of MDD[3]
In individuals aged 15-49 years, data from 2019.

The complexity of emotion management and how it impacts the brain has been investigated for decades but remains a hot topic. Emotional experience, such as stress, can have a negative or positive valence, for example, if it is associated with fear or reward, respectively. Indeed, stress affects several brain regions[10] including the hippocampus (HIPP), prefrontal cortex (PFC), amygdala (AMY), and nucleus accumbens (NAC). Stress-induced neurocircuitry alterations in these regions modulate emotions and mood in humans and related behaviours in rodents [11]. The reward circuit, a key component in emotion regulation, is comprised of frontal and limbic regions centered around the ventral tegmental area (VTA) to NAC projections, receiving inputs from the PFC, HIPP and AMY. These regions respond to environmental stimuli and react depending on their rewarding or aversive nature, resulting in an emotional response [12]. The reward circuitry is affected in stress and mood disorders and functional or structural shifts can be investigated by neuroimaging techniques. Abnormalities in connectivity or neurodegeneration in these regions lead to behavioural dysfunction like hyperarousal, attention deficit or altered emotional processing [13,14]. Cerebral blood flow, an indirect measure of neuronal activity observable by functional MRI (fMRI), is altered in multiple reward-associated regions such as the PFC and related with symptoms in MDD patients [15–18]. Specifically, over-firing in reward circuits or alterations in synaptic plasticity of neurons correlate with symptoms of anhedonia and sadness [12,19].

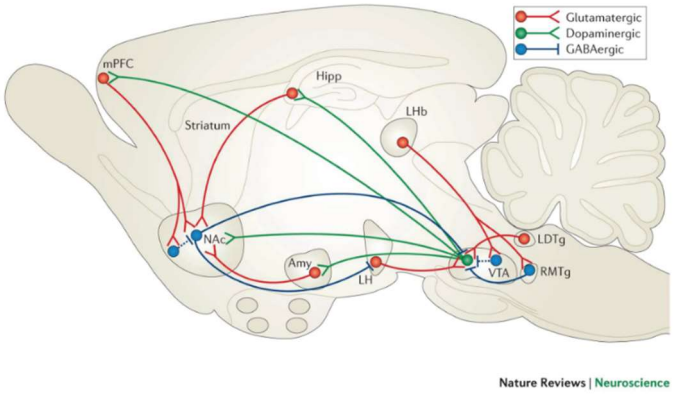

Figure 1. A simplified schematic of the reward circuit in the rodent brain.
From Russo & Nestler (2013). Abbreviations: Ventral tegmental area (VTA) and nucleus accumbens (NAC), medial prefrontal cortex (mPFC), hippocampus (Hipp) and amygdala (Amy), lateral dorsal tegmentum (LDTg), lateral habenula (LHb) and lateral hypothalamus (LH), RTMg, rostromedial tegmentum.

Other brain regions, namely the locus coeruleus and the anterior cingulate cortex (ACC), receive increasing attention for their roles in modulating arousal, cognition, and reward-

related memory (for in-depth reviews, see [20,21]). Indeed, heightened activity occurs in limbic regions such as the AMY, insula, and areas of the ACC [22–24]. Hyperactivity in ACC regions is related to local depletion of the serotonin precursor, tryptophan (TRP), and experiencing transient sadness [25]. Concurrent heightened AMY activity is implicated in rumination and maladaptive processing of negative stimuli [26].

Resting-state activity levels reveal diminished frontal lobe function, specifically in the dorso- and ventro-lateral PFC and the OFC. Decreased activity in cortical regions may underlie cognitive dysfunction, such as depreciated emotional state and interest by limiting executive control over affective processing [26]. Indeed, in MDD patients, positron emission topography (PET) and fMRI studies show altered region-specific connectivity. Decreased functional connectivity occurs between ACC and the medial PFC, key default mode network regions [27]. The default mode network is a set of cortical regions controlling baseline cognitive functions until they are attenuated by a task requiring active attention. Low functional connectivity here occurs during self-reflection induced sad mood states [16]. In MDD patients, depression severity correlates with reduced blood perfusion in the default-mode network [15]. Moreover, extinguishing default mode activity during cognitive tasks is linked to better performance in healthy individuals. Studies found increased activity in these regions linked to negative rumination processes in patients, indicative of a loss of active control of attention. Altogether, reduced PFC function and connectivity between limbic regions may underlie rumination and poor executive functioning in MDD [18].

1.2 Hypotheses of Depression

1.2.1 Biogenic amine hypothesis of depression

Frontline MDD treatment involves pharmaceutical therapy targeting the monoaminergic systems, based on the well-established monoaminergic hypothesis of depression [28]. This theory has been the most prevalent for decades, suggesting deficits in cortical and limbic monoamine signalling, neurotransmitters serotonin (or 5-hydroxytryptamine [5-HT]), dopamine, and norepinephrine, underlies the biological basis of depression. However, the precise mechanisms behind monoamine circuit deficits and how they induce psychopathology is unclear. Common antidepressants act by inhibiting neurotransmitter reuptake, enhancing the bioavailability at the synapse. However, many patients are resistant

to treatments with classical antidepressants, putting into question the extent of the role of central monoamines in depression pathogenesis [29]. In recent years, depression research has diverted away from neurocentric hypotheses and opened to exploring the contributions of other systems, viewing the disease as more of a whole-body disorder.

1.2.2 Neuro-endocrine dysfunction hypothesis

Dysfunctional glucocorticoid signalling is recognized in the context of MDD, playing a pivotal role in the distortion of mood and emotional regulation [30–33]. Via the release of stress hormones, threatening events directly modulate brain circuits involved in emotional processing and cognitive functions. For instance, the formation of memory and reward-making decisions [34] as well as sleep functions are affected in humans [35]. Acutely, stressors activate rapid-acting sympathetic and para-sympathetic signalling, preparing the "fight or flight" response [36]. Activation of the hypothalamic-pituitary-adrenal (HPA) axis initiates downstream pathways of hormone release with final production and delivery of cortisol from the adrenal cortex [2]. Cortisol principally promotes mobilization and utilization of energy [37]; however, a negative feedback loop also allows it to extinguish the acute stress response. Short-term cortisol signalling in the HIPP and hypothalamus improves recall of emotionally relevant information and promotes vigilance, respectively [38,39]. While both mechanisms are protective acutely, repeated exposures or prolonged duration can lead to maladaptive shifts to the HPA axis and these critical brain regions involved in emotion and cognitive processing. Indeed, the HPA axis is overactivated in MDD, leading to abnormal excitation or inhibition of key brain regions involved in maintaining stress-response adaption [13,40]. Evidence of impaired

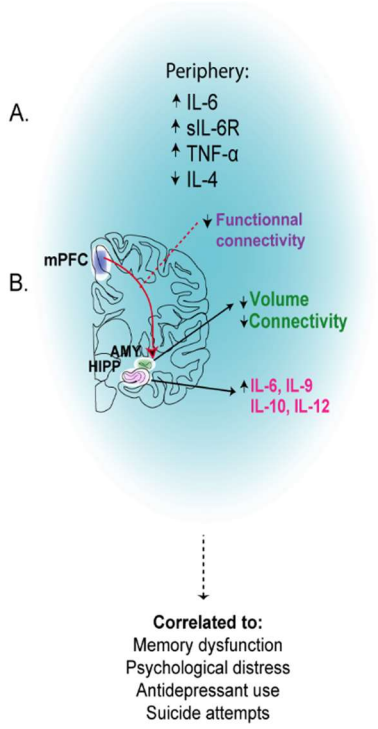

Figure 2. Effects of peripheral and central inflammation on emotion in MDD. **A**) MDD has been associated with a shift toward a pro-inflammatory profile in the periphery. **B**) Decreased volume and connectivity of emotion-regulating brain regions, namely, the HIPP and the AMY, and increased local expression of brain cytokines and chemokines were reported in MDD patients, possibly contributing to the emergence of symptoms. **Adapted from Doney *et al.* 2021**

glucocorticoid-mediated feedback inhibition in MDD resolves with successful treatments with antidepressants [40].

1.2.3 Neuro-immune hypothesis

Maladaptive central and peripheral inflammatory responses are associated with affective disorders. The BBB is a highly selective physical frontier between the central nervous system (CNS) and peripheral circulation, formed by endothelial cells, a basement membrane, astrocytes, and pericytes (**Fig. 2A**). Still, peripheral immune activation influences the CNS through several communication systems: transport systems, BBB permeable regions, and neural transmission. Overactivation or prolonged activation of these pathways could create a highly reactive environment with ramifications to neuronal circuits of mood regulation [32,41–43]. HPA axis activation is also integral to neuro-immune responses as cortisol reduces immune system activity during acute stress to augment energy resources. Suppressing specific adaptive immune responses preserves energy for a crisis, however, extended stress exhausts the negative feedback loop and is implicated in immune signalling dysregulation [43,44,45]. Downstream effects of these shifts can be chronically increased inflammation [44]. Dysregulated peripheral and central innate immune responses are described in MDD patients [46–48]. Therefore, investigation of stress-associated activation of inflammatory pathways is crucial in this context.Peripheral immune cells and factors can communicate with microglia, the primary central immune cells, directly from entering the brain or indirectly through more permeable regions of the neurovascular network, the circumventricular organs. Psychological stress induces many of the inflammatory signals such as activation of peripheral immune cells: monocytes, lymphocytes and mastocytes [49,50], which are linked to damage and disease. Inflammation processes initiated in the periphery can signal to the brain regions implicated in emotional processes (**Fig. 1**) [16,51]. Administration of specific pro-inflammatory cytokines or endotoxins is sufficient to induce behavioural symptoms associated with depression [52,53]. Indeed, increased circulating cytokines are a hallmark of MDD [49,54]. Increased peripheral inflammation is also widely recognized in the pathogenesis of various maladaptive stress responses, yet mechanistic insight of these relationships is still lacking. Transport systems allow peripheral pro-inflammatory or anti-inflammatory signals such as cytokines and chemokines across the BBB. Cytokines modify BBB transporter expression, increasing their uptake into the brain while promoting the trans endothelial

migration of immune cells [55]. Normally, tight junctions and other dynamic barrier-forming proteins hold together the endothelial cells in the brain's vasculature [56]. However, specific pro-inflammatory cytokines can alter permeability by redistributing tight junction proteins as demonstrated rodent models of depression [57]. A detrimental increase in BBB permeability occurs through loss of tight junction proteins, promoting infiltration of circulating inflammatory mediators into the brain and development of depression-like behaviours [54].

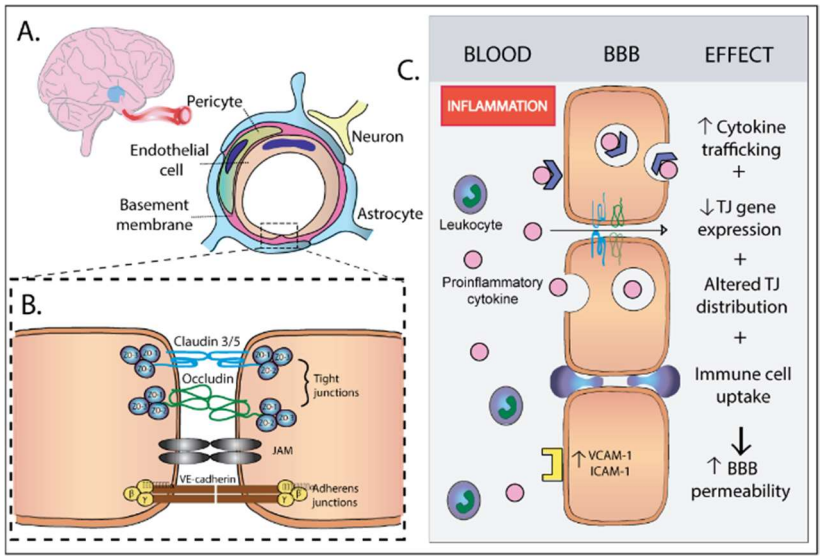

Figure 3. Blood-brain barrier in MDD.

Furthermore, other signalling molecules may be involved in immune/ inflammatory signalling to the brain. Historically, peripheral 5-HT has been discredited in MDD pathology since it does not cross the BBB, however, many 5-HT precursors and metabolites do. Novel roles for peripheral 5-HT metabolism are implicated in inflammatory, immune and metabolic signalling pathways [58,59]. Accordingly, alterations in peripheral 5-HT signalling could have direct outcomes on the brain by altered precursor availability, and indirectly by interactions with inflammatory and immune pathways [60,61]. Unraveling the interactions between peripheral and central inflammation with brain regions affected in mood disorders could provide strategies for future targeted therapies [52,62–65].

In line with the neuro-immune hypothesis of depression, MDD has a high comorbidity with inflammatory bowel diseases (IBD) such as Crohn's disease and ulcerative colitis amongst other inflammatory gastrointestinal conditions [66,67]. It is suggested that inflammation-driven

gut barrier dysfunction may affect emotion regulation and vice versa. In gastrointestinal (GI) disorders, elevated pro-inflammatory cytokines promote permeability in the intestinal tract by suppressing tight junction mediated barrier function [68] (**Fig. 3A-B**) as described above for the BBB (**Fig. 2A-C**). Stress has been linked to the deterioration of the intestinal barrier via alterations of gut–brain signalling [69,70]. Furthermore, the microorganisms composing gut bacteria, the microbiota, play a critical role in maintaining health [71] and influence the brain through complex bidirectional signalling pathways known as the gut-brain axis (GBA) [72–77]. Alternatively, stress, diet, and other environmental factors can disrupt the microbiota homeostasis triggering downstream signalling pathways at the intestinal epithelium to the lamina propria beneath where immune cells elicit a pro-inflammatory response [78]. Accumulating evidence implies a contribution of dysregulated GBA signalling in MDD pathology [43,74,77,79].

1.3 Treatment strategies and limitations

MDD is a highly heterogeneous disorder not only in the experience of symptoms, but also the variability in genes, environment, and lifestyle implicated in its onset. Currently, the diagnosis of psychiatric disorders is based on clinical interview which poses many opportunities for misdiagnosis. Current research is driving to develop more advanced strategies including machine learning for clinical assessment [80], biomarkers (**Table A3**) and neuroimaging to improve disease characterization for precision medicine [81]. Conquering our understanding of disease heterogeneity in MDD is crucial for tackling the low treatment efficiency in this disorder.

1.3.1 Sex differences

Women are twice more likely than men to be diagnosed with MDD [31]. Sexual dimorphism is also reported in the prevalence of various symptoms [82] and in treatment response [83,84]. Females more often exhibit anxiety, somatic symptoms and symptoms from the subtype of "atypical depression", defined in the Diagnostic and Statistical Manual of Mental Disorders (DSM)-5 as hypersomnia, leaden paralysis, hyperphagia/weight gain and interpersonal rejection sensitivity [85–87]. Meanwhile men reportedly have more symptoms of psychomotor agitation, suicidal ideation and greater comorbidity of alcohol and drug use [87]. Gene network analysis of pertinent brain regions for MDD demonstrate major transcriptional

rearrangements in patients, though these patterns have limited overlap between men and women. Moreover, these results were confirmed in mouse models, highlighting to importance of defining sex-specific molecular mechanisms [88,89]. Importantly, vascular alterations also display sex-specific effects as tight junction expression was downscaled in the NAC of human postmortem brains from women and men with MDD. However, these reductions in the PFC were specific to women [90]. Nevertheless, there is still an important lack of data regarding sex differences for these conditions. Until the early 2000s, sex was generally not considered as a factor that could affect health and illness, and clinical and preclinical studies mainly used male subjects for homogeneity purposes [2]. More recently, sex has been increasingly considered a significant experimental variable [31,91,92] which could contribute to the adaptation of diagnostics and treatments.

1.3.2 Treatment resistance

Pharmacological treatment with antidepressants is the frontline approach for MDD and amongst these, selective serotonin reuptake inhibitors (SSRIs) are most prescribed. However, only 50-60% of patients respond to the first-line antidepressant drugs, and the trial process to find the most efficient therapy is long and arduous. Treatment resistance represents a major challenge; approximately half of patients do not respond to currently available antidepressant treatments [1]. Even less respond fully to treatments, and therefore therapeutic responses are often measures of individual symptom remission, as opposed to overall recovery [93]. More than one-third of those affected with MDD are resistant to conventional pharmacologic, psychologic, or somatic treatments [1,2]. It is therefore essential to research new avenues of therapeutic interventions.

1.3.3 Prospective biomarkers of Major depression

Currently, there are no reliable biomarkers for detecting MDD, however, many prospective molecules are being investigated both in the periphery and in the brain. Pro- and anti-inflammatory cytokines along with other inflammatory markers, neurotrophic factors, oxidative stress markers, stress hormones, endocrine markers, genetic and epigenetic markers, as well as neuroimaging strategies are all suggested as candidates for future diagnosis and treatment monitoring of MDD [94] (**Table A3**). Many markers have revealed differences between MDD and healthy individuals but none are defined as a diagnostic tool

of this condition [94]. Therefore, there is an urgent need for a deeper understanding of the causal biological mechanisms to promote the discovery of biomarkers that would improve diagnosis and development of new therapeutic strategies.

Neuroimaging studies have been utilized for many years for investigating connectivity, functional and metabolic adjustments in brain regions associated with MDD. However, heterogeneity in the results is a limitation for many imaging studies [17,95]. Disparity in these findings may be attributed to experimental methodologies, individual differences in compensatory mechanisms or symptom heterogeneity [96]. Still, a majority concurs on overactivated limbic circuits paired with reduced top-down regulation, though the precise mechanisms still need to be elucidated. A novel approach implements dynamic contrast-enhanced MRI for BBB assessment. Kamintsky et al. (2020) imaged bipolar disorder patients, who presented BBB dysfunction corresponding to disease severity [97]. A lack of clinical imaging studies investigates BBB permeability in affective disorders and hopefully this study is the antecedent, prompting future investigations. Comparing BBB imaging reports with peripheral inflammatory factors could also help to uncover pertinent biomarkers.

Maladaptive responses of the peripheral immune system are observed in MDD patients [49,98], particularly in treatment-resistant patients characterized by elevated circulating pro-inflammatory cytokine levels [99–101]. Thus, cytokines have the exciting prospective to give a profile beyond symptoms based on self-reported questionnaires (**Fig. 1**). Many inflammation markers have been investigated [102,103]; some modulated uniquely in MDD, while others across multiple disorders (**Table A3**) [104]. It is unclear if peripheral levels reflect central changes, though some evidence supports this theory [105]. Comparative studies of biomarkers according to symptoms could generate specific signature panels useful for precision of diagnosis of MDD vs. other comorbid psychiatric conditions [106]. Further, blood biomarkers could help provide individualized selection of pharmacotherapies or for monitoring treatment response. Indeed, specific panels of immune-endocrine proteins are significantly associated with treatment response to various types of antidepressants [107,108].

BBB markers also hold great promise as putative biomarkers as many have been reportedly upregulated in affective disorders, revealing dysfunction of this important protective structure (**Table A3**). BBB disruption correlates with many psychiatric symptoms, therefore,

biological indicators in the periphery could reflect increased interactions of inflammatory cytokines and other toxic molecules with the brain, particularly in mood regulation brain regions [32,33]. BBB integrity is primarily maintained by tight junction claudin-5 (CLDN5), a central tight junction protein. CLDN5 is compromised in multiple brain regions in MDD patients, highlighting its potential use as a hallmark of this disorder [32,33,109]. S100 Calcium Binding Protein B (S100B), and soluble cell adhesion molecules, Intercellular adhesion molecule-*1* (ICAM-1) and Vascular cell adhesion protein *1* (VCAM-1) are BBB-related molecules that indicate pro-inflammatory environment, trafficking of inflammatory cells, microbial pathogenesis, and antigen presentation [110,111]. Studies have highlighted a potential role of these markers as measurable in the periphery in MDD [107,112–118,119].

Shared profiles of upregulated pro-inflammatory cytokines in the blood such as interleukin-1 beta (IL-1β), tumor necrosis factor-alpha (TNFα), IL-6 and interferon-gamma (IFN-γ), [66,120], occur in GI disorders and MDD, which could be related to increased intestinal permeability [56,68](**Fig. 3**)**.** Indeed, markers of gut dysfunction have also been noted in MDD [121–123] (**Table 3**). Permeability of the epithelial layer may increase interaction of antigens with immune cells, propagating a pro-inflammatory response (**Fig. 3D**). Microbial translocation, from the intestinal lumen into the systemic circulation in the absence of acute infection, is proposed as a mechanism behind the chronic inflammation in MDD [121,124,125]. Indeed, the passage of bacterial products and immune factors as indirect measures of bacterial translocation has been reported in MDD [121,124,125] (**Table 3**). Identifying gut-related biomarkers may be helpful for patients experiencing more somatic symptoms and targeting treatments towards more peripheral physical symptoms to bolster the therapeutic efficiency of psychological treatments.

1.3.4 Future therapeutic interventions

As resistant patients are often characterized by elevated inflammatory tone, clinical trials with anti-inflammatory treatments have been conducted [54] with seemingly positive effects for symptom reduction, despite inconsistent reports [126]. Nonsteroidal anti-inflammatory drugs like Celecoxib have potential benefits when used alone or in combination with other anti-inflammatory drugs [126]. Cytokine neutralizing drugs, like Infliximab which targets TNF-α, reduce depression and anxiety-like behaviors in pre-clinical and clinical trials [127,128]. The

potential of anti-inflammatory treatments is still controversial but efficacy and benefits seem apparent in subpopulations of individuals more affected by inflammation [129].

To be discussed in following sections, the microbiota has the potential to influence emotion regulation in the context of MDD through various pathways including inflammatory, endocrine, neural, and metabolic signalling. Attempts to reinstate a healthy microbiota with probiotics in IBD has shown positive results on gut symptoms [130–132] while also improving depression and quality of life scores [133]. Coincidently, fMRI analysis convey that probiotic treatments can dampen brain responses to negative emotional stimuli in the AMY and in the frontal and temporal cortices [134]. In MDD patients, supplementation of probiotics had beneficial effects on Beck Depression Inventory scores with an elevation in circulating BDNF that was inversely related to symptoms severity [135,136]. Since peripheral BDNF levels are linked with antidepressant response in MDD patients [137,138] this finding could be pertinent in understanding neuroregulatory potential of these types of therapeutics. Combination treatment with SSRI and probiotics was able to improve cognitive performance in MDD patients [139]. Thus, probiotics could be an interesting option to complement psychotherapies and/or pharmacological treatments possibly improving efficiency.

While current treatments target central neurotransmitters, therapeutics directed at peripheral symptoms may help to bolster positive effects. For instance, MDD patients with GI dysfunction have more severe depressive symptoms [140], therefore interventions directed at ameliorating intestinal distress may be of value. Recently, GI disorder research has expanded towards investigating nanoparticles due to the insufficiencies of conventional treatments. Nanoparticles have a variety of forms including extracellular vesicles for drug delivery and scaffolding structures for growth supportive environment with many implications for their therapeutic potentials across many neuroscience research disciplines [141]. A type of nanoparticle superstructures are synthetic peptides, which are amphiphilic compounds with hydrophilic and lipophilic properties, have extraordinary self-organizing capabilities. For applications in GI disorders, pairing these hydrogels with treatments have the advantage potential to target the therapy on the affected region itself instead of the systemically, while mitigating local and peripheral inflammation and microbiota dysbiosis [142]. Indeed, in colitis models, these developments were mediated by re-establishment of tight junction expression,

reducing local inflammatory cytokines and restoring the gut microbiota diversity [143]. Through their molecular architecture, these products can precisely fit the needs of the controlled environment [144], though digestion processes complicate this particular application considering the dynamic surface, motility, pH gradients among other factors in the GI tract [145]. Still, implications for these types of treatments could be relevant for reducing GI inflammation in specific MDD patients.

1.4 Animal models of depression

Ample choices of animal models of mood disorders are available, developed according to human etiology [146]. However, limitations in the validity, efficiency and translational relevance restrict their use and interpretation. As chronic stress is the main environmental risk factor for MDD [9,147–149], it is commonly used in animals to induce anxiety- and depression-like behaviors [32,49]. Other models, namely, genetic, surgical or pharmacological approaches exist, each providing unique benefits and challenges [150]. In my masters project, I focus on stress models, which have increased in popularity due to their relevance for studying major aspects of human disease which limit treatment efficacy, such as sex differences, individual differences and underlying factors influencing vulnerability vs resilience to develop a disorder.

1.4.1 Chronic stress models

Chronic social defeat stress (CSDS) is a mouse model of depression whereby mice are exposed daily to a novel dominant mouse, enduring bouts of physical stress from the aggressor mouse. This paradigm produces two distinct phenotypes of stress response: stress susceptible and resilient mice [151]. As in humans, not all stressed mice develop depression-like behaviours following CSDS, highlighting the potential for uncovering biological mechanisms of resilience. These subgroups display distinct behavioural changes reminiscent of depressive symptoms in humans [5]. Susceptible mice exhibit increased social avoidance, anxiety-like behaviour, anhedonia, despair, weight changes, metabolic disturbances, and corticosterone reactivity [152]. Though resilient mice resist developing many of these features, there is still evidence of stress exposure, indicated by elevated anxiety-like behaviours and corticosterone reactivity [152]. In mice, chronic psychosocial stress increases between-network functional connectivity, measured by MRI and magnetic resonance spectroscopy (MRS).

Modifications included AMY to PFC and AMY to ACC circuits reflecting the clinical condition [27]. These neuroimaging reports in mice add translational interest in this model [153]. Furthermore, the previously reported reduction in BBB integrity in this mouse model occurred only in the brain of stress-susceptible, but not resilient, mice [32]. This research contributes to the current theory that behavioural vulnerability and resilience occur based on individual differences in neuroimmune and neuroendocrine reactions to chronic stress [63]. The heterogeneity of stress effects also makes this model particularly valuable for studying the basis of individual differences that exist in humans, as well as deciphering mechanisms underlying resilience and susceptibility to chronic stress. Identification of molecular and epigenetic changes associated with stress responses [33,154–157] has become increasingly popular in recent years in that respect.

Another leading stress paradigm is the chronic variable stress (CVS) model, during which mice are exposed to a repetitive sequence of three stressors, most often tube restraint, tail suspension, and foot shocks. Each stressor endures about 1 hour daily and lasts from 6 days to several weeks [158,159]. Afterwards, a battery of tests is run to assess anxiety and depression-like behaviors [49]. CVS also induces a pro-inflammatory immune profile similar to those produced by CSDS [63]. In this paradigm, females and males develop depression-like symptoms at different time points making it a strong model for investigating sex differences [49]. Similarly, in the chronic mild stress paradigm, animals are submitted to multiple stressors from a few weeks to months, in different frequencies for each stressor [160]. Stress such as food or water deprivation, wet environment, new cage partner, temperature changes, light during the dark phase, or flashlight, is randomly presented to the animals preventing their habituation [161]. This paradigm is like CVS, though longer lasting and without a repeated order of stressors.

1.4.2 Sex differences in animal models

As in humans, limited data exists defining biological changes specific to females, in rodent models of chronic stress and depression-like behaviours [162]. In various chronic stress paradigms, sex differences in stress reactivity have been exemplified [163]. For instance, in the CVS model, female mice are more vulnerable than males and demonstrate earlier emergence of depressive behaviors [54]. Alternatively, considerably less females display social avoidance

(characteristic of stress susceptibility) after CSDS than males [164]. In CSDS, difficulties in conducting the female model occurs due to barriers faced in replicating behaviours consistent with the male paradigms. Recently, more efficient female models have been developed to overcome these specific difficulties using chemogenetic activation of the ventromedial hypothalamus in aggressors to induce female-female aggression [164] or applying male odorants to increase resident male aggressivity towards females [165]. Still, they present challenges such as the confounding variable of sexual motivations of aggressor and perception of stressor by defeated mice [164]. Female rodent models involve slight adjustments to the male protocols; however, they may be affected differently and have distinct behavioural responses. For the development of future paradigms with improved face validity, careful consideration should be taken to the sensitivities of each sex [162].

Social stress induces neurovascular pathology associated with depressive symptoms in male mice [33], however, previously this was not addressed in female mice. Recently, it was demonstrated that chronic social and subchronic variable stress (SCVS) also promotes BBB alterations in mood-related brain regions of female mice [90]. However, the outcomes were most prominent in the PFC in female mice compared to the NAC in males. Furthermore, comparative assessment of the endothelium cell-specific transcriptomic profiles of the male vs. female PFC identified distinct pathways and genes involved in susceptibility and resilience to stress [166].

Animal models are crucial to our current understanding of the mechanisms involved in MDD. Chronic stress models are some of the most feasible for recapitulating depressive and anxiety-like behaviours relevant to that seen in patients while involving stress which is an important cause of mood and anxiety disorders [167]. It would be unethical to expose humans deliberately to a chronic stressful environment and the technical approaches such as MRI and postmortem studies are limited for looking at molecular and structural levels. Moreover, animal models are valuable for promoting inflammatory body reactions such as response to lipopolysaccharide (LPS) injection [168]. Therefore, we chose chronic stress models of depression in our study to explore stress-related gastrointestinal pathology inspired from reports in GI disorders within the context of applications to MDD. To set up the framework of how stress is connected to intestinal barrier dysfunction via alterations of gut–brain

signalling and implications for MDD, the following sections overview basic intestinal functioning in health and diseased states. We highlight relationships that may exist between gut barrier and BBB leakiness and why their simultaneous investigation in this project is relevant to contribute to the understanding of consequences of exacerbated peripheral inflammation in the pathophysiology of mood disorders. Finally, we elucidate potential biological mechanisms that may underpin emotional distortion with intestinal permeability in the context of MDD.

2 Intestinal homeostasis and dysfunction

2.1 Intestinal barrier: structures and function

The gut barrier is formed by the mucus layer, the epithelia and a connective tissue layer, known as the lamina propria [169]. The epithelial cell monolayer faces the luminal side, interacting with the environment and regulating absorption. Macro-structures of the epithelial surface consist of elongated villi that protrude into the lumen and crypts of proliferating and regenerating cells at the base between them (**Fig. 3A**). These structures contain four main cell types: enterocytes, enteroendocrine cells, goblet cells, and Paneth cells [170]. The epithelium provides a dynamic and semi-permeable barrier with tight junction complexes,

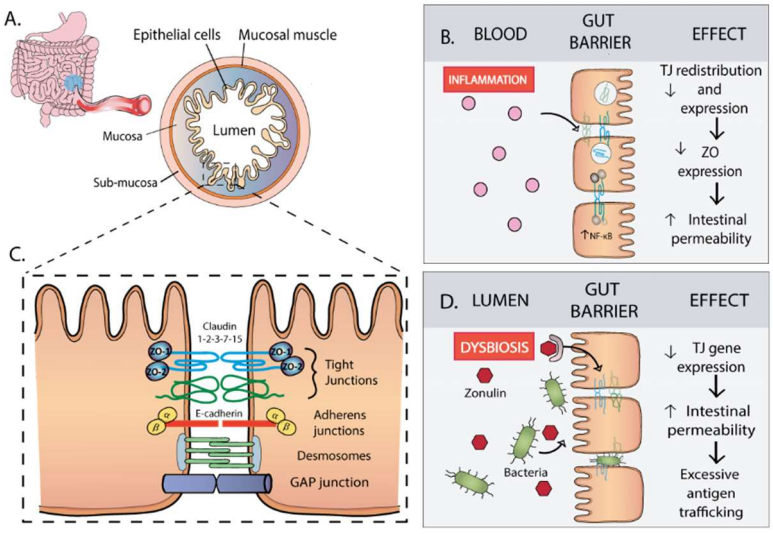

Figure 4. Intestinal barrier leakiness in MDD.
a) The gut barrier is formed by the mucosa, composed of an epithelial cell monolayer, a connective tissue layer, and the mucosal muscle (**b**). Epithelial cells maintain intestinal integrity through tight junction complexes **c**). Peripheral inflammation in MDD is linked to tight junction downregulation and redistribution **d**). MDD is associated with dysbiosis, linked to increased intestinal permeability. Together, these mechanisms may induce excessive bacterial translocation to the bloodstream and heightened pro-inflammatory cytokine production by gut-associated lymphoïd tissue, exacerbating the peripheral inflammatory response. Adapted from Doney *et al.* 2021

linking adjacent cells, mediating the extent of the various functions. Combined, junction complexes and the overlying mucus layer, maintain a healthy functional barrier which allows the passage of nutrients, water and ions, but limits entry of pathogens and bacterial toxins from the lumen [78].

The intestinal barrier is at the forefront of immune-environment interactions where the specialized cells play a critical role in maintaining health through diverse functions [71]. Not only is this separation important for limiting exposure to pathogens, but it also keeps the healthy microbiota contained, preventing dissemination to the blood and rest of the body. Specialized epithelial cells recognize bacteria-derived molecules whether they be noxious components such as LPS or peptidoglycans, or commensal bacteria-derived products like effector molecules which have beneficial action. These molecules are detected through the engagement of pattern recognition receptors like toll-like receptors (TLRs) and nucleotide-binding oligomerization domain protein (NOD)-like receptors. Activating these receptors incites cytokine and chemokine signalling for recruitment of immune cells [171]. Most often, these signalling pathway activations fluctuate to balance of the microenvironment, however, sustained reactions or particularly high noxious insults can become unmanageable allowing aggressive inflammation, pathogenic overgrowth, and downregulation of tight junctions, creating a long term “leaky-gut environment”.

The claudin family of proteins is highly involved in regulating intestinal barrier permeability, with upwards of 27 members currently recognized [172]. Claudins exert diverse functions and have different tissue expression [173]. Combinations of claudins together with other tight junction associated proteins form multiprotein tight junction complexes, the function of which is determined by the overall composition [174]. Claudin-5 is predominant in the vasculature of the brain, whereas claudins 2, 3, 7, and 15 are the most highly expressed in the intestine [175]. The exact function of intestinal claudins remains elusive. Broadly, tight junctions are grouped into "tightening" or "leaky" categories, which depict the main functions of sealing the barrier or forming charge-selective paracellular channels, respectively. Tightening claudins include claudin-1, -3, -4, -5, -6, -8, -12, -18, and -19 [176–179], while leaky claudins consist of claudin-2 and claudin-15 [180–182]. Specific function of claudins and their role in pathogenesis in respective tissues is still poorly understood. Some barrier proteins are

expressed in both the BBB and the gut barrier, such as zonulin (pre-haptoglobin-2), zonula occludens (also known as tight junction protein or Tjp), occludins (Ocln), and potentially Cldn1 and Cldn3, though the presence of the latter in the BBB is still debated [178,183–185]. Therefore, shared mechanisms related to their influence on increased permeability could be occuring.

2.2 Microbiota and gut-brain axis signalling

The microbiota critically maintains health in numerous ways, including the facilitation of nutrient absorption, mediating immune functions, and providing a barrier to prevent pathogens from entering into circulation [71]. Generally, a diverse microbiota is associated with the integrity of the epithelial barrier [122] and preservation of intestinal metabolic and immune homeostasis [186]. The microbiota influences the brain through the GBA, involving the inflammatory/immune axis, the HPA axis, enteric nervous system to CNS signalling and modulation of neurotransmitters and other bioactive molecules such as monoamines and brain-derived neurotrophic factor (BDNF) [187].

A prominent role for the microbiota is the development and maintenance of the intestinal barrier through the production of metabolites that serve as an energy source for epithelial cells and modulation of tight junction expression. Thereby, microbiota richness and diversity are critical for maintaining dynamic functions as each species plays an essential role. Indeed, a microbiota comprising high species diversity is linked to better health [122,186,188]. Commensal species break down carbohydrates to form short-chain fatty acids (SCFAs): acetate, propionate, and butyrate. SCFAs provide an energy source as well as exert local and systemic immunomodulation [189]. Hence, SCFAs boost intestinal epithelial cell integrity by enhancing anti-inflammatory cytokine production [190], modulating the mucous layer [191] and activating regulatory T cells [192]. In healthy humans, predominant bacterial phyla include *Firmicutes*, *Bacteriodetes,* and *Actinobacteria* [193]. Commensal *Firmicutes* such as *Faecalibacterium prausnitzii* (*F. prausnitzii*) and *Eubacterium rectale/Roseburia* spp., produce the most butyrate in the human intestine [194], while *Bacteroidetes* are mainly responsible for making acetate and propionate [189].

Through the GBA, commensal microbes can also influence the central immune system, such as microglia proliferation and function [195]. Microbiota depletion in animal models by

antibiotics or in germ-free mice compromises microglia maturation and ability to respond to microbial pathogens such as endotoxins [195]. Reversal can be seen by reinstating a healthy microbiota or by supplementation with butyrate [195]. GBA communication to microglia may be occurring by two predominant routes, the vagus nerve or signalling molecules such as SCFAs in the peripheral circulation [196,197]. The mechanisms by which the microbiota and GBA signalling influence microglia phenotypes and activity in the CNS are complex systems that are not well understood. However, this is a promising direction for research unraveling how stress-induced dysbiosis, the altered gut microbiota, could promote low-grade systemic and central inflammation.

2.3 Intestinal Dysfunction

2.3.1 Gastrointestinal disorders

A compromised intestinal barrier function is a hallmark of IBD, as well as other GI and metabolic disorders. Altered protein expression at the junctional complexes such as claudins are at the center of barrier dysfunction, resulting in increased paracellular and transcellular permeability of the epithelial layer [174] (**Table 2**). Gut barrier permeability or "leaky gut", may increase the interaction of antigens with immune cells of the lamina propria, encouraging robust pro-inflammatory responses. LPS is a product of the cell wall of gram-negative bacteria and is a well-known endotoxin. Evidence points to increased endotoxin levels in circulation in IBD cases, hinting that enhanced intestinal permeability in these patients increases bacterial translocation and endotoxemia [198,199]. Indeed, macrophages containing *E. coli*, *Listeria* and *Streptococci* and their products were found in the lamina propria and mesenteric lymph nodes of patients with Crohn's disease, indicating a barrier breach [200]. Mucosal breaches may progress the pathology to the gut vascular barrier and into circulation promoting systemic inflammation.

Indeed, IBD patients have increased local inflammation in the intestine but also increased markers of systemic inflammation. Heightened circulating cytokines such as IL-1β, TNFα, IL-6, IFNγ [66,201] among others, occur in ulcerative colitis and Crohn's disease. Similar to the impacts on the BBB, upregulated pro-inflammatory cytokines can be implicated in increased intestinal permeability [56], propagating the response through local effects on tight junctions. Pro-inflammatory cytokines TNF-α and IL-1β downregulate tight junction protein 1 (Tjp1)

through activation of the NF-κB pathway and subsequently increases intestinal paracellular permeability [202,203] (**Fig 3)**. IL-6, IFNγ and TNF-α have been shown to regulate *CLDN2* expression [204,205] whereas the two latter can abate *CLDN3*, and redistribute *CLDN4* [206,207], *CLDN1*, *TJP1* and *OCLN* [206] with marked increase in the gut paracellular permeability. Many studies have identified claudin fluctuations in specific intestinal disorders (**Table 2;** [175]), however, the precise mechanisms by which claudins' expression and localization is regulated in the intestinal epithelium during pathological states remains unclear.

Disease	Claudin changes
IBD	Claudin 1↓
Ulcerative colitis	Claudin 2↑, 7↓
Crohn's disease	Claudin 2↑↓, 4↓, 5↓, 8↓, 12↓↑
Celiac disease	Claudin 2↑,3↓↑, 5↓, 7↓, 15↑

Table 2. Claudin expression changes in the intestines associated with gastrointestinal diseases.
Adapted from Lu et al. 2013.

2.3.2 Microbiota dysbiosis in gastrointestinal disorders

Disrupted microbiota homeostasis triggers downstream signalling pathways at the intestinal epithelium to the lamina propria beneath where immune cells elicit a pro-inflammatory response [78]. Dysbiosis in IBD patients presents as an overall loss of alpha diversity which is a measure of species richness and variety [208]. Indeed, decreased composition of the phyla *Firmicutes* and *Bacteroidetes* occurs, leaving a higher ratio of *Proteobacteria* and *Actinobacteria* [209]. Depleted 'healthy' microbes *Bifidobacterium* and *Lactobacillus* species are reported with proliferating invasive species such as *Escherichia coli* and *Enterococci* [209]. Dysbiosis profiles of GI disorders are linked to pathogenesis of the disease by promoting intestinal inflammation and permeability, upregulating mucosal adherence and invasion, microbial translocation, inefficient clearance of invasive bacteria and loss of immunologic tolerance to commensal bacteria [210].

Patients with active IBD have lower SCFA producers, such as species of the order *Clostridia* and genus *Bacteroides* [208], and lower SCFA concentrations in fecal extracts [211,212]. SCFAs modulate genes involved in tight junction expression, regulates epithelial cell proliferation, differentiation, and mediates the intestinal inflammatory response [189]. Specifically, SCFAs

are ligands for G-protein coupled receptors that activate anti-inflammatory signalling cascades [189].Therefore, limited SCFA production impairs pathways implicated in barrier permeability and may play a role in the establishment of aberrant immune responses in IBD patients. Indeed, altered tight junction expression is reported in colon biopsies from Crohn's disease patients with upregulated CLDN2 and downregulated CLDN5 and CLDN6 [213]. Furthermore, butyrate induces tight junction encoding genes through transcription factor signal transducer and activator of transcription 3 (STAT3) and Specificity Protein 1 (SP1) [189], therefore a lack of butyrate-producing species may contribute to increase barrier permeability by limiting activation of this pathway.

The gut microbiota is also sensitive to stressors [214] and HPA axis activation can influence population densities [215]. In mouse models, chronic stress alters microbiota diversity [216] and promotes intestinal permeability [217]. The resulting dysbiosis upsets colonization resistance, the ability of commensal bacteria to resist the expansion of opportunistic pathogens [218]. Overexpansion of pathogenic strains suppresses healthy species populations and overall biodiversity [219–221], which is associated with negative health issues [188] including reduced intestinal barrier integrity [122,186]. Glucocorticoid secretion resulting from HPA-axis activation increases gut permeability, promoting recruitment of Helper T cells as well as other immune cells to interact with bacteria in the mucus layer and produce pro-inflammatory cytokines [68]. Aberrant stress response is suspected to play a role in the development and relapse of IBD patients, contributing to disruption of host-microbial balance, intestinal permeability, and uncontrolled flux of antigens across the intestinal barrier challenging the peripheral immune system.

2.4 Microbial antigens and intestinal tight junctions

Many pathogenic microbial species have the capacity to compromise tight junctions either by direct interactions or translocating through the epithelial cell membrane to target downstream signalling pathways. Indirectly, microbial antigens can trigger a pro-inflammatory response with repercussions for tight junction expression. Certain opportunistic pathogens modulate CLDN2 expression to alter the environment in their favor. Cldn2 is a component of ion channel complexes that allows the passage of water and small cations, while preventing the passage of anions and large molecules [222]. Cholera toxin (CT)

from species *Vibrio cholerae* (*V. cholerae*) and shiga toxin from pathogenic *E. coli* gain access to cells and disrupt ion flux by binding Cldn2 [223]. Exposing cells *in vitro* to CT upregulated Cldn2 expression, decreased transepithelial resistance (TEER) and facilitated further antigen absorption [224]. These mechanisms could be at work in IBD pathology as inflated Cldn2 expression in the jejunum (JEJ) was identified in these patients [225]. Cldn2 dysfunction is also believed to play a role in intestinal food allergies in patients and intestinal antigen-specific hypersensitivity in mice [224]. Similarly, another species, *Clostridium perfringens* enterotoxin, binds directly to CLDN3 and 4 with detrimental outcomes. This toxin joins the tight junction complex to modulate the pore function, ultimately allowing uncontrolled influx of calcium and compromising the paracellular barrier [225].

LPS from members of gram-negative enterobacteria are also known to be immunogenic [124,226] with implications for barrier permeability through the zonulin pathway. Zonulin is a protein that regulates endothelial and epithelial permeability [123,227,228] and therefore regulates permeability of both the BBB and the intestinal barrier [229]. Zonulin acts by dissociating Tjp1 and Ocln from the junctional complex [228,230,231], which, among other effects, can mediate the transport of paracellular antigens [232]. The zonulin analog, zonula occludens toxin (*Zot)* is an enterotoxin produced by *V. cholerae* [233], which can activate these pathways, dissociating tight junction protein complexes in intestinal epithelial cells [234]. Other enteric pathogens, including *E. coli* and *Salmonella typhi* can release zonulin from the intestine [235]. Considering zonulin is also expressed in the BBB, circulating levels of these toxins achieving the peripheral could influence permeability there as well. Indeed, in mice, imbalances of healthy gut microbes are associated with increased permeability of the BBB [236] indicating that perhaps, inflammatory gut responses can even trigger BBB permeability, promoting depression behaviors. Therefore, stress-induced dysbiosis may compromise the intestinal barrier, feeding into a loop of exacerbated pro-inflammatory environment [237].

3 Gut Barrier dysfunction and potential mechanisms (in MDD)

3.1 Intestinal dysfunction and dysbiosis in MDD

Similar inflammatory pathologies appear in MDD and GI disorders, characterized by low-grade systemic inflammation, increased intestinal permeability [238] and altered gut microbiota [239,240]. Reportedly, the prevalence of depression is 40% higher in patients with IBD [67].

Approximately 49% of people with IBD suffer from depressive symptoms [241] and symptomatic IBD patients have the highest depression and anxiety scores compared to non-active patients. Furthermore, psychological score were coincident with heightened intestinal expression of IL-1β and IL-6 and extracellular matrix protein, MMP-9 [66]. Additionally, fMRI studies of patients with IBD demonstrate aberrant brain function in emotion processing and regulation regions [242,243] reflecting changes seen in patients with MDD [27,140,244,245]. Specifically, in IBD patients with high anxiety symptom severity, diminished functional connectivity occurs between the medial PFC with the ACC, a core node of the limbic system with a unique integrative role in emotion regulation [243].

In MDD, dysbiosis, or altered gut microbiota, has been reported [239,246,247] along with increased intestinal permeability [238] (**Fig. 2D**). Reduced alpha diversity and shifts in prominent species also occurs [248,249], some of which correspond to reports in inflammatory GI disorders [211,250]. Depressed patients have expanded populations of predominant phylum: *Bacteroidetes, Proteobacteria* and *Actinobacteria,* compared to healthy subjects and many further transformations at lower taxonomic ranks [251]. These changes may have negative downstream action on the GI system as assessment of microbiome in MDD patients, KEGG (Kyoto Encyclopedia of Genes and Genomes) analysis, highlighted enriched pathways related to pathogenic enterotoxins [237]. For MDD, distinct signatures within global communities are associated with specific symptoms [239,252,253]. Hence, treatments for re-establishing commensal populations could be beneficial for mood symptoms both retroactively and as prophylaxis.

3.2 Peripheral serotonin

Approximately 95% of all 5-HT is found in the gut, produced mostly by enterochromaffin cells and certain microbial species [252,254]. TRP is the sole precursor of 5-HT; however, more than 90% of the dietary source is degraded through the pre-dominant kynurenine (KYN) pathway [255]. The KYN pathway is strictly immune-related and evidence suggests that stress and inflammation increase KYN production by activating TRP-degrading enzymes, diverting away from 5-HT synthesis [256–258]. Supporting this hypothesis, IBD patients have higher circulating KYN:TRP ratio [66]. Hyper-function of the KYN pathway and its downstream metabolites paired with TRP depletion may be implicated in mood disorder pathogenesis as

it is other stress-related disorders but specific roles are still unclear [58]. KYN and other TRP metabolites can activate the aryl hydrocarbon receptor (AHR), resulting in immunoregulatory effects such as regulatory T-cell induction that suppresses inflammation [259,260]. Therefore, KYN upregulation may have a protective purpose during acute stress, though overextending these pathways, resulting in TRP depletion could have negative influence in the context of chronic stress. Many studies have found low circulating TRP in MDD patients with connections to emotional and cognitive symptoms [261–263] such as suicidal ideation [264]. KYN pathway metabolites have different neuroactive potentials in the brain, linked to neuro-toxic or neuroprotective developments [265] and this balance can be reflected by metabolite ratios in the blood and thus TRP pathways' activity [266]

It is suggested that depleted serum TRP levels leads to immune activation in inflammatory disorders through Indoleamine 2,3 dioxygenase-1 (IDO-1) [267], **Fig. 5**. IDO-1 is primarily expressed in innate immune cells and is the main rate-limiting enzyme in TRP to KYN conversion and further downstream metabolites [267]. Elevated IDO-1 expression modulates the TRP catabolism pathway to favor KYN synthesis over 5-HT in the periphery and brain [263]. Human IDO-1 is highly upregulated in the gut epithelium during inflammation, injury, and infection [268], induced by pro-inflammatory factors [269] or directly by LPS [270,271]. Furthermore, KYN levels increasing along with inflammatory factors in the cerebral spinal fluid have a positive link to depressive symptoms [257]. This evidence highlights the relationship between TRP/KYN metabolites and the immune system in the manifestation of mood symptoms. Indeed, low serum 5-HT is detected in MDD patients in conjunction with higher IDO-1 and amplified pro-inflammatory cytokines [272]. Interestingly, SSRI treatment, reduced concentrations of IDO-1 which positively correlated with symptom improvement [272]. Therefore, IDO-1 function is proposed as a mechanism connecting inflammation and TRP in the context of MDD (**Fig. 5**). However, more work to confirm mixed findings on peripheral IDO-1 expression or KYN pathway activity in MDD [261,262], as this suggesting a missing link in the underlying mechanisms.

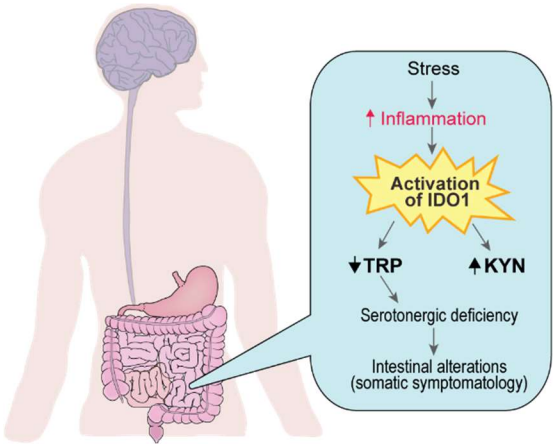

Figure 5. Tryptophan metabolism alterations by stress in MDD.
Modified from Doney et al., 2021.

3.3 Peripheral markers of gut dysfunction and link with MDD

Markers of gut dysfunction have also been noted in MDD [121–123,273–275]; as summarized in **Table 3**. Serum levels of intestinal cell proteins can indicate gut damage, such as Intestinal Fatty Acid binding protein (FABP2) [70,276], which is augmented in GI disorders with levels correlated with antibodies to bacterial products LPS and flagellin [276]. Increased levels have also been implicated in MDD [121,122] (**Table 3**) and been associated with predicting better clinical outcomes [277]. Yet, limitations exist for these markers, for lack of spatial and temporal specificity [70,276]. Otherwise, elevated serum zonulin in patients with GI disorders coincides with intestinal tissue expression [278,279]. Levels are also significantly mounted in patients with MDD and anxiety [123] (**Table 3**). However, disputes of the tissue and protein specificity of current tests limit the application of these measurements at this time [280,281]. Still, it will be interesting to compare in future studies circulating tight junction and cytokine profiles along with brain functional assessments to possibly reveal commonalities between gut disorders and MDD.

Bacteria in the external environment as well as the internal intestinal environment constantly shed LPS fragments. The intestinal barrier is constantly patrolled by immune cells to maintain homeostasis and protect against infection. Indeed, LPS elicits a strong immune reaction and can trigger pro-inflammatory and microbicidal responses through activation of TLRs on immune cells [282]. Specifically, toll-like receptor 4 (TLR4) is well-known for recognizing LPS along with co-receptor cluster of differentiation 14 (CD14). Binding of the

immune complex with LPS initiates downstream pathway activation of transcription factor NFkB, upregulating production of TNF-α and IL-1β, as well as VCAM-1 and ICAM-1 [283]. Increased permeability in the gut is proposed to allow higher levels of translocation of microbial products into the periphery. Indeed, serum detection of specific microbes by testing for pathogen-associated molecular patterns has proven insightful in GI disorders [284–286]. Proliferating pathogens may be entering circulation in MDD as clinical trials have found higher serum levels of LPS in patients [123,287] and in women with post-partum depression [288]. Furthermore, high levels of circulating IgM and IgA directed to pathogenic enterobacteria are related to symptoms of fatigue, autonomic, and GI symptoms [124]. Serum levels of endogenous immune responses to microbes provides an alternative to be tested, however, CD14 can be released by immune cells via non-LPS dependent mechanisms. Peripheral and central TLR4 levels have been found to be elevated in MDD patients [48] and related to levels of anxiety [289], however, elevated levels are implicated in many pathologies[246]. Finally, lipopolysaccharide binding protein (LBP) [123,284,290], indicates immune activation secondary to microbial translocation [291]. LBP activation of TLRs leads to cytokine production through the NOD2 pathway. LBP is reported to be elevated in IBD patients[198],[292] and in MDD patients [121].

Type	Markers	Changes (MDD)	Description	References
Intestinal proteins	FABP-2	↑	Plasma biomarker of gut epithelial cellular dysfunction.	117,119,275,291
	Zonulin	↑	Modulatory protein of TJ permeability	119
		—		118
	TFF3	↑	columnar epithelium, reflects mucus layer thickness and epithelial healing rates	110
	Citrulline	↓	nonessential amino acid synthesized by enterocytes of the small intestine	271,292
Microbial toxins	LPS	↑	Endotoxin	119,280,281
Immune proteins	LBP	↑	Soluble PRR molecule Marker of microbial translocation	117,291
	LPS-specific Ig	↑	Antibody to endotoxin	273,293
	ASCA	↑	Antibodies to *Saccharomyces cerevisiae* (commensal intestinal organism) Increased levels related to inflammation.	294
	CD14, sCD14	—	Marker of LPS-induced monocyte or macrophage activation	118,272

Table 3. Serum intestinal permeability markers associated with MDD.
Abbreviations: ASCA, Anti- Saccharomyces cerevisiae antibody; CD14, cluster of differentiation 14; FABP-2, Fatty Acid-Binding Protein 2; IgA, Immunoglobulin A; LPS, lipopolysaccharide; LBP, lipopolysaccharide binding protein; PRR, pattern recognition receptor; sCD14, soluble CD14; TJ, tight junction; TFF3, Trefoil factor 3.

3.4 LPS and low-grade systemic inflammation

LPS treatment causes expression of certain depression-like symptoms in rodents [293] and is clinically relevant to human depression [121]. A single injection of LPS induces symptoms of anorexia, reduction of locomotion and social interaction, paralleling event in chronic stress models [53]. Administration of LPS is frequently applied in neuroinflammation-associated diseases studies in mice [53]; ergo this model enables a focus on the inflammatory-related pathways associated with depression-like behaviors [52,294–296]. Indeed, in mice, intraperitoneal LPS administration significantly upregulated pro-inflammatory cytokines and chemokines, including IL-1α, IL-1β, IL-9 and MCP-1 in the blood and the PFC, HIPP and striatum [297]. Furthermore, LPS is linked to BBB disruption as LPS administration enhances uptake of tracer molecule 99mTc-DTPA in mice, along with brain levels of pro-inflammatory cytokines associated with innate immune cell trafficking [168,297,298]. However, the BBB is relatively resistant to impacts of LPS on permeability, with some brain regions being more vulnerable than others. Only high doses (>3 mg/kg) induce BBB damages [297] as measured by permeability of small molecule 14C-sucrose (340 Da) and three peripheral injections of 3mg/kg LPS significantly and consistently permeabilizes the BBB to large molecule albumin as well [168].

In humans, intravenous or intramuscular injection of LPS alters mood and produces related behavioural symptoms in humans [41], implicating peripheral LPS in cognitive symptoms through inflammatory actions on the brain. A PET imaging study shows healthy humans injected with LPS have amplified uptake of translocator protein (TSPO), an inflammatory marker [299] ; and MDD patients displayed elevations of TSPO volume in PFC, correlating with depression severity and duration [300,301]. However, discordant reports do not observe this elevation, although alternate methodologies could be liable these discrepancies [302,303]. Still, bacterial translocation may impact BBB integrity directly or even be able to migrate into the parenchyma itself [304]. Mice peripherally injected with pathogenic *S. aureus* exhibit evidence of BBB uptake, with inflammation and significant levels of brain bacterial counts [305]. The microbial endotoxin peptidoglycan, can translocate from the gut into the blood and ultimately

cross the BBB [306]. Peptidoglycan triggers pattern recognition receptors and in mice, manipulation of the microbiota induced corresponding fluctuations of peptidoglycan-recognition proteins in the brain [306]. Substantiating these effects, knockout of these receptors in mice causes alterations in social behavior [306]. This is an intriguing novel concept of a pathway linking the microbiota directly to the brain, however, the mechanisms behind peptidoglycan entry into the brain is unclear. A proposed route is through extracellular vesicles, which can cross the BBB and stimulate trafficking of immune cells into the CNS [307]. LPS treatment in mice stimulates the trafficking of immune cell extracellular vesicles across the BBB [308] and differentially modulates extracellular vesicles uptake into different regions of the brain [309]. Elevated systemic LPS-positive extracellular vesicles have been detected in the serum of IBD patients [310], therefore, even though limited knowledge exists on the mechanisms of these vesicles, they could be valuable to investigate for roles in MDD.

Though common mechanisms behind barrier disruption have been receiving increasing attention, there are many unanswered questions. Stress activates inflammatory and immune pathways, as well as other GBA systems such as peripheral nerve neurotransmitters and direct neural innervation, influencing the gut in the context of stress and mood disorders. This may drive dysbiosis and lead to greater systemic inflammation. Contrarily, gut dysbiosis could be a source of inflammation that impacts the brain of individuals at risk for psychiatric illnesses. The origin and directionality of these dysregulated systems are debated however the clinical relevance remains promising. Uncovering novel pathways and mechanisms linking the BBB and/or the intestinal barrier in the development of maladaptive stress responses and neuropsychiatric disorders may elucidate new ways to bolster current treatments and promote resilience to the development of depression.

Hypotheses and objectives

Study 1: Sex-specific effects of chronic stress on intestinal barrier integrity

In this study, we compare the outcomes of chronic stress on intestinal barrier characteristics, microbiome, local and systemic inflammation, and depression-like behaviours in male and female mice. We chose chronic stress models in mice to examine how stress can influence manifestations of gut permeability through examining tight junction expression. In the social stress model, we predict that stress-induced changes will occur in tight junction expression that may impair gut barrier integrity, playing a role in vulnerability or resilience to chronic stress. Recent work from our team identified tight junction changes to the BBB in regions implicated in stress response and symptoms of depression. To compliment these findings, we propose that recent preclinical and clinical findings as well as mechanistic insights raise the intriguing possibility of a direct implication of BBB and/or the intestinal barrier in the development of maladaptive stress responses and neuropsychiatric disorders. Until recently, the BBB and gut barrier had not been compared directly, however, a study evaluating these in female rats facing social isolation stress [311]. Specifically, they demonstrated shared modifications in *Ocln*, *Tjp1* and *Cldn5* gene expression when comparing the PFC and the most distal part of the small intestine (the ileum) in females. Therefore, we expect to also see some coinciding changes between the expression in the brain and the gut for our gene targets.

We conduct our experiments in both males and female mice as we predict sex-specific effects due to our previous study [90]. Furthermore, the prevalence of MDD is two-fold higher in women, however, until recently, chronic CSDS experiments have been conducted exclusively in male mice highlighting to importance decipher sex-specific mechanisms. Sex differences in stress reactivity have been exemplified in other chronic stress paradigms [163], where female mice are more vulnerable than males and demonstrate earlier emergence of depression-like behaviors [54]. Thus, we also conducted our intestinal barrier analysis across different stress models in both sexes, to consider the potential influence of stressor type or duration.

Many pathways of interaction between the gut and the brain may be involved in intestinal barrier dysfunction during chronic stress. However, the microbiota plays an important role in mediating GBA communications on many fronts. For that reason, we characterize the

composition of the microbiota across males and females from various stress models to potentially identify any profiles related to a specific sex or stressor type. Indeed, chronic stress-induced dysbiosis allows for the proliferation of pathogenic species that can promote inflammation locally but also in the periphery. Implications of microbes themselves, microbial products or microbe metabolites have direct implication for tight junction in the gut but are also proposed to affect barrier proteins of the BBB. Therefore, based on the literature, it is hypothesized that stress transforms microbiota composition and gut-barrier integrity in a sex-specific manner, playing a role in vulnerability or resilience.

Objectives:

1. Investigate intestinal permeability of stressed mice vs. unstressed controls by focusing on expression of genes involved in permeability-related functions to those previously determined in the BBB such as tight junction proteins.
2. To study the potential role of the microbiota in mediating vulnerability vs. resilience to chronic stress by analyzing microbiome from various stress models and evaluating sex differences.

Study 2: Investigation of a novel therapeutic intervention to prevent stress -induced alteration to intestinal barrier integrity

The recent years, research for the development of nanoparticles for biomedical applications has hit with increasing popularity, with potential for treatment of leaky gut. Specifically, a gel obtained by self-assembly of a non-synthetic dairy peptide (sequence: LIVTQTMK) has been generated and studied within the laboratories of Drs. Pouliot and Doyen. This peptide has unique characteristics in that, it is very hydrophobic, soluble only between pH 2 and 3 and forms a translucent gel at basic pH (pH 7 and 11). Due to its non-conventional properties, it is thus possible to consider using this peptide as a protective agent for the natural intestinal wall. In this study, we contribute to the investigation of this peptide treatment by testing the therapeutic potential in chronic stress animal models of depression. We attempt to support the structure of the gut by adding this treatment to the diet of the mice experiencing the chronic stress paradigms. By restoring gut integrity, we attempt to promote resilience to stress by reducing peripheral inflammation and downstream repercussions on the brain.

Sex differences in stress reactivity to various types of stressors prompted the decision to conduct our peptide trials in a CSDS cohort males while complimenting it with a SCVS model in females to optimize the efficiency of the first rounds of testing this treatment. First, we confirm behavioural responses of the male cohort following CSDS in the SI test to divulge any potential behavioural consequences from consuming the peptide treatment. We expect that if consuming the peptide can negate any negative effects of chronic stress on the gut, that we may see a ratio higher in resilient mice compared to susceptible in the treatment group of this cohort. Whereas any adverse impacts of the treatment may be displayed as increase anxiety/ depression-like behaviour. Next, we characterize the tight junctions and other genes related to barrier integrity in the JEJ, identified from study 1, in these cohorts to determine any treatment-related responses. Indeed, if this treatment coats the intestinal wall thereby supporting it against any stress-induced permeability we expect that gene expression changes identified from study 1 will be countered in this study for the animals receiving the peptide treatment.

Previously, studies from our group have identified BBB changes in mood-related brain regions in male and female mice following chronic stress [90]. Specifically, the NAC and PFC feature alterations in the BBB depicting leakiness. There are also sex-specific regional outcomes as males experienced more modifications in the NAC while female changes were predominantly PFC. These results prompted us target the NAC and PFC for transcriptional profiling of genes in our cohorts of mice receiving peptide treatment. We expect the previously identified gene alterations will be replicated and potentially display treatment effects if the treatments indeed alter behaviour and/or have intestinal integrity promoting outcomes.

Consequences of stress on the gut such as barrier dysregulation and dysbiosis, could lead to unwanted circulating molecules in the blood and triggered immune response. Thus, we examine a potential marker of gut permeability, lipopolysaccharide binding protein (LBP) in our male and female models. We expect that this marker will be promoted by experiencing chronic stress. Further we expect to see minimized signs of intestinal permeability in peptide supplemented stressed mice, which will be reflected by reduced circulating levels of LBP. Elevated serum inflammatory markers have previously been reported following various

mouse models of depression [63,312], therefore we also examined serum in male mice after social defeat stress for a panel of inflammatory markers to potentially identify any other indirect markers of aggravated inflammation that may be subsided through successful treatment with the peptide polymer.

Objectives:

1. Explore potential treatment outcomes on expression of genes involved in permeability-related functions in the JEJ.
2. Confirm previously highlighted BBB tight junction related changes in the NAC and PFC of stressed mice and identify any potential downstream effects of peptide treatment.
3. Investigate gut-related biomarkers as novel targets in the context of chronic stress.

Methods

Animals

Experimental mice were naïve male (~25g) and female (~20g) C57BL/6 mice, 7–9 weeks of age at arrival (Charles River Laboratories, Québec, Canada). Sexually experienced retired male CD-1 breeders (~40 g), 9-12 months of age were used as aggressors (AGG), residents for the social defeat procedures (Charles River Laboratories, Québec, Canada). All mice were group housed in 27 × 21 × 14 cm polypropylene cages upon their arrival and left undisturbed for one week of acclimation at the housing facility of CERVO Brain Research Center prior to any procedures commencing. Mice maintained on a 12-h light–dark cycle (lights on from 0800 to 2000 h) with temperature (22 °C) and humidity (63%) kept constant were provided free access to water and food (Teklad Irradiated Laboratory Animal Diet, Madison, USA). All experimental procedures were approved by the animal care and use committee of Université Laval (2018-052) and met the guidelines set out by the Canadian Council on Animal Care.

Chronic Social Defeat Stress

As previously described [151], in the CSDS model, a C57BL/6 mouse is repeatedly subordinated by an AGG mouse for daily bouts of social stress. Before the experiment, AGG mice are screened for aggressive behaviours against a C57BL/6 mouse for 3 days. Then the AGG is designated a social defeat cage (26.7 cm width × 48.3 cm depth × 15.2 cm height, Allentown Inc.) separated in half with a clear perforated Plexiglas divider (0.6 cm × 45.7 cm × 15.2 cm). The experimental C57BL/6 mice are placed in the home cage of an unfamiliar CD-1 male for bouts of physical stress lasting 5 minutes daily over a period of 10 consecutive days. After each physical stress period, the mouse is returned to the other side of the clear plastic divider, leaving the experimental mouse exposed to overnight sensory contact with the AGG mouse. Control animals were housed 2 per social defeat cage, one each side of the Plexiglas divider and kept in the same room as experimental mice. After the last day of social defeat, the experimental mice are single-housed for 24 hours before conducting the social interaction (SI) test.

In the female CSDS paradigm, the procedure is adjusted as described by Harris *et al*. [165]. Before each defeat, ~30ul of urine collected from a CD-1 male was applied to the tail base

and vaginal region of the female mouse. The odour from the urine induces dominant behaviour from the AGG mouse. For urine collection, CD-1 mice were placed in metabolic cages (Life Science Equipment) during the dark phase of the light/dark cycle. Urine was collected the following morning, filtered, aliquoted in 0.5 mL tubes and stored at -80°C until use.

Social Interaction Test

Following CSDS, mice are characterized for vulnerability to stress by way of SI test to establish behavioural phenotypes [151]. In this test, the mouse's propensity to socialize is evaluated. In two trials, this test assesses exploratory behavior of mice in an open field, first alone and then in the presence of an unfamiliar CD-1 mouse contained in a small wire cage within the open field (42 cm x 42 cm x 42 cm, Nationwide Plastics). Movements are tracked by an automated system (AnyMaze™ 6.1, Stoelting Co.) during each 2.5-minute trial. The time spent in the interaction zone, the region surrounding the small cage, compared to the rest of the arena is assessed. The SI ratio is the score obtained by dividing the time spent in the interaction zone with AGG present divided by the time when AGG is absent. Equal time spent when in Trial 1 and Trial 2, giving an SI ratio of 1, is typically used as the cutoff point, dividing those mice with a ratio below 1.0 to be classified as stress-susceptible (SS), while mice with a ratio above 1.0 are considered resilient (RES) [151].

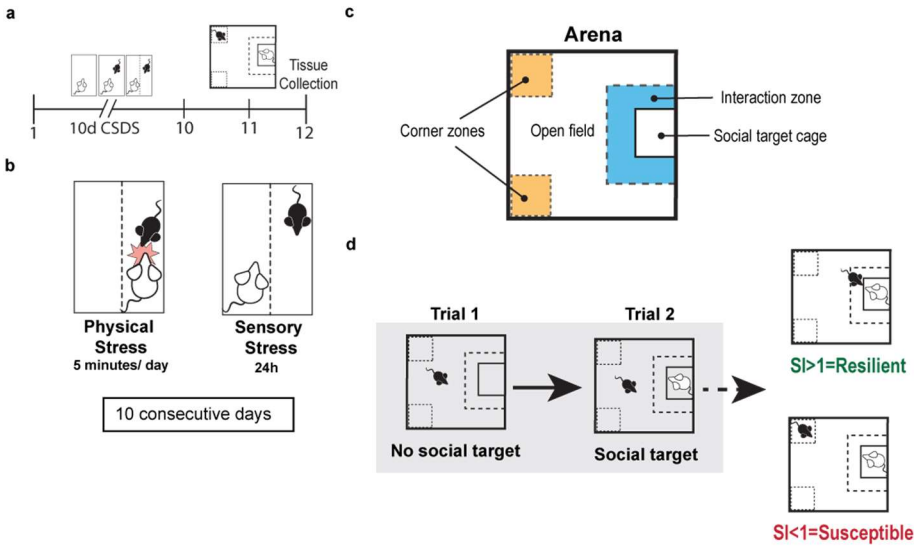

Figure 6. Chronic social defeat paradigm and social interaction test procedure
a) Timeline of social defeat paradigm, social interaction test and tissue collection. **b)** Experimental mice spend 5 mins/day subjected to physical stress by an aggressor mouse. **c)** Arena used for social interaction test highlighting the different zones. **d**) Mice are recorded for exploratory behaviour in the social interaction test in two trials: to determine behavioural phenotype.

Chronic Variable Stress

As previously described [159], mice are exposed to a series of alternating variable stressors (restraint stress, tail suspension, foot shocks) for 1 hour per day unpredictably for 28 days. Stressors were administered as follows: 100 mild foot shocks of 0.45mA at random intervals for 1 h (10 mice/ chamber), a tail suspension stress for 1h and restraint stress, where animals are placed inside a 50ml falcon tube, for 1h within the home cage. After day three the stressors restart with foot shocks and cycles through in this pattern for 28 days.

Subchronic Variable Stress Model

The subchronic variable stress model (SCVS) paradigm consists of the same procedure as CVS but for a total duration of 6 days only. Female C57BL/6 mice were used as experimental mice and stressors were administered as previously described for the CVS paradigm. Stressors were administered daily for 6 days, as follows: 100 mild foot shocks for 1h (days 1 and 4), a tail suspension stress for 1h (days 2 and 5) and restraint stress for 1h (days 3 and 6).

Peptide treatment

Two cohorts of animals: 1 group of male 10d CSDS (**Fig. 6a**) and 1 group of female SCVS (**Fig. 6b**) received a dietary supplement of a non-synthetic dairy peptide in gel form throughout stress paradigm. The peptide treatment is mixed into a neutral hydrogel (ClearH2O; Hydrogel, 70-01-1062) at a dose of 200mg/kg. Water was withdrawn from the cage so that mice will opt for hydrogel as their main source of hydration. Mice are fed a neutral hydrogel with or without (vehicle/treatment control group) peptide treatment for the duration of the stress paradigm and until the day of sacrifice. Treatment commences 6 days prior to the first stress exposure as an adjustment period. Mice are monitored daily for body weight, physical state, and consumption of hydrogel.

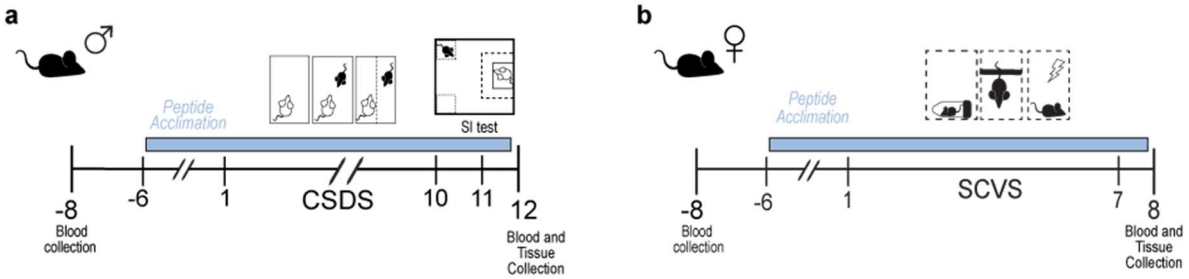

Figure 7. Timelines of CSDS male and SCVS female paradigms receiving peptide

Serum, tissue, and feces collection

Blood and feces samples were collected 48h before the start of the stress protocol. Blood was collected by the submandibular bleeding method and one or two fecal pellets were collected from each mouse by voluntary defecation into a sterile microtube (Eppendorf, Germany). Fecal samples were immediately placed on dry ice during collection and blood was left at room temperature in nuclease-free microtubes for 1h before processing for serum extraction. Blood samples were centrifuged for 2 min at 10 000 RPM at which point the separated serum was collected and transferred into a new tube. This process is repeated with centrifugation at 10 min at 3000 RPM and supernatant was collected. Serum was stored at -80°C for subsequent determination of protein levels.

Blood, feces, and tissues from the same mice were collected 24h (for SCVS or CVS) or 48h (for CSDS) after the last stressor. Trunk blood was immediately collected after euthanasia by rapid decapitation. Post-sacrifice, the small intestine is removed, placed in a petri dish on ice and cut into 3 sections. The most anterior portion and distal section is discarded, and the mid-section (jejunum) is kept and flushed with 0.1 M phosphate-buffered saline (PBS 1X). Two biopsies were taken with Unicore 2.00mm punch (Harris, 7093508) from intact intestinal segments, placed immediately in nuclease-free microtubes on dry ice and stored at −80 °C for subsequent analysis. The remaining intestinal segment is prepared for subsequent protein analysis following a modified version of the swiss roll protocol [313]. Briefly, intestinal segments were cut open longitudinally along the mesenteric line. Tissue is rolled over a wooden skewer with mucosa facing outwards and then transferred into a tissue mold filled with Tissue-Tek® O.C.T. Compound (Sakura, NC1862249) to embed samples. The tissue is snap frozen in isopentane on dry ice and stored at -80°C.

Additionally, from the mice who underwent chronic stress paradigms with peptide treatment, tissue was collected from brain regions for transcriptional profiling. Brain samples from the NAC and PFC were collected and processed as described previously [32]. Briefly, bilateral 2.0mm punches were collected from 1mm coronal slices on wet ice after rapid decapitation and immediately placed on dry ice and stored at –80 °C until use.

Transcriptional profiling of mouse tissue

Quantitative polymerase chain reaction (qPCR) using SYBR green chemistry was performed on JEJ tissue samples for evaluation of gene expression of targets related to intestinal permeability, such as tight junctions, tight junction associated proteins and inflammatory markers. Total RNA was extracted with TRIzol (Invitrogen, 15596026) homogenization and chloroform layer separation. Tissues were processed using PureLink® RNA Mini Kit (Invitrogen, 12183018A) following manufacturer's protocol for Purifying RNA from Animal Tissues. Yields and purity (ratio of absorbance at 260 and 280 nm) of extracted RNA was assessed by NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific, ND-2000). Complementary DNA (cDNA) was synthesized using Maxima™ H Minus cDNA Synthesis Master Mix, with dsDNase (Thermo Fisher Scientific, M1681) from 5µg of RNA. The cDNA was applied as a template for qPCR reaction using PowerUp SYBR Green Master Mix (Applied Biosystems, A25742) containing ROX™ Passive Reference Dye. QPCR was performed with Applied Biosystems QuantStudio 5 Real-Time PCR System (ThermoFisher Scientific, MA, USA). Oligonucleotide primers are listed in **Table A1** and primers that amplify *Actb* and G*apdh* were used as reference genes. Analysis was done by way of the ΔΔCt method.

Microbiome Analysis

Total DNA was extracted from fecal samples using a Stool DNA Isolation Kit (Norgen Biotek, 27600) following manufacturer's protocols. Extracted DNA yields and purity were assessed by a Qubit Fluorometer (Invitrogen, Q33238) and only samples with high purity (absorbance ratios A260/A280 greater than 1.8 and A260/A230 greater than 1.6) were kept for downstream analysis. The V3 and V4 hypervariable regions of the 16S ribosomal RNA gene (16S rRNA) were amplified from bacterial DNA and sequenced using a MiSeq Illumina system. For analysis of microbiome data of each mouse, QIIME 2.0 [314] was used to initially process the data, followed by MicrobiomeAnalyst to calculate diversity indices using methods as previously described [315]. The parameters evaluated were alpha diversity, number of taxonomic units within the ecosystem, represented by Chao1 and Shannon indices and beta diversity, difference in the abundance of taxonomic units between samples ecosystems.

Immunohistochemistry of Cldn3

Swiss rolls of JEJ tissue samples from male CSDS mice were sectioned on a cryostat

(CryoStar™ NX50 cryostat, Thermo Scientific), cut into 7 µm thick sections at −17°C and mounted on Superfrost Plus slides. Slices were rinsed in PBS 1X and incubated for 30 mins in blocking solution, consisting of 10% normal donkey serum in PBS 1X. Slides were incubated overnight at 4°C with primary antibodies (**Table A2**) in solution, 1% bovine serum albumin (Life Sciences, SH3057401) and 0.01% Tween 20 (Fisher BioReagents, BP337-100) in PBS 1X. The slices from male mice were double stained with primary antibody CD326 for visualization of epithelial cells and tight junction Cldn3. Sections were washed three times with PBS 1X and incubated with secondary antibodies (see **Table A2)** for 1h at room temperature. Slices were washed three times in PBS 1X and stained with 4′,6-diamidino-2-phenylindole (DAPI) for nuclei visualization. Finally, slides were mounted with ProLong Diamond Antifade Mountant (Invitrogen, P36961), and cover slipped. Six 0.5µm thick *z*-stack images were acquired using an Axio Observer.M2 microscope (Carl Zeiss) with a 20X objective. Processing of images was done with Imaris (Bitplane, Zurich, Switzerland) for volume quantification and intensity colocalization. To measure the intensity of Cldn3 expression within a region of interest in the intestinal epithelium, solid surfaces were created based on CD326 fluorescence.

Two JEJ tissue swiss roll slices per female mouse were double stained with primary antibodies for Cldn3 and actin filaments (F-actin) with the same protocol as described above. Ten 0.250 µm thick z-stack by six tiles were acquired with a 40X lens. Images were imported into Imaris for 3D reconstruction. For volume quantification of Cldn3 expression within the region of interest in the intestinal epithelium, solid surfaces were created based on Cldn3 fluorescence and F-actin fluorescence. Masks were made of the volume renderings and colocalization analysis was performed.

ELISA and Multiplex assay of LBP and inflammatory markers

The quantitative detection of serum lipopolysaccharide binding protein (LBP) was performed by Mouse LBP ELISA assay (Abcam, ab213876) according to the manufacturer protocol. Serum was diluted 1:100 for LBP detection, measured on an Eon Microplate Spectrophotometer (BioTek Instruments Inc., Winooski VT) and calculated from a serial dilution curve using Gen5 Data Analysis Software. Serum levels of inflammatory molecules were determined by cytokine multiplex assay (Bio-Rad, M60009RDPD) according to

manufacturer's protocol. The Bio-Plex murine cytokine kit measures 23 cytokines: IL-1α, IL-1β, IL-2, IL-3, IL-4, IL-5, IL-6, IL-9, IL-10, IL-12 (p40), IL-12(p70), IL-13, IL-17, eotaxin (CCL11), G-CSF, GM-CSF, IFN-γ, KC (CXCL1), MCP-1 (CCL2), MIP-1α (CCL3), MIP-1β (CCL4), RANTES (CCL5), and TNF-α. The multiplex plate was read with Bio-Plex 200 system Luminex (Bio-Rad, 171000201) and analyzed using the Bio-Plex Manager™ 6.0 software.

Statistical Analysis

All data are presented as means ± standard error of the mean (S.E.M.). Comparisons between group was performed using t-test, one-way ANOVAs and two-way ANOVAs with Bonferroni post hoc follow up test when required. Values of $P<.05$ were regarded as statistically significant. Graphs and statistics were generated using GraphPad Prism Software version 8 (GraphPad Software Inc.). Normality was determined Shapiro–Wilk tests and Levene's test for homogeneity of variances. Data was analyzed using the nonparametric Kruskal-Wallis test when it did not meet the assumption of normality, or the Mann-Whitney U test for pairwise comparisons. Visual representation of average and S.E.M. with heatmaps was created using Matlab-based software. Individual values were used to compute correlation matrices and p-values were determined by Matlab-based software (MathWorks). Comparison of microbiota alpha diversity was performed with Microbiomeanalyst using the phyloseq package [316] and further comparisons using the Mann-Whitney U test. Beta diversity was performed using Bray–Curtis (dis)similarity matrices using the phyloseq package. The principal coordinate analysis (PCoA) was performed to visualize the distance matrix and the importance of the modifications at the community level was assessed using one-way permutational multivariate analyses of variance (PERMANOVA) tests.

Chapter 1: Results of sex-specific effects of chronic stress on intestinal barrier integrity

Chronic social defeat stress induces social avoidance in a subset of mice

To investigate the implications of stress on gut barrier characteristics, tight junction expression in the JEJ of male and female mice was observed after CSDS exposure. Specifically, a comparison of any identified alteration with levels of avoidant behaviour in the SI test was determined if chronic stress-induced gut barrier alterations may be playing a role in vulnerability or resilience. CSDS induced expression of both phenotypes– SS and RES–based on behaviour in the SI test (**Fig. 8a**). Among the 29 male mice that experienced social defeat, 16 had SI ratios <1 and were classified as SS (55.2%) and 13 had SI ratios of ≥1 and were classified RES (44.8%). These ratios of resulting phenotypes aligns with other CSDS reports [32,152]. Average SI ratios per group among CTRL (non-stressed) (1.566 ± 0.2726), SS (0.4888 ± 0.3018), and RES (1.422 ± 0.3835) mice, differed significantly from one another, (*H(2)= 29.20*, *P<.0001*). Follow up comparisons indicated less interactions with the novel social target for SS mice compared to both CTRL (*P<.0001*) and RES (*P<.0001*) **Fig. 8b**. Time in corners confirmed avoidance behavior in the SS group when aggressor was present F(2, 37)= 9.567, *P<.0004* (**Fig. 8d**) while total distance travelled was consistent between groups (**Fig. 8d**). Representative heatmaps of normalized time spent in the arena of each phenotype in males are presented (**Fig. 8e**).

Of the total female mice (n=31) that experienced social defeat, 11 were classified as SS (35.5%) and 13 RES (64.5%). This is a lower amount of SS and more RES then typically seen but is not uncommon due to the aforementioned potential confounders in the female CSDS paradigm. Average SI ratios per group among CTRL (1.406 ± 0.2919), SS (0.5118 ± 0.2778), and RES (1.352 ± 0.2115) mice were distinct (*F (2, 39) = 47.43*, *P<.0001*). Again, there was a decline in SS mice compared to CTRLs (*P<.0001*) and RES (*P<.0001*) (**Fig. 8f**). As in males, baseline time spent in corners (**Fig. 8g**) and distance traveled (**Fig. 8i**) were consistent for all groups in females. Corner time with AGG present differed between phenotypes (*F (2, 39)= 22.92*, *P<.0001,* **Fig. 8h**). Follow up test confirmed that SS mice spent more time in corners compared to both CTRL (*P=.0001*) and RES (*P=.0001*) mice, highlighting the socially avoidant behaviours.

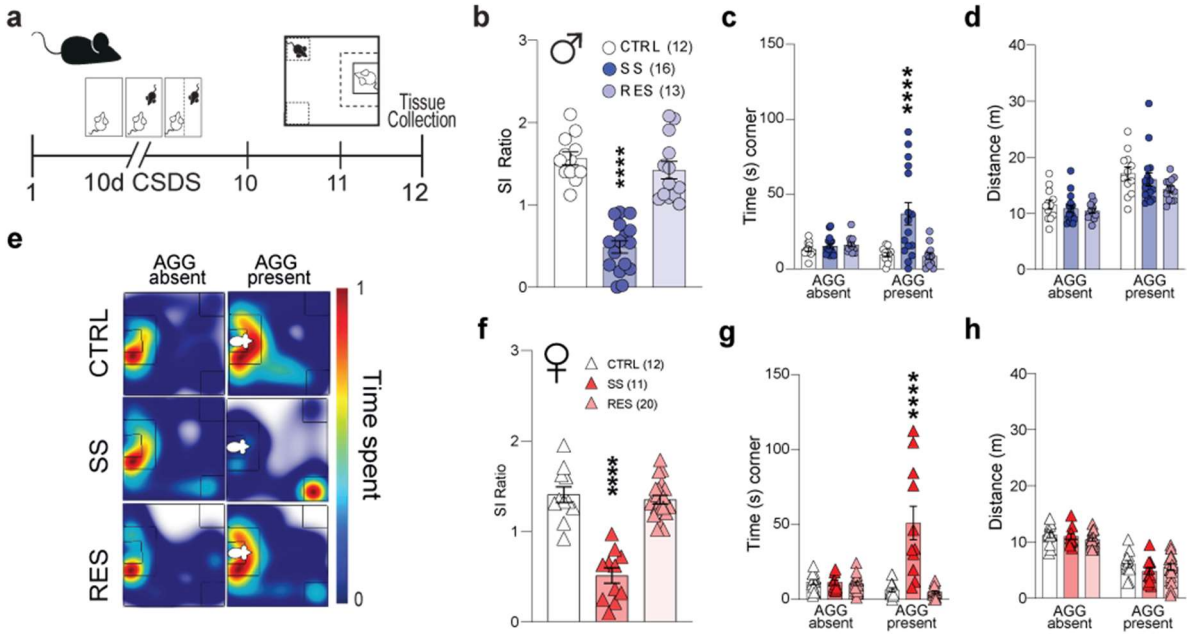

Figure 8. Chronic social defeat stress induces a depression-like phenotype (social avoidance, anhedonia) in a subset of mice (males and females).
a) Timeline of the CSDS paradigm. **b, f**) Ratio of time spend interacting with novel social target declined in SS mice for both male and female mice. **c, g**) Cumulative time (in seconds [s]) spent in corners with social target present was augmented in SS mice for both males and females. **d, h**) Cumulative distance traveled (meters [m]) in arena with social target present were consistent between groups. **e**) Representative heatmaps of normalized time spent in the arena during social interaction test in males. Data are assessed by one-way ANOVA followed by Bonferroni's multiple comparison test and represented as mean ± S.E.M.; ***p <.01,*

Social defeat stress induces changes to intestinal tight junction expression with sex-specific effects

Using qPCR for mRNA expression we performed transcriptional profiling of various genes of interest involved in intestinal barrier integrity (see **Table A1** for a list of all primers used). Gene targets involved tight junctions (*Cldn3*, *Cldn7*, *Cldn12*) and tight junction-associated proteins (*Tjp1, Tjp2, Tjp3, Ocln,* MARVEL domain-containing protein 2 *[Marveld2]*), as well as proteins involved in serotonin metabolism (*Ido1* and *Ahr*) and mucus layer formation, Mucin-2 (*Muc2*). Gene expression profiles in the JEJ of stressed mice following 10d CSDS and unstressed controls (CTRL) and were compared (**Fig. 9a**). In males, exposure to CSDS altered the mRNA expression of various tight junctions in the JEJ (**Fig. 9b**). *Cldn3*, an important intestinal tight junction, was upregulated in male mice after CSDS (*P=.0002,* **Fig. 9c**). Further, we compared SS to RES mice to identify individual differences in the intestinal barrier as a response to chronic stress. Between groups there were marked differences for *Ocln* ($F(2,36) = 6.603$, $P=.004$), *Tjp2* ($F(2, 34) = 11.33$, $P<.001$) and *Marveld2* ($F(2,34)=$

8.598, *P=.02*) expression (**Fig. 9b**). Post hoc comparisons confirmed SS mice had lower expression of *Tjp2* (*P=.006*, *P<.001*) and *Ocln* (*P=.02*, *P=.007*), **Fig. 9b**. *Marveld2* expression levels were unaltered in SS mice but raised in RES mice compared to both CTRL and SS (*P=.02*, *P=.047*, **Fig. 9b**). In males, tight junction mRNA expression was linked to the extent of social avoidance (**Fig. 9d**). Fold changes were positively correlated with SI ratios for *Cldn12* (*P=.006*, r=0.44), *Tjp1* (*P=.03*, *r=0.36*), *Tjp2* (*P=.004, r=0.46,* **Fig. 9e**) and *Ocln* (*P<.001, r=0.55*), **Fig. 9d**. A trend (*P=.0845, r=-0.29*) indicated that mice with the most avoidant behaviour had the highest *Cldn3* expression, though it did not reach significance (**Fig. 9e**).

In female mice, exposure to CSDS altered mRNA expression of only *Tjp1* (*P=.001*) in the JEJ, out of all the genes tested (**Fig. 9f**). Post hoc comparison revealed group differences *(H(2)=9.837 P=.007*), specifically, RES mice had more *Tjp1* than controls *(P=.006,* **Fig. 9f)**. Unlike in males, *Cldn3* was not elevated in female mice after CSDS (**Fig. 9g**) and did not follow the same pattern of trend with SI ratio (**Fig. 9i)**. However, *Ocln* diminished with SI ratio *(P=.04*, r=0.31) for all mice and the same relationship was detected for *Cldn7* (*P=.02*, r=0.41) and *Ido1* (*P=.04*, r=0.24), but uniquely in stressed mice (**Fig. 9h**). These specific tight junction changes in respect to depression-like behaviours were not observed in male mice and could be unveiling a pro-susceptible phenotype in females. There was a main effect of sex on *Cldn3* (*F (1, 54) = 7.508*, *P=0.008,* **Fig. 9j left**) and *Tjp1* (*F (1, 53) = 10.88*, *P=.002,* **Fig. 9j center**) expression in SS and RES mice. Post hoc tests confirmed that female mice had lower *Cldn3* expression than males in both SS and RES groups (*P=0.0499, P=0.0499*). Similarly, SS males had lower *Tjp1* expression than SS females (*P=0.01*). There was an interaction between the factor sex and behavioural phenotype on *Tjp2* expression (*F (1, 54) = 4.332*, *P=0.04*). Specifically, promoted *Tjp2* expression was only occuring in male RES mice (*P=0.01,* **Fig. 9j right**). Therefore, CSDS modified tight junction expression in both male and female mice, these changes were specific to each sex.

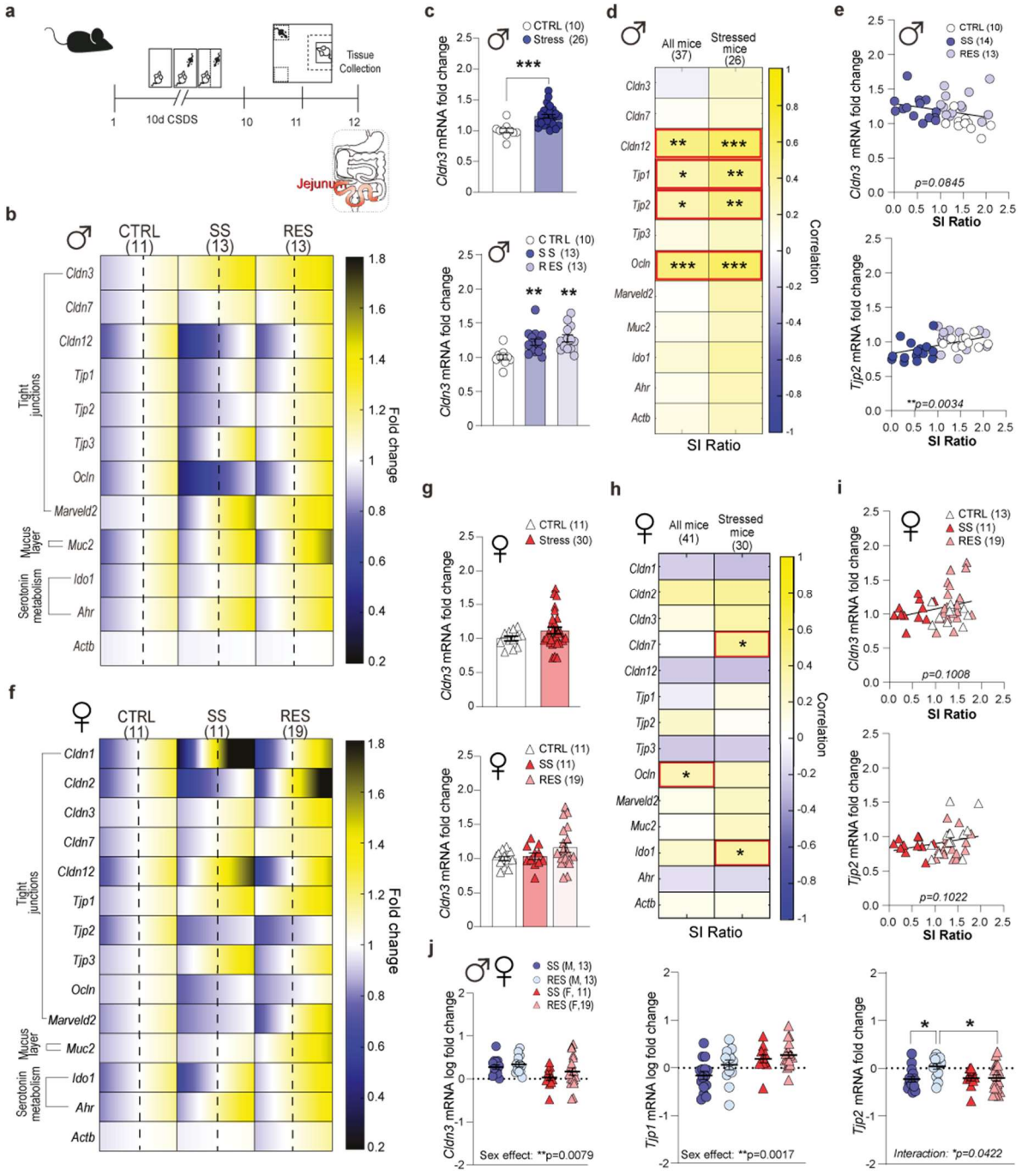

Figure 9. Chronic social defeat stress induces changes to intestinal tight junction expression.

a) Experimental timeline of social defeat stress paradigm, social interaction test and jejunum tissue collection. **b**) Quantitative PCR revealed significant shifts in jejunum of SS and RES mice compared to controls for gene expression of targets related to tight junctions. The range of color indicates individual differences within a group; S.E.M. from the average represented by the dashed line. **c)** Heightened *Cldn3* in stressed males was independent of phenotype group. **d**) Social stress impacts on mRNA expression of tight junction proteins in the jejunum of male mice as a function of group condition. Red boxes highlight genes with significant correlation with social avoidance. **e**) Social avoidance escalated along with *Tjp2* expression and showed a trending relationship with *Cldn3*. **f)** Tight junction changes as a function of phenotype in female mice. **g)** *Cldn3* expression is unchanged in female mice following social stress. **h**) *Ocln*, *Cldn7* and *Ido1* expression correlated

with social avoidance behaviours. Red boxes highlight genes with significant correlation with social avoidance. **i**) Sex-specific effect of stress on the *Cldn3* and *Tjp1* expression. **j**) There is an effect of sex as a factor on *Cldn3* and *Tjp1* gene expression, and an interaction occurs between the factor Sex and behavioural phenotype on *Tjp2* expression. T-tests and one-way ANOVA followed by Bonferroni's multiple comparison test for between groups analysis; two-way ANOVA followed by Bonferroni's multiple comparison test for comparison between sexes; correlations were evaluated with Pearson's correlation coefficient; **P< .05, **P<.01, ***P<.001.*

Intestinal tight junction expression changes depend on stress type and duration.

It is established that female and male mice are disparate in their reaction to various types of stress. Thus, accounting for observations in the previous section between sexes in tight junction expression following CSDS, we decided to further compare across various stress paradigms (**Fig. 10a, b, c**). Females are susceptible to short-term chronic variable stress (6-day SCVS), whereas males are not. However, both sexes display depression-like behaviours after weeks of stress exposure [88,159]. Therefore, we evaluated the same profile of genes in male and female mice after both SCVS and CVS **(Fig. 10a**).

Notably in males, previously amplified *Cldn3* **(Fig. 10a left**), remained consistent following SCVS (**Fig. 10b left**). Furthermore, in males SCVS exposure did not yield changes in the expression of any other of the genes investigated (**Fig. 10d**). After long-term exposure, in the CVS paradigm, stressed males had declined *Cldn3* (*P=.03,* **Fig. 10c left**), *Cldn7* (*P=.04*), *Cldn12* (*P<.001*), *Tjp2* (*P<.0001*), *Tjp3* (*P<.001*), *Ocln* (*P<.0001*) and *Marveld2* (*P=.02*), **Fig. 10d**. In males, a significant difference in *Cldn3* expression appeared in comparing across the three models (*F (2, 41) = 40.00*, *P<.0001*). Social stress elevated *Cldn3* expression compared to short- and long-term CVS (*P=.001, P=<.0001*), **Fig. 10e top**. At the same time, CVS abated *Cldn3* relative to CSDS and SCVS (*P=<.0001, P=.0023),* **Fig. 10e**. The effects incurred by social stress were not specific to the SS or RES phenotype (**Fig. 10e bottom**)**.** The opposing effect of CSDS vs. CVS on *Cldn3* expression may reflect a habituation mechanism in play where an original gain in *Cldn3* expression begins to get suppressed after long term stress in male mice.

Alternatively, in female mice, SCVS modulated expression of many tight junction genes. Previously unchanged *Cldn3* (**Fig. 10a right**) was lower for stressed mice (*P=.008,* **Fig. 10b right**). Further, *Tjp2* (*P=.009*) and *Tjp3* (*P=.009)* expression diminishes (**Fig. 10d**), while opposite expression modifications ensued for *Cldn12* (*P=.009*) and *Tjp1* (*P=.02*), **Fig. 10d**. The SCVS-induced *Tjp2* reduction was maintained after long-term variable stress (*P=.04*)

though to a lesser extent, which may indicate a slow habituation attempt to reinstate normal levels. Otherwise, after SCVS, a trend of inflated *Ahr* expression was seen (*P=.0519*), that was significant after CVS (*P=.001*). Also, a decrease in *Ido1* expression arose after CVS exclusively (*P<.0001*). Overall, when comparing stressed mice *Cldn3* expression across stress paradigms in females, a difference was noted (*F(2,43)=5.581*, *P=.007*). Specifically, a reduction (*P=.007,* **Fig. 10f top**) occurs uniquely after SCVS that is distinct from levels in CSDS mice (**Fig. 10f top**). Furthermore, when distinguishing RES vs. SS groups, the SCVS group *Cldn3* levels are significantly reduced from the RES mice only (*P=.009*, **Fig. 10f bottom**). This effect indicates that perhaps, though SS and RES groups do not differ significantly to each other for *Cldn3* expression, there could still be a protective mechanism elevating *Cldn3* in the resilient animals. Variable stress may affect females more intensely than CSDS, with a downregulated *Cldn3* that seems to subside after a longer-term exposure which could be a compensatory mechanism.

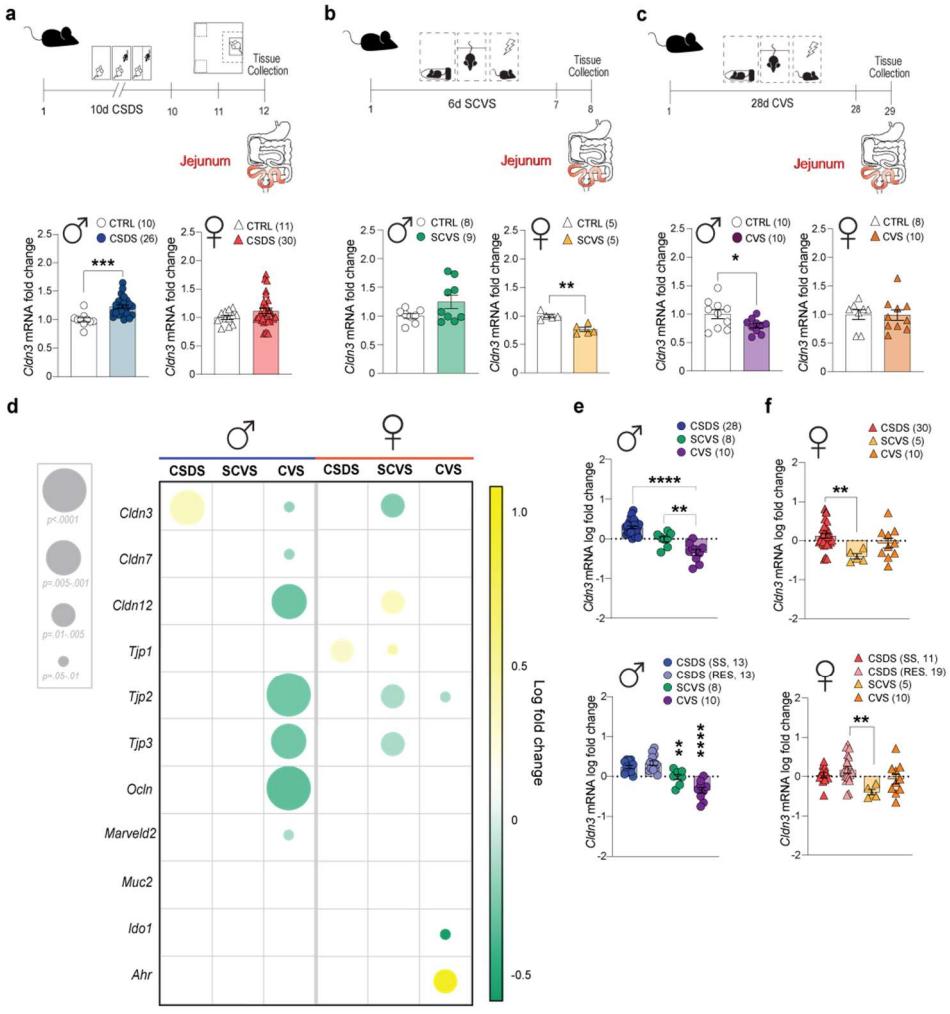

Figure 10. Changes in intestinal tight junction expression are dependent on stress type and duration
a) Experimental timeline of chronic social defeat with graphs of *Cldn3* gene expression comparing control and stressed groups of male and female mice. **b**) SCVS experimental timeline with comparison of Cldn3 gene expression results below of male and female mice. **c**) Experimental timeline of CVS with Cldn3 gene expression results between control and stressed groups of male and female mice from this paradigm. **d**) Representation of gene expression changes in the JEJ of stressed mice across stress models in both males and females. Circle diameter represents the statistical significance of the gene expression change. Circle colour represents directionality of change with green as a downregulated gene and yellow, an upregulated gene **e**) Direct comparison of *Cldn3* gene expression changes in male stressed mice exposed to different stress types; CSDS, SCVS and CVS [top], with CSDS phenotypes separated to SS, RES [bottom]. **f**) Direct comparison of *Cldn3* changes in female stressed mice exposed to different stress types; CSDS, SCVS and CVS [top], with CSDS phenotypes separated to SS, RES [bottom]. T-tests and one-way ANOVA followed by Bonferroni's multiple comparison test for changes between groups. **P< .05, **P<.01, ***P<.001, ****P<.0001*

Impact of chronic stress on the intestinal microbiota

Due to the relationship between intestinal microbiota and tight junctions of the intestine, we analyzed microbiota populations in various stress models for both male and female mice to determine if dysbiosis could be playing a role in the altered expression that we saw in certain groups. Feces samples were collected from mice prior to and after CSDS and similarly, samples were collected from unstressed controls (**Fig. 11a**). Alpha diversity at the feature-level, as measured by Shannon and Chao1 indexes, remained consistent following CSDS in female mice (data not shown). Furthermore, no differences were detected between phenotype groups, SS or RES (**Fig. 11b**). To quantify potential differences in microbial communities, beta diversity was measured by Bray-Curtis index (**Fig. 11c)**. There were no differences in the presence or absence of feature taxonomic abundances in communities from stressed mice compared to unstressed controls (**Fig. 11c**). Examination of relative abundances throughout taxonomic ranks highlighted CSDS induced transformations to various populations following CSDS. Mice exposed to social stress had higher levels of phylum *Bacteroidetes* (*P=.048*, **Fig. 11d top**), class *Bacteroidia* (*P=.045*, **Fig. 11e left**) and order *Bacteroidales* (*P=.045,* **Fig. 11e right***)*. Conversely, socially stressed mice also had lower abundances of order *Rhodospirillales* (*P=.04,* **Fig. 11e right**). Comparison of phenotypes uncovered distinct populations of phylum *Bacteroidetes (H(2)=7.096, P=.02)* class *Bacteroidia (H(2)=7.096, P=.02)* and order *Bacteroidales (H(2)=7.096, P=.02)*. Post hoc analysis confirmed SS mice had higher *Bacteroidetes* (**Fig. 11d bottom**)*, Bacteroidia* and *Bacteroidales* than controls (*P=.02,* **Fig. 11f**)*.* At the family and genus level, a trend was detected between groups for *Prevotellaceae* (*P=.065*) and *Prevotella* (*P=.065,* **Table 6**) that

did not reach significance. No correlation was noted between any relative abundances and SI ratio.

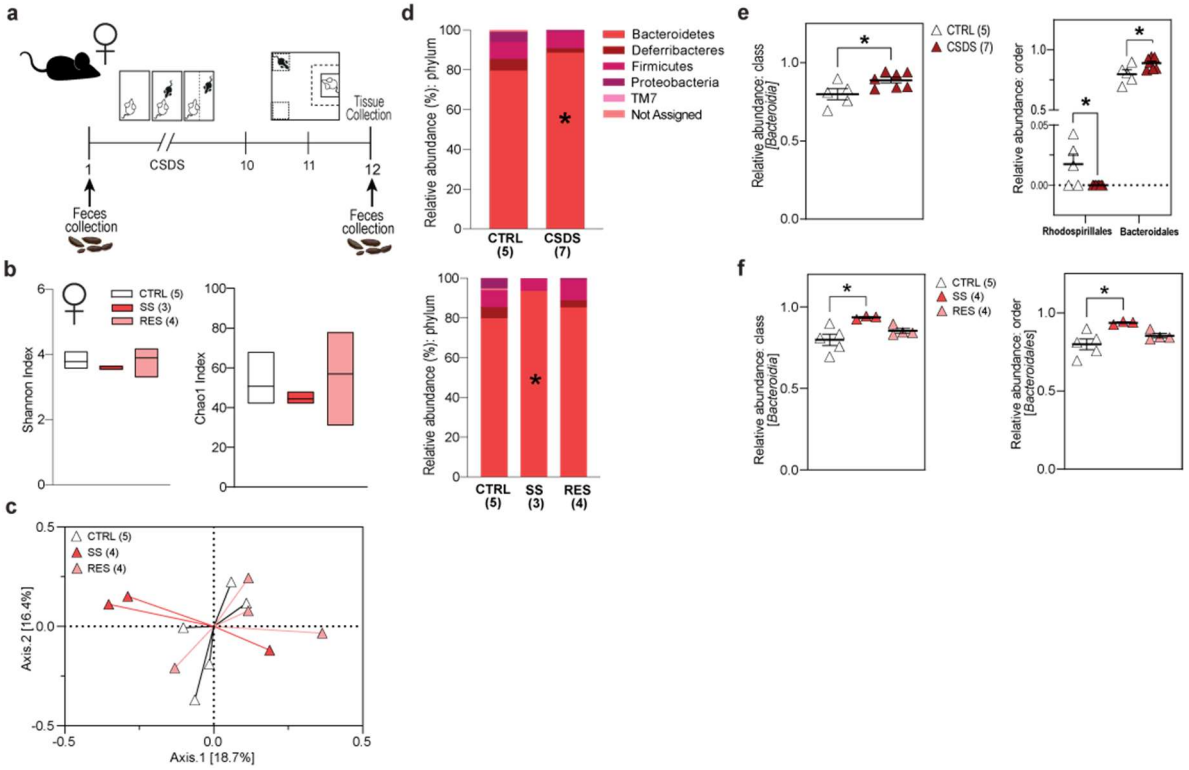

Figure 11. Social stress induces microbial composition changes in females.
a) Alpha diversity between groups boxplots with line inside indicating median, lower boundary representing the 25th percentile and upper boundary representing the 75th percentiles. **b**) Representation of beta diversity as PCoA plot for visualization of Bray-Curtis dissimilarity matrix between communities. Each point represents the entire microbiome of a single sample. **c**) Among predominant phyla, stressed mice had more *Bacteroidetes*. **d**) Elevations of *Bacteroidia* following CSDS, with altered *Rhodospirillales* and *Bacteroidales*. **e**) Phylum *Bacteroidetes* is higher in SS mice. **f**) *Bacteroidia* and *Bacteroidales* populations are raised in SS mice. Relative abundance data represents median ± SD; number of animals (n) is indicated on graphs. Mann-Whitney tests and Kruskal-Wallis tests followed by Dunn's multiple comparison used for two-group comparisons. *$P<.05$.

	Genus	Females		
		CTRL	SS	RES
CSDS	**Bacteroides** spp.	16.11±1.66	15.75±3.27	18.63±0.88
	Not Assigned spp.	47.73±5.37	52.14±2.86	42.99±4.67
	Prevotella spp.	**7.69±1.90**	**13.85±1.50**	**6.12±0.22**
	Alistipes spp.	20.72±2.65	16.10±1.51	22.20±0.81
	Mucispirillum spp.	5.54±2.36	0.00±0.00	3.46±1.70
	Clostridium spp.	2.21±1.40	2.16±1.21	4.50±0.81

Table 4. Relative abundance (%) of top genera in female mice after chronic social defeat stress.
Data shown as group mean relative abundance ± SEM. *$P<0.05$. Values in bold represent a trend that was noted between phenotypes for abundance of *Prevotella spp.* that did not reach significance.

Comparison of fecal microbiota from female and male mice post-SCVS was performed (**Fig. 12a**). Short-term variable stress did not alter overall microbial diversity in male mice (**Fig. 12b**) and direct community comparison did not reveal any distinctions in SCVS exposed males from unstressed controls (**Fig. 12c**). The relative abundances of major phylum were also consistent (data not shown). However, SCVS exposed males had fewer *Alphaproteobacteria* (*P=.02;* **Fig. 12d**) and more *Rikenellaceae* (*P=.02;* **Fig. 12e**). At the genus level, enlarged abundance of *Alistipes* (*P=.02*) was detected in stressed mice (**Table 5**). In female mice, both measures of alpha diversity had increases in mice post-SCVS that that did not reach significance (Shannon, *P=.052*; Chao1, *P=.058*; **Fig. 12f).** Beta diversity was unchanged throughout community comparisons in females (data not shown). However, SCVS-exposed mice had altered ratios of major phylum, with reduced abundances of *Bacteroidetes* (*P=.02*) and subsequent rise in *Firmicutes* (*P=.02*), **Fig. 12**. Furthermore, diminished levels of class *Bacteroidia* (*P=.02,* **Fig. 12h**) and subsequent taxonomic levels; *Bacteroidales* (*P=.02*), and *Bacteroideceae* (*P=.048)* appeared in stressed mice (data not shown). Enrichements of class *Clostridia* (*P=.048*, **Fig. 12h)**, order *Clostridiales* (*P=.048*) and family *Lachnospiraceae* (*P=.048; data not shown*). Finally, a decline in *Bacteroides* genus was discovered in the SCVS group (*P=.048,* **Table 5**).

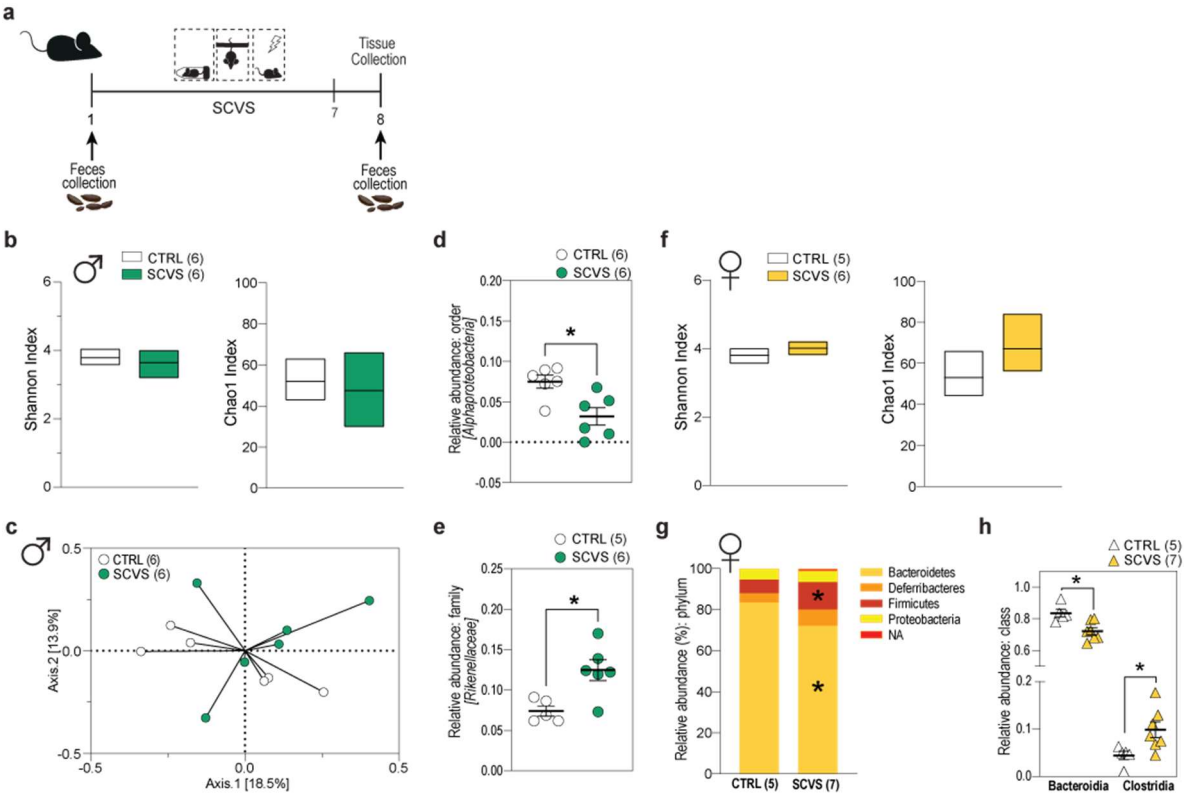

Figure 12. Subchronic variable stress alters prominent microbiome phyla in males and females
a) Experimental timeline of SCVS with fecal microbiota analysis. **b**) Alpha diversity box plots between groups. Line inside the box indicates median, lower and upper box boundaries represent the 25th and 75th percentile respectively. **c**) Principal coordinate analysis plot shows no obvious (dis)similarity amongst microbiome communities of male control and SCVS groups. **d**) Decreased relative abundance of *Alphaproteobacteria* in stressed mice. **e**) Expanded *Rikenellaceae* in stressed male mice. **f**) Measures of alpha diversity between mice after SCVS stress in females. **g**) Relative abundances of dominant phylum are altered in female stressed mice. **h**) Lower relative abundance of *Bacteroidia* and heightened *Clostridia* populations in female SCVS exposed mice. Number of animals (n) is indicated on graphs; data represents mean ± S.E.M. for relative abundance; Mann-Whitney test performed for two-group comparisons; Beta diversity assessed with PERMANOVA. **P<.05.*

	Genus	Males		Females	
		CTRL	SCVS	CTRL	SCVS
SCVS	**Not Assigned** spp.	57.16±2.70	52.04±4.96	51.83±3.54	57.29±2.56
	Bacteroides spp.	12.73±1.42	13.25±1.09	13.77±2.26	**8.54±0.89***
	Prevotella spp.	8.70±0.89	11.37±2.78	7.58±1.01	5.34±1.02
	Alistipes spp.	7.30± 0.64	**12.49±1.25***	19.05±1.25	15.91±2.14
	Mucispirillum spp.	6.78±2.15	5.79±2.11	4.54±1.98	8.12±1.94
	Clostridium spp.	5.21±2.55	5.06±1.19	2.70±1.29	4.42±0.09
	Lactobacillus spp.	0.00±0.00	0.00±0.00	0.54±0.33	0.79±0.30

Table 5. Relative abundances (%) of top genera in male and female mice after subchronic variable stress.
Data shown as group mean relative abundance ± SEM; values marked in bold are significantly changed; **P<.05*

Analysis of microbiota composition following CVS (**Fig. 13a**) did not yield detectable differences in overall species richness or diversity after CVS in males (**Fig. 13b**). Although, microbiome community compositions exhibited dissimilar features compared to unstressed controls (*F=1.4754*; R^2=0.11826; *p<.042*) as measured by Bray-Curtis beta diversity index (**Fig. 13c**). Loss of *Bacteroidetes* in CVS-exposed males was noted at the phylum level (*P=.02;* **Fig. 13d**). Stressed mice had reduced levels of class *Bacteroidia (P=.02),* and enhanced *Clostridia* (*P=.02)* compared to controls (data not shown). At the order level, a decrease in *Bacteroidales (P=.02;* data not shown) and an increase in *Clostridiales (P=.02;* data not shown*).* As well, a rise in *Lachnospiraceae* family occurred (*P=.02)* and decrease of *S24_7 (P=.02)*, **Fig. 13e.** At the genus level, extended *Clostridium* populations were noted (*P=.04*), but all other genera remained unchanged (**Table 6**). In female mice, CVS did not induce alter alpha diversity between groups (**Fig. 13f**). Beta diversity analysis could not distinguish between female stressed and control mice microbial communities (**Fig. 13g**). No significant differences were detected in the relative abundances of various phylum or at lower taxonomic levels. Genus levels were similar between stressed and control animals (**Table 6**).

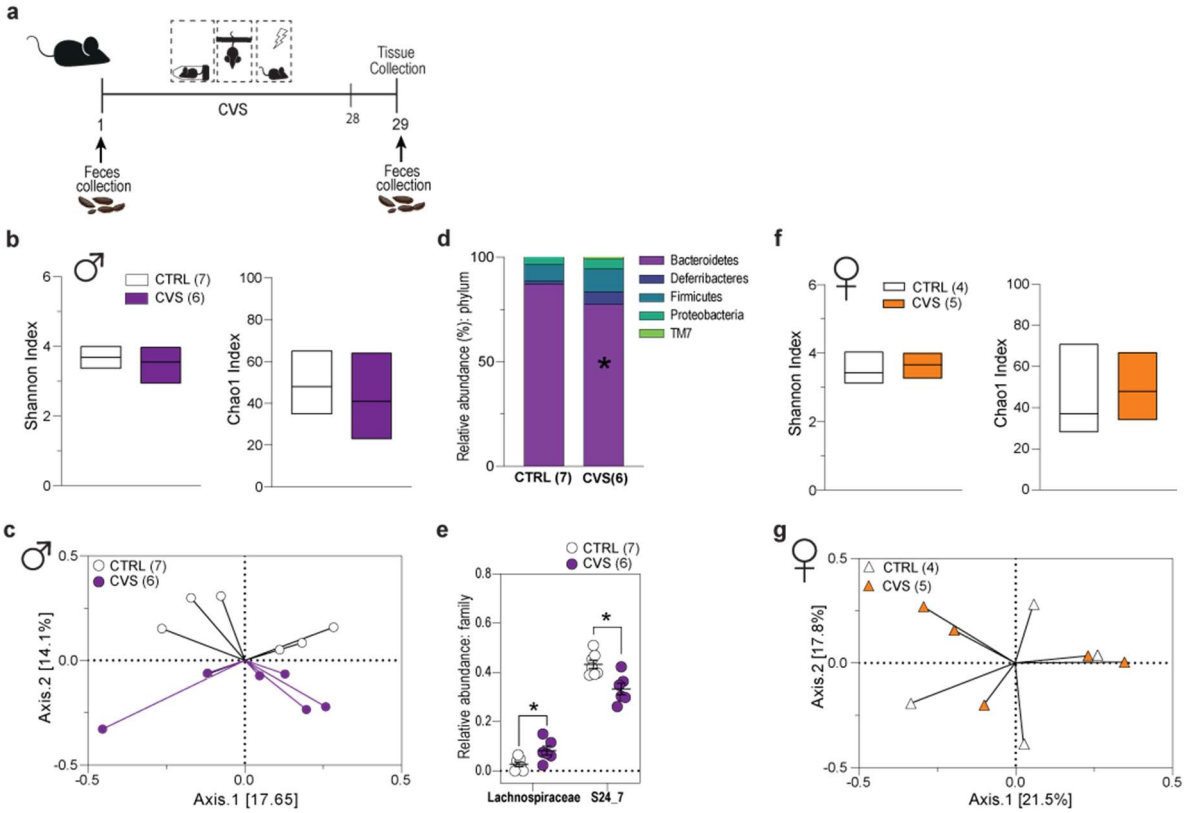

Figure 13. Chronic variable stress alters microbiome communities in males specifically.
a) Experimental timeline of CVS with fecal microbiota analysis. **b**) Measures of alpha diversity are unchanged in males after CVS. Line inside the box indicates median, lower and upper box boundaries represent the 25th and 75th percentile respectively. **c**) Beta-diversity of fecal bacterial communities in male CVS and unstressed controls illustrated by PCoA plot of Bray-Curtis distance analysis shows two distinct clusters. **d**) Overall relative abundance of phylum communities. **e**) Altered relative abundances of *Lachnospiraceae* and *S24_7* families in stressed males. **f**) Measures of alpha diversity remain the same in female mice. **g**) PCoA plot showing no obvious (dis)similarity amongst microbiome communities of female control and CVS groups. Number of animals (n) is indicated on graphs; data represents mean ± SEM for relative abundance; Mann-Whitney test performed for two-group comparisons; *$P<.05$.

	Genus	Males		Females	
		CTRL	CVS	CTRL	CVS
CVS	**Bacteroides** spp.	12.81±1.49	10.86±1.87	15.09±4.07	9.45±0.86
	Not Assigned spp.	59.10± 1.88	51.68±3.10	47.47±7.18	52.43±7.61
	Alistipes spp.	15.34±2.81	14.73±2.05	18.29±2.80	20.57±2.20
	Prevotella spp.	10.45±0.98	12.35±1.38	10.41±1.61	9.94±2.88
	Clostridium spp.	0.80±0.59	**4.23±1.70***	2.39±1.21	2.41±1.49
	Mucispirillum spp.	1.50±0.87	6.14±2.49	5.35±2.65	4.53±2.41
	Lactobacillus spp.	0.00±0.00	0.82±0.42	1.01±0.84	0.66±0.66

Table 6. Relative abundance (%) of top genera in male and female mice after chronic variable stress.
Data shown as group mean ± SEM. Values marked in bold are significantly changed *P<.05.

Discussion – Chapter 1

Chronic social defeat stress-induced social avoidance

The first objective of this study was to characterize the expression of various genes related to intestinal barrier function in the intestine of male and female mice after exposure to different stress conditions. Also, to see if any alterations would be attributed to the SS or RES phenotypes. Our cumulative distribution of SI values for male mice was consistent with the expected ratios of SS to RES mice following social defeat stress [32,152]. We had a lower amount of female SS mice than is typically reported [165,317], however, this was not unexpected. The male CSDS model has high validity, whereas it is recognized that confounding variables still exist due to the modifications made when implementing the female specific version [317]. The urine application to females induces similar behavioural responses as in the typical male-male model (social avoidance and anxiety-like behaviors), however, still produces slightly lower levels of aggressive behaviour from the AGG males. As well, in females, the overnight sensory exposure does not enhance susceptibility as does in males, in fact, it may even enhance resilience [164]. Finally, having a male social target during the SI test in females may results during the classification of SS and RES phenotypes. Despite the utility of this model, the potential sex-specific outcomes of social stressors and even stressors in general need to be taken into consideration, the fact of which motivated us to conduct the biological tests across other stress models.

Chronic stress induced changes to intestinal tight junction expression

Our results support the expectation that altered expression of intestinal tight junctions would result from exposure to chronic stress. We saw many changes in tight junction Cldn3, which is a part of the "tightening" tight junction group and contributes to barrier permeability throughout the intestinal tract. Previously, in rodents Cldn3 expression correlates with direct measures of permeability, assessed by TEER [179]. *In vitro*, Cldn3 overexpression reduced paracellular permeability to inorganic cations and anions, the solute fluorescein (332 Da) and to the macromolecule FD-4 (FITC dextran, 4 kDa) [178]. The specific role of Cldn3 in development and pathology is unclear, although alterations are implicated in IBD and celiac disease [318]. Increased intestinal Cldn3 expression is postulated to be host response to repair the epithelial barrier during intestinal injury [319]. This aligns with our findings that CSDS

elevated *Cldn3* expression in males. Although 28-day CVS caused a reduction in *Cldn3*, therefore potentially long-term stress exposure is enough to overcome the reparative mechanisms.

Other transitions in tight junctions depended on the type of stress exposure. Males seemingly experienced more overall tight junction expression modifications after social defeat stress. A similar study produced no changes following CSDS to *Cldn3* among other tight junctions tested in the colon, therefore our findings in the JEJ could indicate region-specific effects of stress [320]. Otherwise, no studies were found investigating the small intestine in mice with the same stress experiences as ours. In rats, chronic psychological stress did not affect the levels of CLDN1, OCLN and TJP1 protein levels in the JEJ [321]. Another similar study of acute stress demonstrated upregulated *Cldn1*, *Cldn5*, *Cldn8*, *Ocln* and *Tjp1* in the JEJ. These shifts did not continue after chronic stress, though permeability persisted (measured by TEER) [322]. We did not see *Tjp1* modulations from SCVS although our paradigm differed substantially from their subacute stress. In our mice, *Tjp1* was unchanged after CVS which aligns with their findings, though we did see a considerable decline in *Ocln* which may be attributed to the intensity or duration of stress since our protocol lasted two weeks longer. As well, the reports differ by the type of stressor employed, social stress compared to water avoidance and crowding may be involved in the results. Also, previous reports indicate different breeds of mice have distinct behavioural and intestinal tight junction changes [320], so the reported findings in rats may not translate exactly to our findings in mice.

Surprisingly, we did not detect any tight junction changes in females with specific associations the extent of depression-like behaviour. As in MDD, women have a higher risk of developing IBDs, therefore, we expected many tight junction alterations. Of course, there are many other options for gene markers that could be changing in a sex-specific manner aside for the genes of interest that we chose. For instance, the other claudins listed in **Table 2** as implicated in GI disorders, or any of the many claudins that still have unspecified or unidentified functions. Other options for identifying female-specific changes could be investigations into gene markers of molecules involved in the serotonin/ inflammatory pathway. A study highlighting sex differences in IBD indicated that progesterone modulates the colonic 5-HT system. Here, both serotonin transporter and 5-HT levels were lower in

women with IBD compared with healthy controls [323]. Due to novel implications of peripheral serotonin metabolism in inflammatory, immune and metabolic signalling pathways [58,254] we did incorporate serotonin metabolism markers IDO-1 and AHR in the intestinal qPCR analysis. IDO-1 is highly upregulated in the human gut epithelium during inflammation [268–271] and linked to symptoms in MDD [257,261,262,272]. Therefore, we predicted stress exposure will promote IDO-1 expression. We did see altered IDO-1 exclusively in females, although it was in the opposite direction as expected, with a decrease in expression after CVS. In males, even without specific stressed-induced fluctuations, we still saw a highly positive correlation of IDO-1 expression and inflammatory markers in the gut, which is consistent with the literature. We had unexpectedly low inflammatory marker expression in the JEJ of stressed males (which will be discussed in the following paragraphs), and this coincided with low IDO-1 expression. Previously, IDO-1 was upregulated in the small intestine of male mice subjected to unpredictable chronic mild stress [324]. However, this protocol lasted substantially longer with analysis of the entire small intestine combined, therefore potentially region-specific effects or those related to stress intensity are involved in the discrepancies between results.

The second serotonin-related marker, AHR, is a transcription factor activated by many dietary and endogenous molecules, including tryptophan metabolites. AHR ligands can also be produced by commensal microbiota organisms [325]. AHR has anti-inflammatory roles for intestinal epithelial cells through regulation of the IL-10 and IL-22 pathways and antimicrobial peptide release. In our mice, a significant increase in *Ahr* expression after CVS was seen that was again exclusive to females. As well, this effect occurred in SCVS-exposed females, though it did not reach significance. CSDS did not alter *Ahr*, indicating potential specificity of changes to a specific type of psychological stress response in females. Inflammatory cytokine-induced expression of IDO-1 has downstream effects on AHR through the production of ligand L-kynurenine. Since stress promoted *Ahr* expression, we would expect higher IDO-1 as well, mediating KYN production which acts as an Ahr ligand. On the contrary, we saw decreased IDO-1 expression in these mice implying there is another mechanism at play. AHR responds to microbial metabolites in an attempt by the host to resist colonization by opportunistic pathogens such as *Listeria monocytogenes* and *Citrobacter rodentium* [326,327]. Indeed, *Lactobacilli spp* produces AHR ligands, initiating innate lymphoid

cells for targeted destruction of the *Candida albicans* pathogen to re-establish homeostasis [328]. In a similar way, AHR can induce IL-22 production, stimulating antimicrobial peptides release in the mucosa [328]. *Lactobacillus* populations were unchanged in our mice, though expansion may not be required to stimulate these pathways. Additional work is necessary to specify functional adaptations, but they provide interesting prospective for what may be occuring as a response to chronic stress. AHR modulation in the gut also has implications for the brain. In an experimental autoimmune encephalomyelitis mouse model, microbiota derived TRP metabolites had far-reaching implications. By activating AHR in astrocytes of the CNS this pathway initiated an anti-inflammatory response, emphasizing the potential positive implications of these gut-brain relationships [329].

Overall, data on intestinal expression of tight junction-related molecules in stress models are limited. Studies that do investigate these topics mostly focus on the colon due to the relevancy to IBD pathogenesis. However, other intestinal dysfunctions occur in the small intestine such as celiac disease and small intestinal bacteria overgrowth (called SIBO) and certain functions of the small intestine make it particularly interesting for our investigation. For instance, the JEJ is the primary site of nutrient absorption, including carbohydrates, lipids, and protein breakdown products [330]. A few investigations have provided stress related evidence of specific jejunal permeability. One such report demonstrated acute stress stimulated Cl- secretion and permeability to ions and larger molecules in the JEJ of rats [331]. No recent studies were found specifically on jejunal function after stress or comparing sections throughout the small intestine. In a preliminary investigation we compared the ileum and JEJ in females exposed to SCVS (data not shown). Indeed, we saw modulations distinct to each region. For that reason, in future studies it would be relevant to incorporate each section of the small intestine.

Chronic stress-induced changes to microbiota communities

In humans with MDD and in mouse models, previous work has shown decreased overall microbiota richness and diversity compared to healthy controls [237,249,332]. Here, the alpha diversity measures remained consistent throughout the models indicating that the richness and evenness of the observed taxa were not altered by any of the stress exposures. CSDS has lowered alpha diversity as well as induced distinct differences in beta diversity between

control and stressed mice previously [332]. However, these studies were conducted in males only vs. ours in females, indicating possible sex-specific effects. Also, they involved a gavage treatment which is highly stressful to mice and could be contributing to the more dramatic transformations. We also quantified beta-diversity to identify if there were differences between microbial communities from stressed mice and unstressed controls. Communities from stressed vs. control mice were distinct from each other in CVS males only. These findings align with a similar study in rats where chronic unpredictable mild stress treatment did not alter alpha diversity but did show dissimilarities in communities at the beta-diversity levels and further modifications within specific taxonomic levels of the composition microbiota [217]. Specifically, three enhanced bacterial taxa were the family *Lachnospiraceae*, order *Clostridiales* and class *Clostridia,* all of which were also represented in our CVS male cohort. These changes were not seen in the male mice after SCVS, indicating it may be a longer-term outcome. However, we also saw these exact changes after SCVS in female mice, contributing to the evidence that 6-day CVS is sufficient to induce depression-like effects in females only [54]. Surprisingly, after CVS in females, no modifications were accounted for though many were expected. This group had a very small sample size and samples had high variance between them. It would therefore be relevant to add more samples to these in the future to confirm the results. Still, it is interesting that we saw a higher number of changes in males resulting from CVS on tight junction expression and microbiota compared to females. Perhaps adaptive mechanisms involving the microbiota in females is bolstering the gut after this type of stress.

Within the altered microbial profiles, we expected certain patterns of upregulated pathogenic species and downregulated commensal bacteria in stressed mice compared to unstressed controls. Certain species of *Proteobacteria* have been known to be opportunistic pathogens [124], therefore we would expect a potential upregulation resulting from stress. In females exposed to CSDS we also saw declines in a group from the phylum *Alphaproteobacteria*. At the order level, *Rhodospirillales* were depleted and there were no further changes at lower taxa. In SS mice, a significant difference was detected between groups for *Prevotellaceae* and *Prevotella*, though it did not remain significant with follow up post-hoc comparison. Previously, in males following CSDS, genus *Prevotella* has been upregulated in the stressed group when tested 3 weeks after the last defeat, highlighting long lasting implication of

stress-induced alterations [216]. Interestingly, structural and functional differences in specific brain regions have coincided with clusters of gut microbes in healthy women [333]. Higher prevalence of *Prevotella* groups were associated with lower HIPP volume, higher NAC volume, and greater white matter connectivity in limbic-cortical-striatal circuits, all of which are features of depression.

Increased abundance of the phylum *Bacteroidetes* was noted in SS mice. This phylum is the main group of Gram-negative bacteria in the intestinal microbiota which fits with the hypothesis that exaggerated production and exposure to LPS may be occuring in stressed animals. However, the endotoxic activity of *Bacteroidetes* LPS is lower than that from other Gram-negative bacteria such as the *Proteobacteria* phylum [334]. *Bacteroidetes* and *Firmicutes* make up approximately 90% of total gut microbiota. The Bacteroidetes/ Firmicutes ratio has been a popular reference to measure alterations in gut metabolism. In MDD, it is proposed increased *Bacteroidetes* and overall reduction of Firmicutes/Bacteroidetes ratio occurs [253]. Therefore, resulting surges in acetate and propionate production and reduction in butyrate may be related to downstream effects.

Exposure to SCVS in male mice unexpectedly reduced *Alphaproteobacteria* (order). However, *Alphaproteobacteria* has shown to depreciate along with the Hamilton Depression Rating Scale-24 item (HDRS24) score in patients with MDD. Specifically, lower abundances were linked to higher severity of depression, aligning with our findings [277]. Therefore, we considered these to be potentially the beginning of modulations in SCVS that had not yet reached an imbalance until a more chronic stress state. However, reduced *Alphaproteobacteria* did not persist following long-term CVS. We also found accumulation of *Alistipes* genus from the *Bacteroidetes* phylum. *Alistipes* are common in the gut of healthy humans, and dysbiosis of *Alistipes* growth has demonstrated both beneficial and harmful effects [335]. However, *Alistipes* translocation to the blood stream is reportedly involved in abscesses of the periphery and in the brain, supporting a potential role as an opportunistic pathogen. Furthermore, *Alistipes* population augmentations have been reported in MDD [336], and are proposed to be linked to clinical symptoms through altering serotonin bioavailability in the gut [337]. *Alistipes* also produce LPS that are associated with gut inflammation in IBD patients [337], implicating them in inflammatory pathways that may be relevant for MDD.

Throughout the taxonomic levels we saw shifts in relative abundances of different groups that were unique to the type of stress paradigm and were sex specific. Unfortunately, throughout the different stress groups, a high percentage of relative abundance were being categorized into the "Not Assigned" category at the genus, sometimes even reflecting a significant difference between groups. Therefore, some relevant changes could be occuring that were not identifiable at this time. Overall, we expected to see distorted alpha and beta diversity since it has been reported in MDD patients and associated with propagating overgrowth of pathogenic species and inflammatory responses [186,237]. However, greater diversity in these patients has also been reported [251,338], while others show no differences at all [239,240]. Developing a cohesive approach across studies will be invaluable for future targeted therapeutic approaches. For instance, probiotics may stabilize local intestinal permeability. Indeed, neonatal mice given *Lactobacillus rhamnosus* exhibited increased Cldn3 mRNA expression and protein levels in the ileum in a dose dependent manner that improved barrier development, through TLR signalling [319]. Administration of *L. plantarum* into the duodenum of healthy human volunteers amplified TJP1 and OCLN protein levels of tight junction structures [339]. These studies highlight the importance of commensal microbes in promoting barrier integrity. Furthermore, treatment at the gut level may have positive effects on subsequent peripheral inflammation and even far reaching to the BBB. Indeed, germ-free mice demonstrate that lacking a gut microbiota caused BBB permeability as established by PET imaging and Evans blue dye extravasation into the cortex, striatum, and hippocampal parenchyma. Here, permeability was linked to altered expression of tight junction proteins (CLDN5, OCLN and TJP1). Further dynamic insight was provided and colonization of germ-free mice with commensal bacterial strains *Clostridium tyrobutyricum* and *Bacteroides thetaiotaomicron* was found to normalize BBB function [236]. These responses were all specific to germ-free mice and not pathogen free mice which indicates that features remain to be identified to understand before harnessing any applications of implementing community modifications for therapeutics response purposes.

Chapter 2: Investigation of a novel therapeutic intervention to prevent stress-induced alteration to intestinal barrier integrity

Investigating potential therapeutic response on the intestine after CSDS in males

In this portion of the study, we contribute to an initiative for the investigation of a peptide treatment by testing the therapeutic potential in chronic stress animal models of depression. Primarily, we wanted to confirm if the tight junction expression adjustments from chronic stress exposure in Chapter 1 would be rescued in mice receiving the peptide intervention. First, male mice were subjected to 10d CSDS without (vehicle) or with access to the peptide treatment (**Fig.14a**). Among the 21 stressed males, 13 became SS (61.9%) and 8 were RES (38.1%), ratios which reflects higher amounts of SS mice than our previous cohorts but still consistent with other reports [151]. Average SI ratios per group among CTRL (1.408 ± 0.2407), SS (0.3823 ± 0.3054), and RES mice (1.281 ± 0.1532) differed significantly from one another, (*F(2, 27)= 53.64*, *P<.0001*). Follow up tests confirmed that SS mice had fewer interactions with the novel social target than both CTRL (*P<.0001*) and RES (*P<.0001*), **Fig. 14b**. Comparison of treatment groups did not identify any treatment response on SI Ratios (*F (1, 24) = 1.023, P=.32;* **Fig. 14c**).

As in previously cohorts, the mRNA expression of various intestinal barrier related genes was assessed following CSDS in males receiving vehicle or peptide treatment. *Cldn3* levels were unaltered in stressed animals (**Fig. 14d left)** as were other genes assessed (data not shown). Similarly, there were no changes detected between phenotype groups (data not shown). However, a positive correlation was seen between *Cldn3* and SI ratio (*P=.042*, *r=0.38,* **Fig. 14d right**). Due to the importance of Cldn3 in the gut and the relevancy to GI disorders, we focused on Cldn3 for further assessed at the protein level. CLDN3 immunostaining was performed together with epithelial cell marker CD326 in the JEJ. No significant difference overall of colocalized volume of CLDN3 within CD326 was detected in the villi (ROI) of stressed mice compared to controls (**Fig. 14e**). However, a trend towards enlarged volume of CLDN3 (μm^3) in mice exposed to social stress occurred that did not reach significance (*P=.11*; **Fig. 14f**).

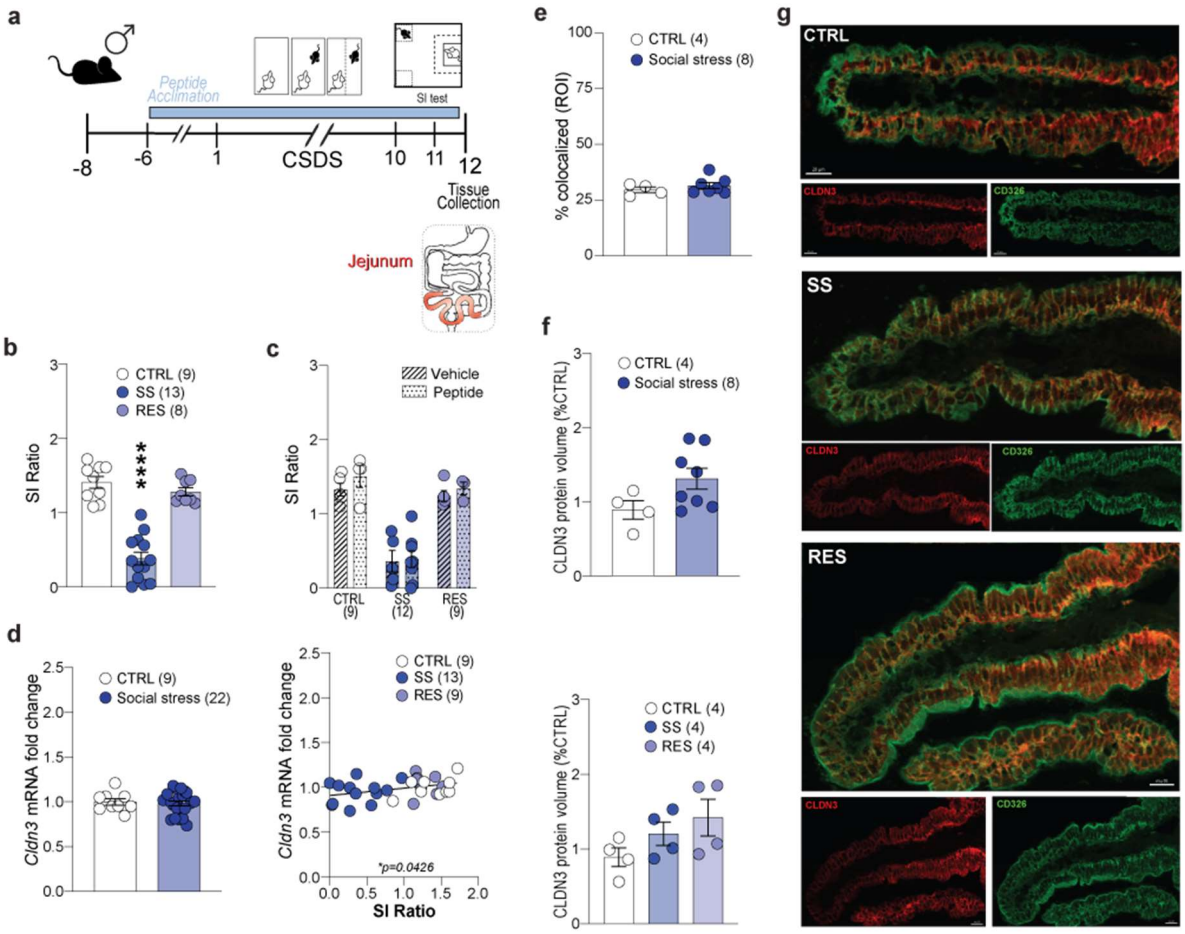

Figure 14. Investigating potential therapeutic response on the intestine after CSDS in males.
a) Schema of CSDS protocol in male mice while receiving vehicle or peptide treatment. **b**) Ratio of time spent interacting with novel social target is decreased in SS mice. **c**) Ratio of time spent interacting with novel social target were consistent between treatment groups. **d**) *Cldn3* levels are unchanged in stressed mice, but still correlate with social interaction ratio. **e**) Volume of co-localized Cldn3 protein with epithelial cell marker CD326 per villi (ROI) did not significantly differ in stressed mice. **f**) Volume of CLDN3 protein was unchanged in stressed mice post-CSDS and between groups. **g**) Representative immunofluorescent images of Cldn3 in each phenotype group. Data represents mean ± S.E.M; number of animals (n) is indicated on graphs. One-way ANOVA followed by Bonferroni's multiple comparison test for two-group comparisons; Two-way ANOVA for analysis of treatment groups; Correlations were evaluated with Pearson's correlation coefficient. *****P<.0001.*

Social defeat alters intestinal expression of inflammatory markers in males

After observing stress-induced alterations in JEJ tight junction gene expression patterns and microbiota we sought to further investigate if the inflammatory mechanisms were at play. Given the functional implications of increased inflammation and inflammatory pathway signalling in MDD and CSDS models, tied to clinical symptoms and depression-like behaviour respectively [49,312,340,341]. Therefore, we examined the JEJ for the relative mRNA expression of various cytokines and chemokines (**Fig. 15a**). Socially stressed mice had

diminished *Tnfα* (*P=.008*), *Il1b* (*P=.025*) and *Cxcl4* (*P=.044*) in the JEJ (**Fig. 15b**). Comparison of phenotypes identified between group differences for *Cxcl4* (*F (2, 26)* = 5.185, *P=.013*), *Tnfα* (F (2, 28) = 8.205, *P=.002*) and *Il1b (F (2, 27) = 6.242*, *P=.006*) as well. Follow up post hoc test confirmed downregulation in SS mice of *Cxcl4* (*P=.019*) compared to controls (**Fig. 15c**). As well, low *Tnfa* and *Il1b* was occuring in SS mice compared to both RES (*P=.002*, *P=.035*) and control mice (*P=.032, P=.01*), respectively **Fig. 15c**. Social avoidance correlated positively with inflammatory factors *Cxcl4* (*P=.001, r=0.57*), *Tnfα* (*P=.002, r=0.55*) and *Il1b* (*P=.0003, r=0.62*), **Fig. 15d**. However, no treatment effects were detected for any of the genes investigated (data not shown). Identified correlations between expression for inflammatory and intestine barrier genes include a positive relationship between *Cldn3* with *Tnfα* (*P=.0043*, *r=0.51*, **Fig. 15e**), *Cldn3* with *Cxcl4* (*P=.023*, *r=0.41*, data not shown), *Cldn7* with *Cxcl4* (P=*.001*, *r=0.58*, **Fig. 15e**), *Tnfa* (*P=.003*, *r=0.53*, data not shown) and *Il18* (*P=.0006*, *r=0.59*, data not shown). Finally, positive correlations were observed between intestinal *Ido1* expression with *Cxcl4* (*P<.0001*, *r=0.78*, **Fig. 15e**), with *Tnfα* (*P<.0001*, *r=0.67*, data not shown), *Il1b* (*P=.0004*, *r=0.62*, data not shown). These fluctuations of inflammatory gene expression in the JEJ and correlations with tight junction gene expression could corroborate the concept that stress induces intestinal permeability through inflammatory pathways.

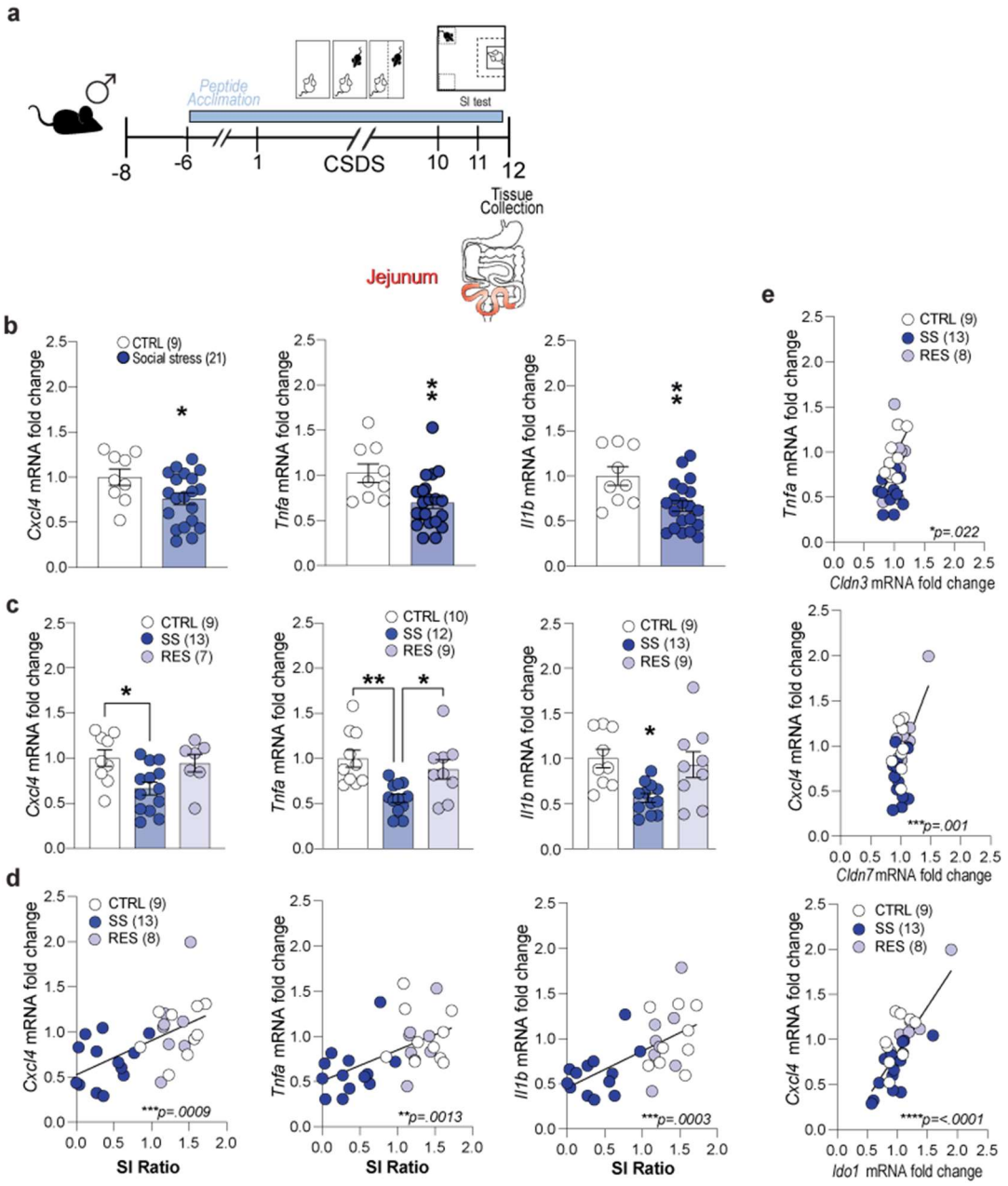

Figure 15. Impact of chronic stress on intestinal inflammatory gene expression.
a) Schema of male CSDS with vehicle or peptide treatment. **b**) Social defeat stress lessen mRNA expression of inflammatory factors *Tnfα*, *Il1b* and *Cxcl4* in the jejunum. **c**) Decreases in *Tnfα*, *Il1b* and *Cxcl4* were specific to SS mice. **d**) Inflammatory markers show a positive relationship with SI ratio. **e**) Correlations of selected inflammatory markers vs tight junctions. Data represents mean ± S.E.M; number of animals (n) is indicated on graphs; One-way ANOVA followed by Bonferroni's multiple comparison test for two-group comparisons; Correlations were evaluated with Pearson's correlation coefficient. **P<.05; **P<.01, ***P<.001, ****P<.0001*

Impact of chronic social stress on peripheral markers in male mice

Adjustments in tight junction expression may reflect permeability alterations, thus we explored peripheral markers as indicators possibly distinguishing mice exposed to stress, or

the distinct phenotypes. We also chose to evaluate inflammatory markers as well as a marker of gut permeability in our mice and check if there were any effects of the treatments. The serum of males (n=28) before and 48h post-CSDS was measured for LBP concentration with an ELISA (**Fig. 16a**). Serum LBP levels were distinct between groups post-CSDS (*H (2) =8.631, P=.013*). Follow up tests validated the outcome was propagated by SS mice compared to CTRLs (*P=.015*) (**Fig. 16a**). The same pattern of group differences emerged when circulating LBP levels were controlled for individual baseline levels (*H (2) =7.141*, *P=.028*). Again, there were specific elevations in SS mice (*P=.023*, **Fig. 16c**). No differences between groups were seen in pre-CSDS serum levels as expected (*H (2) =0.6107*, *P=.74*), **Fig. 16d**). The serum concentration of LBP post-CSDS depreciated with SI ratios (*P=.0006, r=−0.61*), **Fig. 16e**. No effect for vehicle or peptide treatment was detected on serum LBP levels (**Fig. 16f**). However, there was an interaction (stress×treatment, *P=.014*). Increased LBP concentration in SS mice compared to CTRL (*P=.0005*) and RES mice (*P=.005*) was seen exclusively in vehicle-treated mice (**Fig. 16f**). A trend (*P=.0626, r=−0.86*) of a negative association that did not reach significance was observed in vehicle-treated SS mice which does not occur in SS mice treated with peptide (**Fig. 16g**). Considering serum LBP was not elevated in peptide-treated SS mice, this may indicate a potential effect of the peptide treatment in blunting a leaky-gut induced peripheral response.

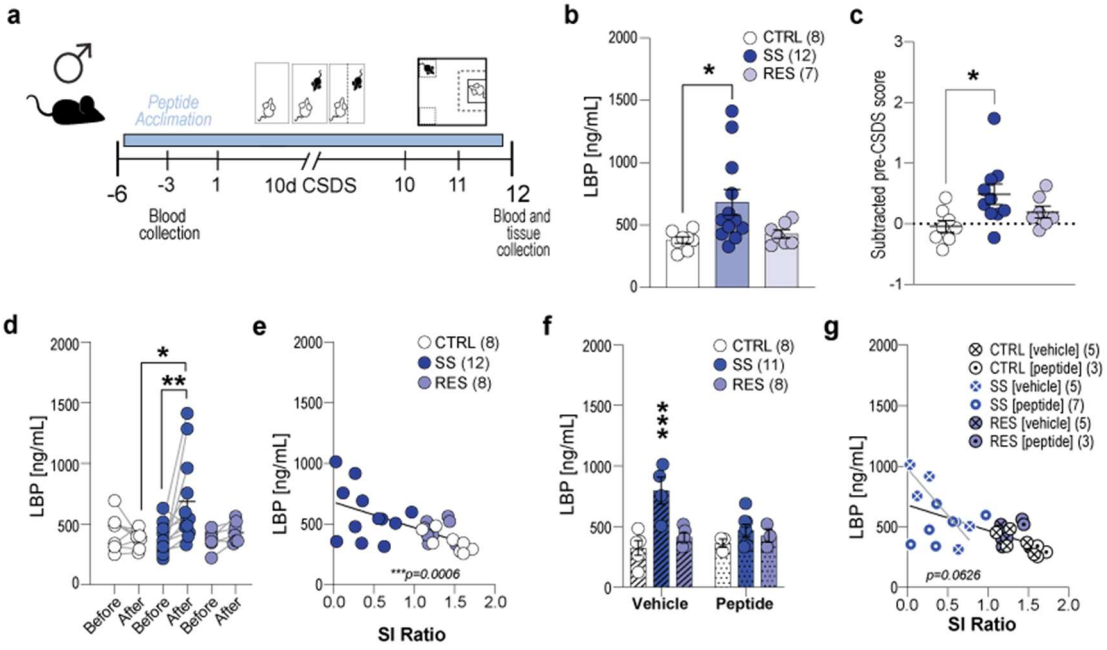

Figure 16. Social defeat alters serum levels of lipopolysaccharide binding protein in male mice
a) Timeline of blood collection during CSDS paradigm for serum analysis for lipopolysaccharide binding protein (LBP) concentration. **b**) Elevated circulating LBP in SS mice. **c**) Upregulation in SS mice is maintained when controlled for baseline LBP levels. **d**) Pre-treatment LBP levels did not differ between groups and only increased in SS after CSDS. **e**) LBP levels correlated negatively with social interaction ratios. **f**) Treatment with peptide normalized social stress-induces LBP elevations in SS mice. **g**) A trend of negative association between serum LBP and social interaction ratio in vehicle-treated SS mice only. Data represents mean ± S.E.M; number of animals (n) is indicated on graphs. One-way ANOVA followed by Bonferroni's multiple comparison test for two-group comparisons; Two-way ANOVA followed by Bonferroni's multiple comparison test for treatment effects; Correlations were evaluated with Pearson's correlation coefficient. **P<0.05; **P<0.01.*

Following LBP analysis, the serum levels of multiple cytokines were also evaluated in these mice using the Bio-Plex Pro mouse cytokine group I 23-plex to compare peripheral inflammation between stress exposure and potentially detect any repercussion of treatment condition. The levels of GM-CSF, IL-2, IL-3 and IL-13 were below the detection limit. Male mice who experienced CSDS display high Granulocyte colony-stimulating factor (G-CSF; *P=.006*), IL-6 (*P=.005*) and keratinocyte-derived chemokine-1 (CXCL1; *P=.005*) compared to controls (**Fig. 17a, b, c**). Also, a trend of elevated IL-1β was notable in CSDS-exposed mice that did not reach significance (*P=.053*, **Fig. 17d**). Between-group differences were demonstrated in G-CSF (*H(2)=8.837*, *P=.007,* **Fig. 17e**), IL-6 levels (*H(2)=8.837, P=.005,* **Fig. 17f**), CXCL1 (*H(2)=11.35*, *P=.003,* **Fig. 17g**), IL-1β (*H(2)=6.459, P=.03,* **Fig. 17h**). These differences were all driven by an increase in SS mice compared to controls (G-CSF, *P=.01*; IL-6*, P=.009;* CXCL1, *P=.002;* IL-1β*, P=.044*). Assessment of treatment groups did not yield significant effects (data not shown). An inverse relationship was seen between SI ratio and serum levels of G-CSF (*P=.005, r=–0.43*), IL-6 (*P=.0005, r=−73*), CXCL1 (*P=.0003, r=–0.63*) and MCP-1 (*P=.018*, *r=−0.51*) **Fig. 17i**. Contrarily, IL-12 levels increased with SI ratio (*P=.013*, *r=0.49*, **Fig. 17i**). Overall, the result conveys that peripheral inflammation coincides with depression-like behaviour after chronic stress.

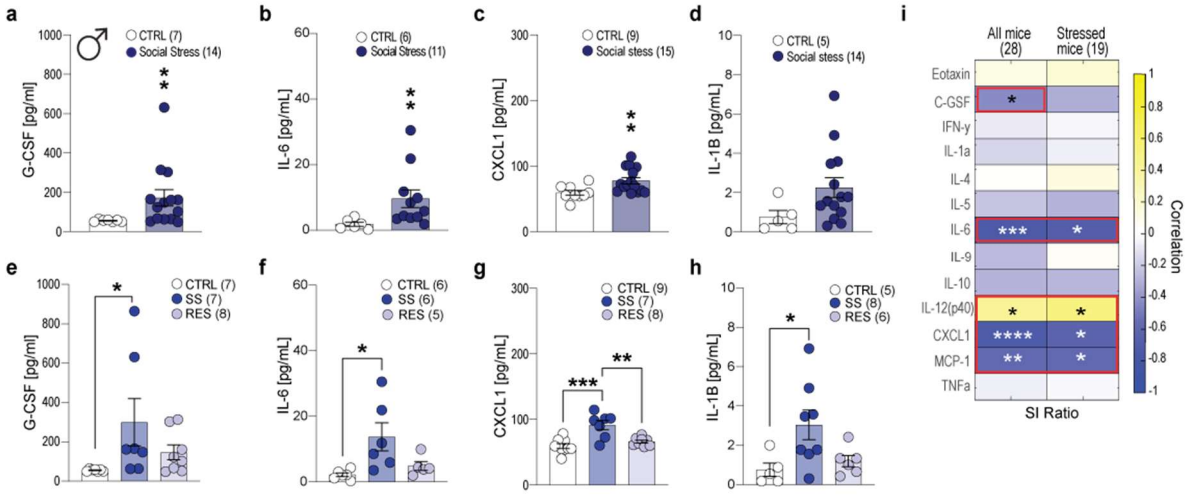

Figure 17. Stress susceptible male mice have increased peripheral inflammation.
Serum analyzed with multiplex for cytokine and chemokine concentrations show increased **a**) G-CSF, **b**) IL-6 and **c**) CXCL1 in male mice following social defeat. **d**) A trend of heightened IL-1β instressed mice that did not reach significance. Upregulation specifically occurs in SS mice for G-CSF (**e**), IL-6 (**f**) CXCL1 (**g**) and IL-1β (**h**). **i**) Social avoidance behaviour correlated with many inflammatory markers. Red squares highlight significant changes. Colour scale indicates directionality and size of correlation. Data represents mean ± S.E.M; number of animals (n) is indicated on graphs. Two-group comparisons were evaluated with Mann-Whitney test or Kruskall-Wallis test followed by Dunn's multiple comparison test; Correlations were evaluated with Pearson's correlation coefficient. **P=.05; **P<.01; ***P<.001, ****P<.0001.*

Social stress alters expression of inflammatory markers in mood-related brain regions

The NAC is an important stress-mediating region implicated in MDD [12] and the BBB in this area is vulnerable in male mice after CSDS exposure [32]. Therefore, we evaluated the expression of tight junctions, tight junction associated proteins and inflammatory markers in the NAC of male mice following CSDS (**Fig. 18a**). We also investigated the PFC to compare regions specific effects since recently, we showed that the PFC is more vulnerable to chronic stress in females and the NAC in males [90]. We also sought to characterize any potential far reaching treatment effects of the vehicle or peptide treatment. In the NAC, stressed males had lower gene expression of *Cldn5* (*P=.015*), *Tjp1* (*P=.007*), *Tjp2* (*P=.039*), *Tjp3* (*P=.016*) and *Marveld2* (*P=.004*), **Fig. 18b**. Notably, *Cldn5* was downregulated in the stressed group, although it was unchanged between SS and RES mice (**Fig. 18c**). Differences between phenotype group mean were identified for *Tjp1* (*F(2,27)=10.97*, *P=.0003*), *Tjp2* (*F(2,28)=3.856*, *P=.03*), *Tjp3* (*F (2, 27) = 4.281, P=0.03*) and *Il33* (*F (2, 28)=* 4.089, *P=.027*) (**Fig. 18b**). Specifically, RES mice had lower *Tjp2* (*P=.029*) and *Tjp3* (*P=.021*) than controls. *Tjp1* expression was lower in both SS (*P=.048*) and RES (*P=.0002*) compared to controls. A trend of lower *Mfsd2a* (*P=.097*) expression in RES mice appeared that did not reach significance (data not shown). For the immune genes investigated, *Il33* was decreased

in SS (*P=.016*). A trend of lower *Il6* (*P=.081*) expression in SS mice occurred that did not reach significance (data not shown). *Il33* expression in the NAC increased along with SI Ratio (*P=.0009, r=0.58*) as did *Il6* expression (*P=.03, r=0.41).* Contrarily, *Cxcl4* expression showed the opposite relationship with the highest levels being associated with the lowest interactions with the social target (*P=.047, r=−0.38*), **Fig. 18d.** Analysis of treatment effects showed that peptide-treated mice had a decrease in *Cldn5* following CSDS (*P=.024*) that was not maintained in vehicle-treated mice (interaction treatment × stress: *P=.037*). A similar pattern was seen for *Marveld2*, with a decrease in peptide treatment mice in stressed group vs. peptide treated unstressed controls (*P=.017*, interaction treatment × stress: *P=.028),* **Fig. 18e**. No other effects of either treatments were seen.

Transcriptional profiling of the same gene panel in the PFC revealed region specific modifications. All markers remained consistent between stressed and CTRL males, as well as between phenotype groups in the PFC (**Fig. 18f**). In peptide-treated mice, we saw an increase in *Ahr* for RES group compared to both SS (*P=.009*) and CTRL mice (*P=.03*), that was not replicated in untreated mice. For *Ahr*, the treatment contributed 10.92% of the between group variation and interaction effect was contributing to another 24.09% (interaction treatment × stress: *P=.0096),* **Fig. 18i**. A significant positive correlation was seen for *Il33 (P=.014*, *r=0.24*), and a negative correlation for *Marveld2* (*P=.023*, *r=−0.41*), **Fig. 18h**.

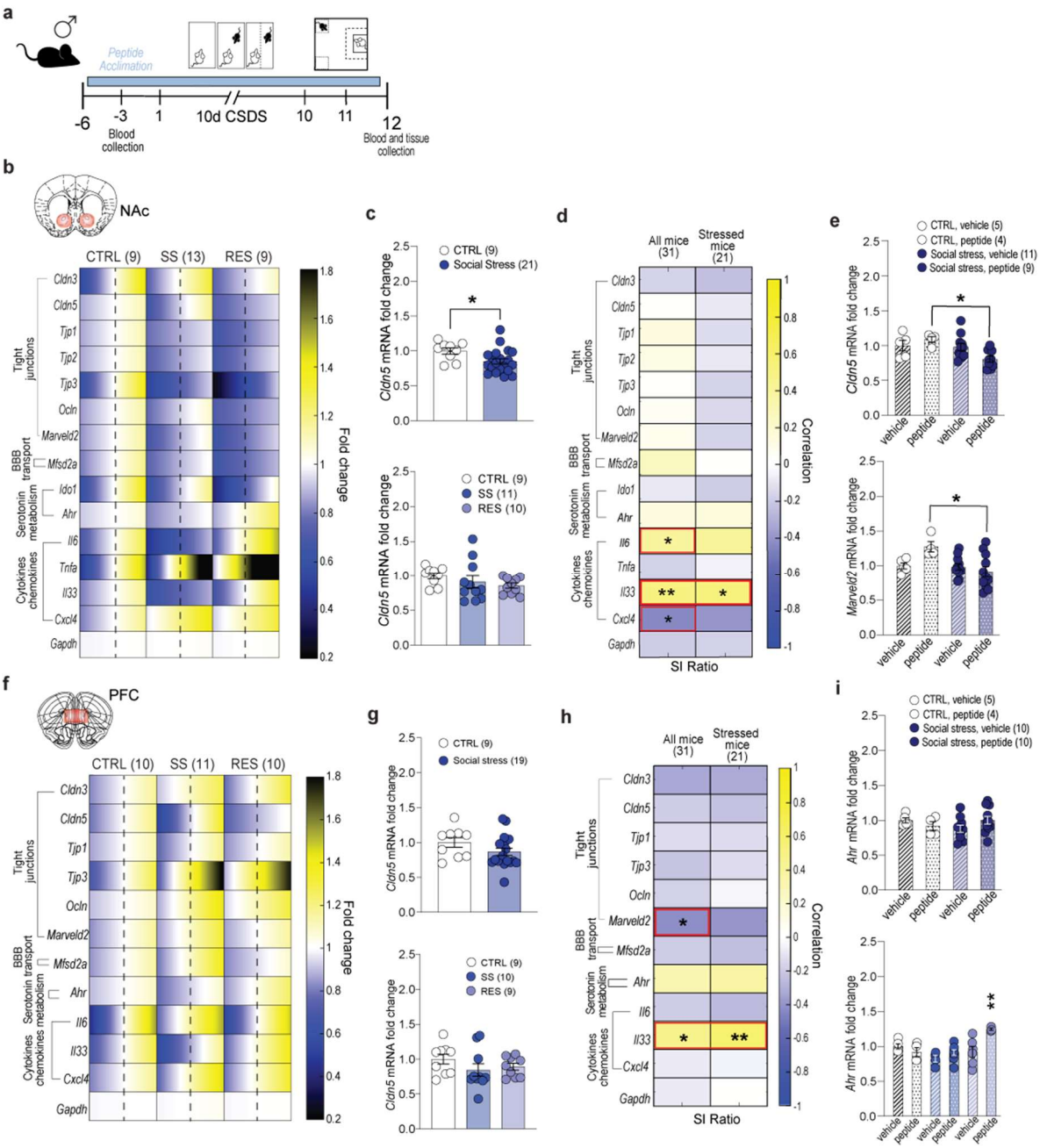

Figure 18. Social defeat induces region-specific changes in neurovascular and inflammatory genes in males.

a) Schema of CSDS protocol with male mice receiving treatment. **b**) Significant changes in the NAC of SS and RES mice for gene expression related to tight junctions, tight junction associated proteins and inflammatory markers. **c**) Decreased *Cldn5* expression in the NAC of stressed male mice. **d**) Several patterns of cytokines and chemokines correlated with SI ratio. Red boxes highlight significant correlations. **e**) Peptide-treated mice have less *Cldn5* and *Marveld2* expression following CSDS compared to peptide-treated controls. **f**) Quantitative PCR of genes in the PFC reveals region-specific changes in stressed male mice vs controls. **g**) *Cldn5* expression was unchanged in the PFC. **h**) Gene expression in the PFC vs. SI ratio. Red boxes highlight significant correlations **i**) An interaction of treatment × phenotype effects *Ahr* expression. Data represents mean ± S.E.M; number of animals (n) is indicated on graphs. Two-way ANOVA followed by Bonferroni's multiple comparison test for treatment groups; 2-group comparisons were evaluated with Mann-Whitney tests; for heatmap, the range of color indicates individual differences within a group; S.E.M. from the average represented by the dashed line. **P<.05; **P<.01.*

Intestinal tight junction expression changes in females after subchronic variable stress with peptide treatment.

As in previous SCVS cohorts, we compared transcriptional profiling in the JEJ with the addition of treatment groups possibly reveal sex-specific effects of treatment efficiency (**Fig. 19a**). SCVS upregulated *Tjp1* (*P=.0016,* **Fig. 19b**) and downregulated *Cldn3* (*P=.03,* **Fig. 19b, c**) in the JEJ. *Cldn5* and *Pai-1* were additionally investigated in this cohort that were previously not looked at in the gut. Decreased *Cldn5* expression (*P=.02*) occurred in SCVS-exposed mice with simultaneous increase of *Pai1* (*P=.016),* **Fig. 19b**. A trending decrease in *Cldn3* was observed in peptide-treated stressed mice compared to vehicle-treated controls but it did not reach significance (*P=.059*, **Fig. 19d**). Otherwise, no significant treatment effects appeared on the genes tested except for *Il1b*. Inflated *Il1b* expression in the peptide-treated mice following stress compared to both control groups (*P=.047*, *P=.021*) was not replicated in vehicle-treated stressed mice (**Fig. 19e**). To confirm if any Cldn3 changes were occurring at the protein level, CLDN3 was assessed by immunostaining with actin filament marker (F-actin). No differences were detected in the JEJ of SCVS-exposed mice compared to control females in the total volume of CLDN3 (μm^3), **Fig. 19f, g**. Further, the volume of CLDN3 was unchanged between treatment groups as well **Fig. 19f**.

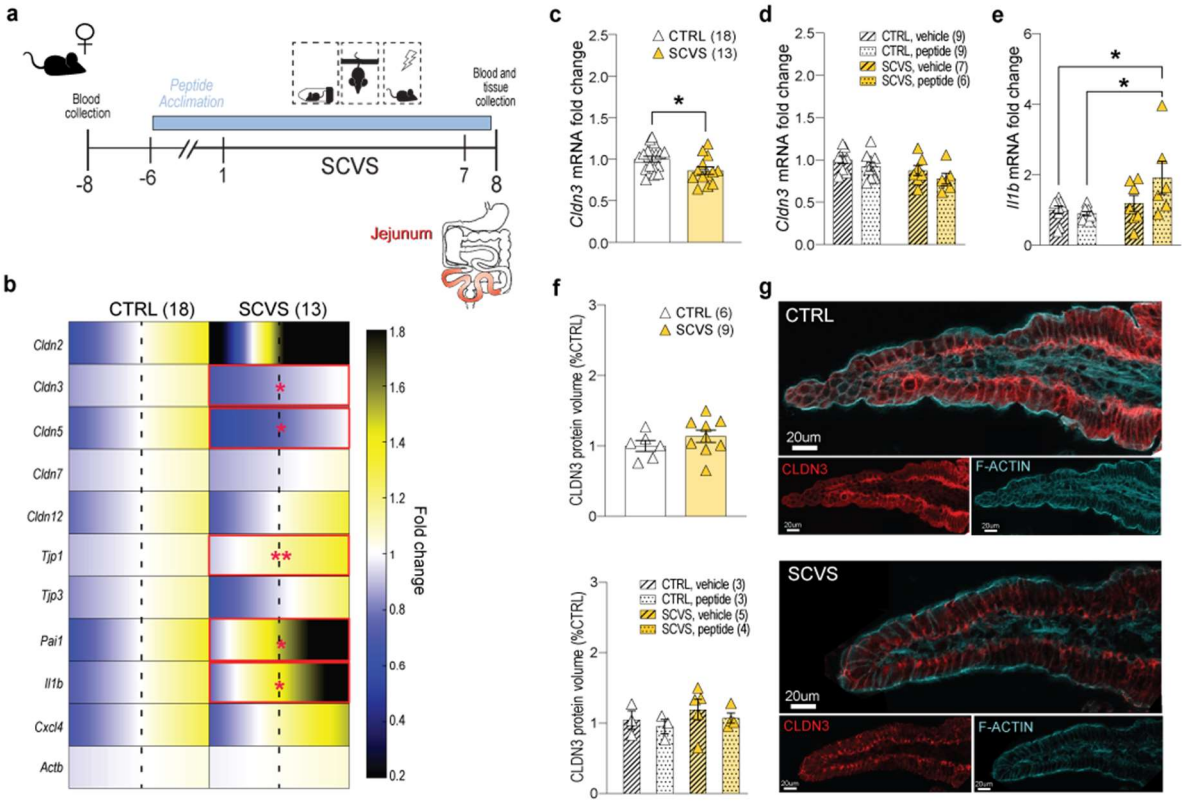

Figure 19. Chronic variable stress does not alter peripheral lipopolysaccharide binding protein in females.
a) Schema of blood collection during subchronic variable stress with female mice receiving treatments **b**) Post-CVS circulating LBP concentration was unchanged between stress and control animals. **c**) No significant differences between stress and control animals were seen when controlled for baseline LBP levels. **d**) No effects of treatment response on LBP levels were revealed. Data represents mean ± S.E.M; number of animals (n) is indicated on graphs. Two-way ANOVA followed by Bonferroni's multiple comparison test for peptide treatment; 2-group comparisons were evaluated with unpaired t-tests.

Subchronic variable stress does not alter serum LBP in females

The serum concentrations of LBP were determined with ELISA in female mice pre- and post-SCVS in groups receiving treatment (**Fig. 20a**). The post-CSDS serum LBP concentration was not distinct in stressed mice compared to controls (**Fig. 20b**). No changes in post-CVS vs. unstressed control concentration were seen when controlled for baseline LBP serum levels (**Fig. 20c**). Furthermore, no significant differences were detected between groups with peptide treatment (**Fig. 20d**).

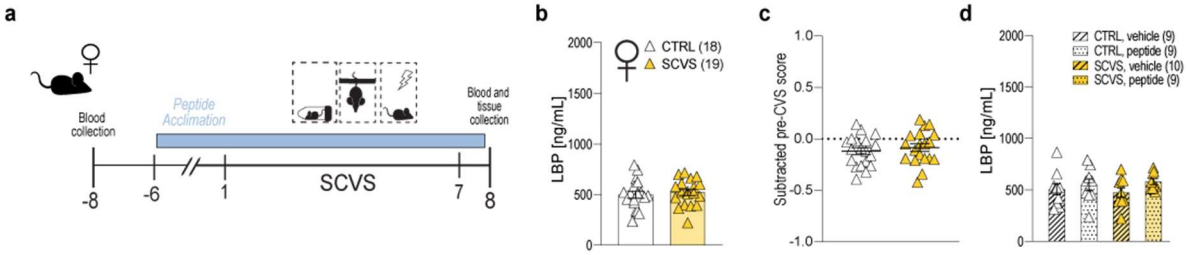

Figure 20. Chronic variable stress alters intestinal inflammatory markers in the jejunum of females
a) Schema of subchronic variable stress with female mice receiving treatments. **b**) Transcriptional profiling reveals changes in the jejunum of stressed mice for gene expression related to tight junctions, tight junction associated proteins, and cytokines. **c**) Decreased *Cldn3* expression in stressed mice. **d**)A trend of lower Cldn3 expression in peptide treated stressed mice that did not reach significance **e**) Increased jejunal *Il1b* expression following SCVS in mice receiving peptide treatment. **f**) CLDN3 protein levels are unchanged following SCVS and no effects of treated are seen. **g**) Representative immunofluorescent staining of CLDN3 and F-actin in SCVS exposed and control animals. Data represents mean ± S.E.M; number of animals (n) is indicated on graphs. Two-way ANOVA followed by Bonferroni's multiple comparison test for treatment groups; 2-group comparisons were evaluated with unpaired t-tests; *$P<0.05$; **$P<0.01$.

Subchronic variable stress alters brain tight junction mRNA expression in females.

Recently, in work from our lab, we showed that SCVS induces behavioural and neurovascular modifications in female mice [90]. Thus, we did transcriptional profiling on genes of tight junctions, tight junction related proteins and inflammatory molecules to confirm these changes in females exposed to SCVS while also exploring any that may be initated by treatment group (**Fig. 21a)**. SCVS altered the expression of various tight junctions in the NAC of females (**Fig. 21b**), including upregulated *Tjp1* (*P=.0098;* **Fig. 21b**) and *Cldn3* (*P=.0008*, **Fig. 21c**) and down-regulated *Cldn5* (*P=.012,* **Fig. 21d**) and *Il33* (*P=.014,* **Fig.**

21e). There was a trend of increased *Cxcl4* in SCVS exposed mice that did not reach significance (*P=.085,* **Fig. 21b**). Comparison of treatment groups revealed a reduction of *Il33* in the vehicle-treated stressed group vs. vehicle-treated unstressed controls (*P=.008*) that was alleviated in the peptide-treated stressed group (interaction treatment × stress: *P=.032,* **Fig. 21f**). Transcriptomic profiling in the PFC shows region-specific effects in females as well (**Fig. 21g**). As in the NAC, SCVS upregulated *Cldn3* expression in the PFC of stressed females (*P=.036*, **Fig. 21h**), however *Cldn5* was not significantly decreased in this region (*P=.176*, **Fig. 21i**). Though there was no treatment effects or interaction effects on *Cldn3*, there was an increase in *Cldn3* in vehicle-treated stressed mice compared to vehicle treated controls (*P=.041*). This alteration is not maintained in the peptide-treated stressed mice, suggesting there may be an effect from the treatment on this gene (**Fig. 21j**).

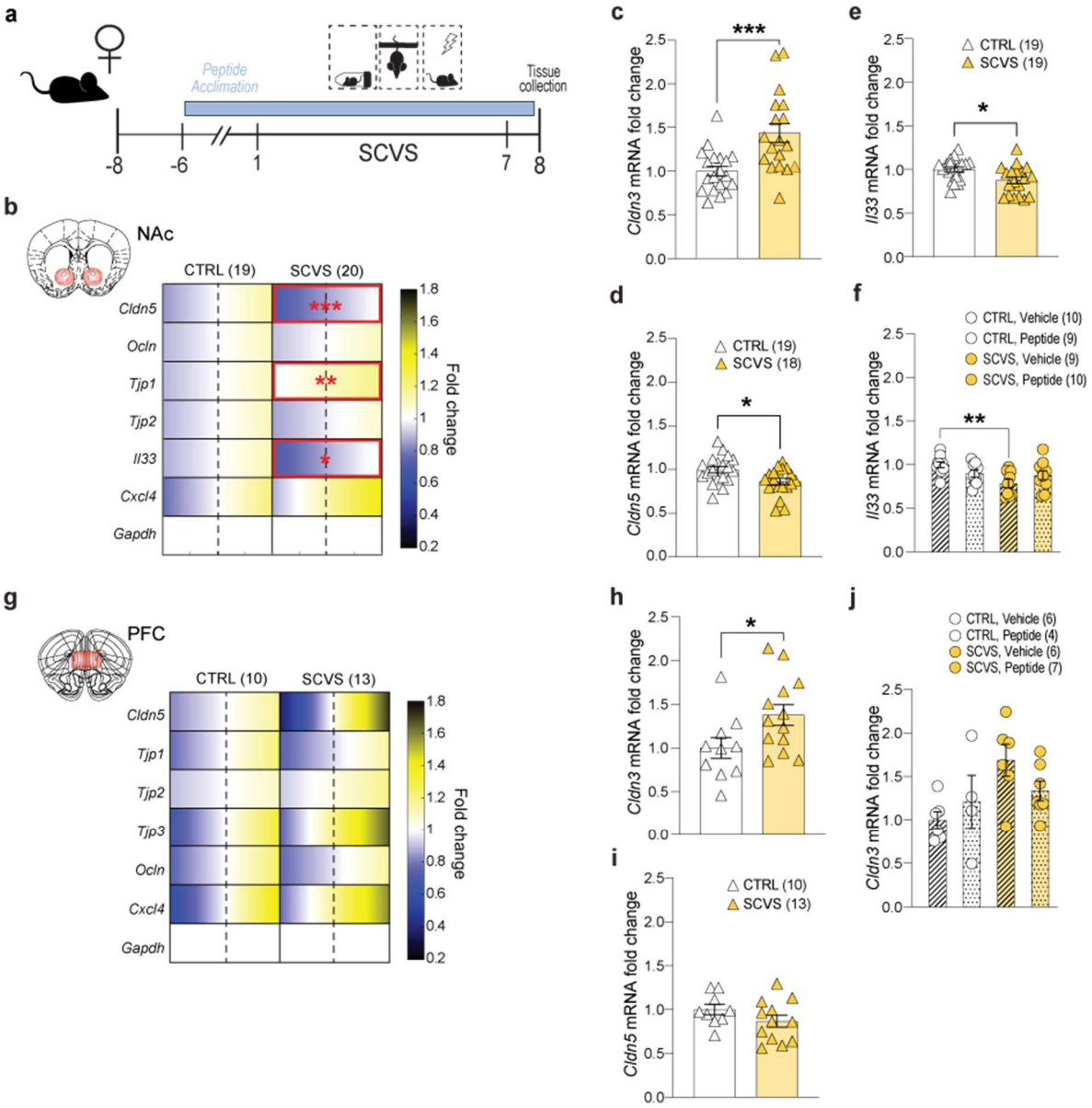

Figure 21. Subchronic variable stress induces region-specific changes in neurovascular and inflammatory genes in females

a) Schema of subchronic variable stress protocol in female mice receiving vehicle or peptide treatment. **b**) Quantitative PCR revealed significant changes in the NAC of stressed mice in gene expression related to tight junctions, tight junction associated proteins and inflammatory markers. Red boxes highlight genes with significant changes. **c**) Enhanced *Cldn3* expression in the NAC of SCVS-exposed mice. **d**) Reduced *Cldn5* and *Il33* (**e**) in the NAC of stressed animals. **f**) Decreased *Il33* in stressed mice shows a normalization with peptide treatment. **g**) Gene expression in the PFC of stressed and SCVS exposed females. **h**) Cldn3 expression was increased in the PFC after SCVS. **i**) Cldn5 expression was unchanged in the PFC after SCVS. **j**) Stressed mice had a rise of *Cldn3* in the PFC compared to controls in the vehicle treated group that was not maintained in the peptide treated group. Data represent mean ± S.E.M; number of animals (n) is indicated on graphs. Two-way ANOVA followed by Bonferroni's multiple comparison test for treatment effects; 2-group comparisons were evaluated with Mann-Whitney tests; for heatmap, the range of color indicates individual differences within a group; S.E.M. from the average represented by the dashed line; **P<.05; **P<.01; ***P<.001.*

Discussion – Chapter 2

Peptide treatment on chronic stress-induced behavioural response and intestinal barrier characteristics

In line with the collaborative effort to test a novel therapeutic approach for protecting intestinal permeability, we introduced a peptide treatment during our chronic stress paradigms. The purpose of the treatment is to structurally stabilize and reduce gut permeability by coating the intestinal tract. We anticipated that any positive effects on the gut may result in a higher ratio of RES mice. In contrast, combined, the vehicle/ peptide-treated cohort had more SS animals then in the previous cohorts. The range of normal SI ratio spread is varied and depending on the source, therefore our results here could still be within a normal range [151,152]. Still, the SI scores of SS mice indicated higher overall avoidance behaviour than the previous cohorts as well. Many factors can influence the level of severity of aggression throughout a CSDS experiment. Here, different individuals conducting the first cohorts vs. treatment cohorts may have contributed to varying intensity. Furthermore, CD-1 aggressor mice show high variability in their levels of aggression and despite conducting aggression test prior to commencing, it is still difficult to maintain consistency throughout each cohort.

The CSDS-induced intestinal barrier gene alterations were anticipated to remain consistent to previous cohorts in the stress vs. control comparison, with altered effects by successful treatment in the peptide-treated group. This was not the case for many genes which may be related to the altered phenotype split in this cohort. The initial CSDS-induced *Cldn3* elevated was not replicated in the treatment cohort. On the other hand, we also saw differential *Cldn3* expression depending on type and duration of stress since long-term CVS reduced expression. This may provide insight on dynamics in the CSDS-treatment cohort, since protocol adjustments made to integrate the treatment added to the duration of the stress and possibly the intensity as well. Indeed, for 6 days prior to stress initiation, all the animals in this cohort had water access removed, acclimated to consuming a hydrogel, were manipulated daily during weighing session and were socially isolated. Thus, the added stressors may have enhanced the CSDS effects to be more comparable to long-term CVS. In this way, the characterization of *Cldn3* may not reflect a typical CSDS or SCVS cohort.

Nonetheless, we performed immunostaining for Cldn3 at the protein level since mRNA fluctuations do not always reflect the same results as protein quantification. Imaging showed unaltered CLDN3 as well, although there was a trend of increased levels in stressed mice with patterns that resembled the previous male CSDS cohort. Regardless, claudin shifts are not simply restricted up or downregulation, rather, tight junction structures and functions are highly dynamic. At the cell border, tight junctions typically display as linear structures, however, adjustments in claudin interactions with underlying scaffold and cytoskeletal proteins alter these shapes. The transition processes are often called ruffles and spikes, as described in a recent review of these morphologies [342]. Briefly, ruffles present as a zig zag shaped border between cells. Heightened ruffles may be indicative of manipulation of tight junction proteins through molecular signalling pathways, induced by various stimuli such as hypoxia or direct mechanical stimulation. These areas have been quantified using the "zig-zag index" and increases often reflect enhanced paracellular permeability [343]. Tight junction spikes are areas where vesicle budding and fusion occur, appearing as inward projections from the border. While analyzing our images, some of these patterns were noticed although not quantified. It would be relevant for future investigations to include this type of analysis to discriminate more dynamic Cldn3 alteration that may be occuring.

In females, shifts previously seen following SCVS such as upregulated *Cldn3* and *Tjp1* in the JEJ were maintained in the treated SCVS cohort. However, some inconsistencies occurred, such as the previously upregulated *Tjp3* and *Cldn12*. In fact, the latter was altered in the opposite direction in this cohort. Similarly, to what was stated in males, this possible was related to level and duration of stress. Moreover, the neutral hydrogel supplemented to all animals is a common supplement that should not have negative effects; however, we cannot rule out downstream implications of this product on the intestine through overall hydration or otherwise. Furthermore, typically mice are group-housed during SCVS, while in this case we needed to monitor the individual consumption of the hydrogel to ensure sufficient hydration or intake of the required peptide dose. Therefore, individual housing was necessary, but it is known that social isolation is a significant stressor for mice, especially in females. Thus, social isolation could have influenced the behaviours and outcomes in this cohort due to aggravated stress compared to our previous cohorts of study 1 and in published studies.

Peptide treatment on chronic stress-induced intestinal inflammation

Otherwise, in male mice, there were no indications that the peptide treatment may have affected tight junction gene expression in the gut. For the first time, in this cohort we also evaluated local expression of inflammatory genes inspired by intestinal biopsy results from an IBD study showing mRNA expression of inflammatory markers were augmented coincident with clinical diagnosis of severity [66]. We suspected that chronic stress exposure would similarly increase intestinal expression of inflammatory markers, specifically in SS mice. We also predicted that these effects would be negated in the peptide-treated group. To our surprise, stressed mice incurred a marked reduction of relative expression for many inflammatory markers (*Tnfa*, *Cxcl4* and *Il1b*) in the JEJ, particularly for SS mice. Furthermore, a positive relationship with SI ratio indicated that the most socially avoidant mice had the lowest inflammatory marker expression. When comparing the treatment groups, we saw no evidence of peptide treatment effects on inflammatory gene expression in the gut. Unfortunately, we did not previously test inflammatory genes throughout our stress cohort comparisons in Study 1, therefore we did not have a baseline from which to compare. Some inflammatory molecules have reparative mechanisms, like Cxcl4 which recruit leukocytes to sites of inflammation [344], so perhaps the decrease in SS mice is indicative of an absence of repair initiation in the gut. Another possibility that should be confirmed is if acute stress would raise these markers in males in case the effect we see after chronic stress is a suppression over time. Unfortunately, we could not identify other investigations of inflammatory markers in the intestine following chronic stress models to derive insight. Thus, we highlight the importance of future studies into these relationships.

The fluctuations to inflammatory genes in males post-CSDS were not maintained in the female JEJ. *Cxcl4* was unaltered in the JEJ and *Il1b* was affected but in the opposite direction compared, which may highlight a sex-specific mechanism. Potential treatment effects on tight junctions in the gut of female mice were not identified. Inflammatory genes changes were limited to inflated *Il1b* in peptide treated animals compared to both unstressed groups. This is contra intuitive to what would be expected if indeed the treatment was reinforcing the intestinal barrier. However, the *Cxcl4* expression was decreased in stressed untreated animals, and seemingly re-established in the stress group receiving peptide, indicating a potential inflammatory modulation effect.

Treatment implications for stress-induced region-specific changes in neurovascular and inflammatory genes in males and females.

A growing body of literature connects aberrant GBA signalling in psychiatric disorders, particularly in MDD. Paired with the evidence linking gut permeability and BBB leakiness in this context [166], we were motivated to confirm whether the peptide supplement would have downstream effects on the brain, precisely on the expression of markers from our previous accounts in the NAC and PFC of male and female mice. Until recently, the BBB and gut barrier had not been compared directly, however, a study emerged compared these in female rats facing social isolation stress [311]. Focusing on the PFC and the most distal part of the small intestine (the ileum) in females, they found shared changes between the two regions in *Ocln*, *Tjp1* and *Cldn5* gene expression. Therefore, we expected our changes to reflect these effects with comparison of genes shared in both BBB and the gut barrier to reflect similar alterations and directionality of effects.

In males who underwent CSDS, we saw decreased *Cldn5* in the NAC, though it was not specific to SS mice as previously seen [32]. In female mice, we also saw decreased *Cldn5* in the NAC after SCVS, corroborating a portion of our findings [345]. Though it *Cldn5* was also previously depleted in the PFC, which we did not see. However, due to processing errors we had to eliminate some PFC tissue samples, therefore the smaller samples size may be involved. The modulation of same directionality for *Cldn5* in the NAC and JEJ of stressed female mice is congruent with the Karailiev et al., 2020 report. Interestingly, we also saw augmented *Cldn3* expression in both brain regions which seemed to be a sex-specific modification as it did not occur in males. Furthermore, changes in the brain vs. the gut for Cldn3 had opposing directionality. Although, *Cldn3* is ubiquitously expressed in the small intestine [178], the cell specificity and role in the BBB is still highly debated. *Cldn3* is considered to have a very low expression in the BBB endothelial cells [185,346,347], however, others suggest it is uniquely expressed in the epithelial cells of the choroid plexus [183]. Mice deficient in Cldn3 still have intact BBB [184], compared to a lack of Cldn5 which is detrimental [348]. Nevertheless, Cldn3 plays an important role in the blood–cerebrospinal fluid barrier of the choroid plexus, a region where leukocytes pass prior to accessing the BBB [346]. Here Cldn3 has been implicated in neuroinflammatory conditions as selective loss of choroid plexus Cldn3 occurs in postmortem tissue from multiple sclerosis patients along with increased

inflammation (CD45+ leukocytes, VCAM-1 and CCl2). Knockout of Cldn3 in multiple sclerosis model enhances clinical signs and exacerbates inflammation in the choroid plexus [346]. These studies highlight a potential role for Cldn3 either directly or indirectly in maintaining BBB integrity. More work on the specific function, localization, and dynamics of Cldn3 and other claudins and moreover, their role in pathogenesis in respective tissues is warranted

Chronic stress on peripheral markers of inflammation and intestinal permeability

Clinical studies of MDD and IBD report augmented inflammatory cytokines in circulation [66]. Therefore, we sought to investigate the expression of inflammatory markers in the blood of stressed mice. We saw elevated concentrations of many inflammatory markers in the serum, mostly propagated by SS mice and not RES. Multiple high values were seen in the SS group and were considered outliers, however, these exceedingly elevated scores corresponded with wound scores of higher severities at the time of sacrifice. Therefore, inflation of inflammation due to wounding acquired during the physical stress likely had an influence. However, with the outliers eliminated, there was still a significant upregulation for CXCL1 and IL-6. The results are compatible with clinical evidence in MDD patients [99,102,138,341](see **Table A3**) as well as SS mice following CSDS [63]. No significant effects of treatment were seen; however, many out of range values occurred from the plate reading, and therefore a conclusion could not be made for many of the markers due to insufficient group size. Still these markers could be important to revisit in the context of assessing a gut-targeting treatment. We saw changes correlating with avoidant behaviour for serum protein levels of C-GSF, IL-6 and CXCL1. The latter two have been reportedly increased in MDD patients [340,349–351] and also in mice following CSDS [63]. Furthermore, IL-6 levels had a strong negative correlation with social interaction behaviour [63], matching our data. Limited investigations exist on serum C-GSF in MDD, but some show no fluctuations in depression [352] while another had significant elevations in treatment-resistant depression patients specifically [100]. Interestingly, C-GSF therapy is used as a supportive treatment in GI disorders and treatment in rats during chronic unpredictable mild stress improves depression-like behaviors likely through effect on neuronal plasticity [353]. This effect shows interplay between the gut and the brain and could be relevant for potential gut related therapeutic target for treatment resistant patients.

As previously discussed, there are many options to consider as potential indirect markers of intestinal permeability or microbial dissemination, each provide certain intrigue and downsides. We chose LBP due to its specificity for LPS endotoxin and its nature of providing a chronic measure. Indeed, LBP rises were specific to SS mice after CSDS in males whereas we did not see shifts in females following SCVS, highlighting its potential as a sex-specific biomarker. However, confirming levels in a CSDS model in females would be required. It is possible that the peptide treatment influenced serum LBP levels. Indeed, elevated LBP was seen in vehicle-treated SS males that was not occuring in peptide-treated SS mice. Furthermore, the trending association between LBP and social avoidance was only seen in vehicle-treated SS mice, supporting the concept that peptide-treatment may be blunting intestinal permeability in SS mice. These effects would need confirmation, but they are compelling preliminary findings.

Conclusions and future perspectives

Previously, work by Dr. Caroline Ménard showed that CSDS disrupts the integrity of the BBB in stress-related brain regions and leads to the establishment of depressive behaviors [32]. Reduced BBB integrity and abnormal blood vessel morphology in the brain of SS, but not RES mice resulted in the passage of peripheral pro-inflammatory cytokines and subsequent expression of depression-like behaviors that highlight a direct link between immune response, neurovascular health, and depression [238]. Therefore, lowering stress-related peripheral inflammation may protect the BBB favoring stress-resilience. Chronic stress alters the gut microbiota diversity, promoting intestinal permeability and exacerbating peripheral inflammation [238]. Though, limited studies investigate intestinal health and permeability in mouse models in the context of chronic stress. Therefore, we believe that by adding intestinal analysis to the knowledge obtained for BBB permeability and peripheral inflammatory markers, we provide a comprehensive assessment of leakiness and inflammatory responses associated with depression-like behaviors vs. resilience in mice.

Microbiome

Investigating the microbiota has become increasingly popular, especially in the context of psychiatric disorders. The microbiota has the potential to influence emotion in the context of mood disorders through pathways including inflammation, endocrine, neural, and metabolites (for review, see [166]). Societal transformations to overall dietary habits have influences the dynamics of our microbiotas over time [354]. Westernized diets have limited the diversity with negative links to both intestinal health and mental health [355,356]. Therefore, targeting the gut to bolster current therapeutic interventions is increasingly relevant. Attempts to reinstate a healthy microbiota with probiotics has shown positive results in GI disorders, improving depression scores while modulating peripheral inflammation [131–133]. Interestingly, fMRI analysis showed that *B. longum* treatments reduced responses to negative emotional stimuli in AMY and in the frontal and temporal cortex of patients with IBD [134].

Contributing to the concept that probiotics may complement psychotherapies or improve their efficiencies in MDD, supplemental *Lactobacillus helveticus NS8* in rodents showed similar effects to the SSRI, citalopram, in reducing stress-induced anxiety, depression-like effects and cognitive dysfunction [357]. Probiotic treatments could have resilience-boosting

effects in the face of chronic stress. Indeed, stress resilience was associated with the emergence of *Bifidobacterium* in the host gut following CSDS. Furthermore, *Bifidobacterium* or *Lactobacillus rhamnosus* as prophylaxis enhanced the total number of RES mice [332,358]. Another application of microbiota monitoring could be for therapeutic response. In MDD patients, a decreased representation of *Faecalibacterium* in actively depressed patients subsided in those who successfully responded to treatment [251].

Microbiome studies hold much promise, but there are still hurdles to overcome. The use of alternate sequencing technologies throughout studies, for instance, 16S rRNA gene-sequencing compared to the more sensitive metagenomic sequencing, introduces subtle differences in result interpretation. Even modern high-throughput sequencing of microbial DNA has limitations, highlighted recently using a mock human gut microbiota for assessment of current databases [359]. The lack of consensus on specific microbiota signatures of MDD is an issue, as many studies disagree on fluctuations even at the most broad levels [186,237,239,251,360]. Developing a cohesive database with improved specificity will be invaluable for future targeted therapeutic approaches, for monitoring treatment response or assessing predisposition to a disorder. Continuing to characterize the microbiota specific to stress responses and MDD pathogenesis may eventually lead us to achieve individualized treatments involving microbiota modulation.

In line with this goal, the purpose of our analysis of microbiota community was to give a comprehensive picture pertaining to individual differences and sex differences in the clinical condition. We demonstrated that different stressors link to microbiota in unique ways, with sex-specific effects. Integrating those outcomes with modulations to the intestinal expression of tight junction and inflammatory markers proposed various mechanisms that could be in place. Reinforcement with microbiota characterization beyond taxonomic variation would be extremely relevant to bridge the gap. Indeed, functional analyses to predict metagenome functional content combined with KEGG mapping could pinpoint specific networks further alluding to mechanistic pathways.

Intestinal barrier

We were able to show tight junction responses to stress that were sex-specific and related to stress type and duration. Our findings support the concept of psychological stress affecting

barrier function. As previously mentioned, morphological changes at tight junction borders occur indicating dynamic remodeling that are not necessarily represented by mRNA expression or quantification of immunostaining. For that reason, in future analysis, we planned a collaborative effort to implement machine learning techniques and super resolution imaging for improved ability to analyze more complex transformations. These techniques should improve viability of analysis to give a deeper understanding regarding the mechanisms responsible for the stress-induced tight junction alterations.

Biomarkers

The lack of reliable biomarkers plagues the field of psychiatry. Indeed, reliable targets to discern specific disorders would enhance diagnostic procedures immensely. Promising markers have been highlighted, but currently none are considering a reliable marker for MDD. Therefore, there is an urgent need to deepen the understanding of causal biological mechanisms to promote the discovery of biomarkers. The interest in the relationship between IBD and MDD has brought clinical studies towards investigations of shared biomarkers. Thus far, studies have produced encouraging results (see **Table 3**); however, limited investigation of these markers has been performed in the context of chronic stress models. Therefore, we investigated serum levels of markers linked to intestinal dysfunction in the context of chronic stress models. Few studies have investigated the effects of stressors on blood biomarkers in microbial translocation, and to our knowledge, none of these included females, therefore our comparison of male and female LBP levels after chronic stress is fitting in this context. LBP as a potential gut-related biomarkers of chronic stress susceptibility showed promising results and we believe that future endeavors in the context of MDD are warranted. In line with our previous and on-going effort in our lab to identify potential biomarkers in MDD [345], we plan to assess blood samples from male and female patients with depression and healthy controls for LBP levels and other gut-related markers with potential in MDD.

Novel therapeutic intervention

In collaboration with Dr. Alain Doyen's team we contributed to the assessment of a peptide polymer in our depression models. Limited alterations to intestinal tight junctions were uncovered during the peptide treatment protocols. Though we did see promising results in the LBP serum test, showing a potential effect on this indirect indication of enhanced barrier

permeability. Although this is an interesting preliminary result, future studies employing functional tests such as TEER would be essential to directly measure of permeability in response to peptide treatment. Limitations of this experiment included testing the effects of the peptide without first ensuring its binding capabilities in the intestine. Another group was testing the treatment in an *in vitro* human digestion simulator system; however, these experiments were run in parallel. Therefore, our testing may have missed specific targets of this treatment due to lack of knowledge involving its specific mechanisms. In the context of our results, further evidence of the basic effects of peptide on the gut should be established before proceeding with more studies on modifications in the context of chronic stress and depression.

As MDD affects many people each year, the need of finding innovative diagnostic tools and effective treatments is crucial. Many non-CNS systems are modulated by stress in mood disorders and resulting in improper functioning. The implication of the peripheral immune system leads to effects on the brain such as dysregulated cognitive functions, escaladed BBB permeability, and infiltration of undesired molecules. Effects stemming from the gut such as barrier dysregulation and dysbiosis could lead to unwanted circulating molecules in the blood, weakening the BBB and altering brain circuits. Similarly, novel implications for serotonin system links both CNS and the gut through many pathways in the GBA, in a way that implicates a whole-body approach compared to our previous neurocentric comprehension. Whether dysregulated GBA signalling in the context of depression starts in the gut or the brain is hard to say. Regardless. hopefully, unraveling these mechanisms will allow us to harness the potential of unlocking a healthy mind through a happy gut!

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Appendices

Appendix A

Gene	Assay ID	Gene	Assay ID
Cldn1	Mm.PT.58.6163880	**Marveld2**	Mm.PT.58.7719303
Cldn3	Mm.PT.58.43310459.g	**Ido1**	Mm.PT.58.29540170
Cldn7	Mm.PT.58.16298059	**Ahr**	Mm.PT.58.11116644
Cldn12	Mm.PT.58.41535303	**Muc2**	Mm.PT.58.29496069.g
Tjp1	Mm.PT.58.29459730	**Mfsd2a**	Mm.PT.58.32675283
Tjp2	Mm.PT.58.16834535	**Gapdh**	Mm. PT.39a.1
Tjp3	Mm.PT.58.43961106	**Actb**	Mm.PT.39a.22214843.g
Ocln	Mm.PT.58.42749240		
Custom Primers			
Gene	**Fwd sequence**	**Rev sequence**	
Cldn2	5'-GTCATCGCCCATCAGAAGAT-3'	5'-CTGTTGGACAGGGAACCAGT-3'	
Cldn5	5'-TTTCTTCTATGCGCAGTTGG-3'	5'-TGAAGTAGGCACCAAACTGC-3'	
mTNFa	5'-CCCTCACACTCAGATCATCTTCT-3'	5'-GCTACGACGTGGGCTACAG-3'	
mTNFRSF1A	5'-GCCCGAAGTCTACTCCATCATTTG-3'	5'-GGCTGGGGAGGGGGCTGGAGTTAG-3'	
mIl1b	5'-GCAACTGTTCCTGAACTCAACT-3'	5'-ATCTTTTGGGGTCCGTCAACT-3'	
mIl6	5'-TAGTCCTTCCTACCCCAATTTCC-3'	5'-TTGGTCCTTAGCCACTCCTTC-3'	
mIl10	5'-ACTCAATACACACTGCAGGTG-3'	5'-GGACTTTAAGGGTTACTTGG-3'	
mIl13	5'-TGGCTCTTGCTTGCCTTGGTGG-3'	5'-CCATACCATGCTGCCGTTGCA-3'	
mIl18	5'-GACTCTTGCGTCAACTTCAAGG-3'	5'-CAGGCTGTCTTTTGTCAACGA-3'	
mIl33	5'-TCCAACTCCAAGATTTCCCCG-3'	5'-CATGCAGTAGACATGGCAGAA-3'	
mCXCL4	5'-CAGTCCTGAGCTGCTGCTTCT-3'	5'-TCCAGGCTGGTGATGTGCTTA-3'	

Table A 1. qPCR primers

Marker	Type	Host	Dilution	Company, Catalog #
Cldn-3	Primary	Rabbit	1:250	Life Technologies, 341700
Cldn-7	Primary	Rabbit	1:250	Life Technologies, 349100
CD326	Primary	Rat	1:300	Invitrogen, 953624
Cy2 (anti-rat)	Secondary	Donkey	1:300	Jackson Immunoresearch, 712-225-153
Cy2 (anti-rabbit)	Secondary	Donkey	1:300	Jackson Immunoresearch, 712-225-152
Alexa 594 (anti-rabbit)	Secondary	Donkey	1:400	Jackson Immunoresearch, 711-585-152
Phalloidin (F-actin)	Primary/Secondary	N/A	1:200	Abberior (Sigma), 30972
DAPI	N/A	N/A	1:1000	Invitrogen, D1306

Table A 2. Primary and secondary antibodies

Type	Marker	Changes	Clinical Symptoms	References
Cytokine	**IL-1β** *(interleukin-1)*	↑	≈ depressive symptoms in bipolar disorder (IDS-30 score)	361 362
		↑	positive correlation with symptom severity (HAM-D)	102,363
	IL-4 *(interleukin-4)*	↓	Decreases in suicide patients	102,104
	IL-6 *(interleukin-6)*	↑	≠ with specific depressive symptoms	102,103
				364
			≈ symptom severity (HAM-D)	138
			≈ response (MADRS) to ketamine	365
	TNF-α *(tumor necrosis factor alpha)*	↑		102,103
			Correlation with symptoms severity (HAM-D)	363
	CRP *(C-reactive protein)*	↑	Correlation with symptom severity (BDI-II)	366
			Correlates with ↓ functional connectivity (AMY, PFC) in comorbid PTSD or anxiety only	367
			baseline CRP ≈ treatment (venlafaxine) response (HAM-D), ♂	138
			Low baseline levels ≈ ↑ escitalopram response (MADRS) High baseline levels ≈ ↑ nortriptyline response (MADRS)	368
		-	Strong correlation with CSF levels Correlation with other inflammatory markers (IL- 6, TNF, sTNFR2) Plasma levels ≈ ↑ anhedonia (♀+♂), CSF levels ≈↑ anhedonia (♀).	105
BBB	**VCAM-1** *(Vascular cell adhesion molecule 1)*	↑/↓	Sex-specific effects (↓♀, ↑ ♂)	114
	ICAM-1 *(Intercellular cell adhesion molecule 1*	↑		115,116
			↑ pre-treatment levels ≈ ↑ response to mixed antidepressants, ↓ response to Venlafaxine	107
	sICAM-1 *(soluble ICAM-1)*	↑	late-life depression	117
			3-days post antidepressant washout	118
	PAI-1	↑	≠ symptom severity (HAM-D, HAM-A) or disease duration	369
			≈ symptom severity (CES-D)	370

	(plasminogen activator inhibitor 1)		♀, ↑ levels ≈ venlafaxine treatment response (HAM-D)	138
		—	↑ pre-treatment levels ≈ better response (HAM-D, IDS) to mixed antidepressants	107
	TPO *(thrombopoietin)*		↑ pre-treatment levels ≈ response (HAM-D, IDS) to mixed antidepressants	
	CCL11/eotaxin-1	↑	≈ suicidal ideation	340 371
		—		349
	MCP1/CCL2 *(Monocyte chemoattractant protein 1)*	↑		351
		—	Possibly related to metabolic effects	372
	MMP2 *(Matrix metalloproteinase 2)*	↓	≈ response to ECT	373,374
				375
		↑		376
	MMP7	↑		376
	MMP9	↑	♀, associated with treatment response	138,374,376
				375
		—	≈ symptom severity (depression, quality of life scores, and social function scores)	377
			↓ in non-relapsing patients after ECT	373
	S100B *(S100 calcium binding protein B)*	↑	≈ symptom severity (HAM-D)	378,379
			≠ treatment response (HAM-D) to antidepressants	380
			≈ attentional processes in remitting patients	381
			≈ treatment response (HAM-D) to celecoxib	382
			≠ with clinical severity (BDI-II and HAM-D)	383
			♀, higher in remitting disorder	383,384
			≈ treatment response	108,385
			≈ memory processes	386
			≠ symptom severity (HAMD-17 or YMRS). ↑ levels in non-medicated patients ↑ levels ≈ family history ≠ overall sex-specific effects (♀ vs. ♂), ↑ levels (♀) controls vs. patients	387
			remission, correlated with lower cognitive performance	388

Table A 3. Potential circulating cytokine and blood-brain barrier metabolite markers of MDD.
Modified from [166]. **Symbols***:* ≈, associated with; ≠, no association. **Abbreviations**: HC, healthy controls; BDI-II, Beck Depression Inventory-II; CSF, cerebral spinal fluid; HAM-D, Hamilton depression rating scale; HAM-A, Hamilton anxiety rating scale; IDS, Inventory of Depressive Symptoms; IDS-C, 30-item Inventory of Depressive Symptomatology Clinician-Rated; YMRS, Young Mania Rating Scale; MADRS, Montgomery–Asberg Depression Rating Scale; CES-D, Center of Epidemiologic Studies Depression Scale.