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Chronic Stress Exposure Alters the Gut Barrier: Sex-Specific Effects on Microbiota and Jejunum Tight Junctions

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ABSTRACT

BACKGROUND: Major depressive disorder (MDD) is the leading cause of disability worldwide. Of individuals with MDD, 30% to 50% are unresponsive to common antidepressants, highlighting untapped causal biological mechanisms. Dysfunction in the microbiota-gut-brain axis has been implicated in MDD pathogenesis. Exposure to chronic stress disrupts blood-brain barrier integrity; still, little is known about intestinal barrier function in these conditions, particularly for the small intestine, where absorption of most foods and drugs takes place.

METHODS: We investigated how chronic social or variable stress, two mouse models of depression, impact the jejunum intestinal barrier in males and females. Mice were subjected to stress paradigms followed by analysis of gene expression profiles of intestinal barrier–related targets, fecal microbial composition, and blood-based markers.

RESULTS: Altered microbial populations and changes in gene expression of jejunum tight junctions were observed depending on the type and duration of stress, with sex-specific effects. We used machine learning to characterize in detail morphological tight junction properties, identifying a cluster of ruffled junctions in stressed animals. Junctional ruffling is associated with inflammation, so we evaluated whether lipopolysaccharide injection recapitulates stress-induced changes in the jejunum and observed profound sex differences. Finally, lipopolysaccharide-binding protein, a marker of gut barrier leakiness, was associated with stress vulnerability in mice, and translational value was confirmed on blood samples from women with MDD.

CONCLUSIONS: Our results provide evidence that chronic stress disrupts intestinal barrier homeostasis in conjunction with the manifestation of depressive-like behaviors in a sex-specific manner in mice and, possibly, in human depression.

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Major depressive disorder (MDD) is the most prevalent mood disorder and a leading cause of disability worldwide (1–3). Core symptoms include low mood, irritability, anhedonia, apathy, difficulty concentrating, and disrupted appetite and sleep (1–3). MDD is highly heterogeneous, and experience, symptoms, and treatment response vary across individuals (1,3). Maladaptive central and peripheral inflammatory responses are associated with mood disorders and have received increasing attention in psychiatry (4–6). Indeed, administration of proinflammatory cytokines or endotoxins can induce depressive symptoms, and elevated levels of circulating cytokines are characteristic of treatment-resistant individuals (3–6). Aligning with the neuroimmune hypothesis of depression, MDD has high comorbidity with inflammatory bowel diseases (7,8), suggesting that inflammation-driven gut barrier dysfunction may affect emotion regulation and vice versa (1). In gastrointestinal disorders, elevated proinflammatory cytokines promote increased permeability in the intestinal tract by suppressing tight junction–mediated barrier function (9). Shared profiles of upregulated proinflammatory cytokines in the blood occur in gastrointestinal disorders and MDD, which could be related to increased intestinal permeability (1,7). Stress has been linked to the deterioration of the intestinal barrier via alterations of the microbiota and gut-brain signaling (10–13). We showed that it can alter blood-brain barrier (BBB) integrity in a sex-specific manner through loss of the tight junction protein claudin-5 (CLDN5), leading to the development of anxiety- and depression-like behaviors (14–17). To our knowledge, the impact of social and variable stress exposure on gut barrier integrity and microbiota with assessment of sex differences has yet to be determined.

Women are two times more likely than men to be diagnosed with MDD (18). Symptoms and treatment responses also differ

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between sexes (19–21). As chronic stress is the main environmental risk factor for MDD (22), it is used in rodents to induce depression-like behaviors and investigate underlying biology. Chronic social defeat stress (CSDS) is a mouse model of depression based on social dominance, which produces two distinct phenotypes: stress-susceptible (SS) and resilient (RES) mice (23–25). SS mice display behavioral changes reminiscent of human depressive symptoms with increased social avoidance, anxiety, anhedonia, despair, body weight changes, metabolic disturbances, and corticosterone reactivity (23–25). Furthermore, loss of BBB integrity occurs only in the brain of SS, but not RES, mice (14,16). Another leading stress paradigm is the chronic variable stress (CVS) model, in which mice are exposed to a repetitive sequence of stressors, namely, tube restraint, tail suspension, and foot shocks, ranging from days to several weeks (26,27). Similar to CSDS, CVS induces a proinflammatory immune profile (28). However, females and males develop depression-like behaviors at different time points making it highly relevant for investigating sex differences (4,27).

The gut barrier is formed by the mucus layer, the epithelia, and a connective tissue layer known as the lamina propria (29). The epithelial cell monolayer faces the luminal side, interacting with the environment and regulating absorption and secretion. In the small intestine, macrostructures 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 (29). The epithelium provides a dynamic and semipermeable barrier with tight junction complexes, linking adjacent cells and the overlying mucus layer allowing passage of nutrients, water, and ions, but limiting entry of pathogens and bacterial toxins (29,30). The intestinal barrier is at the forefront of immune-environment interactions, where the specialized cells play a critical role in maintaining health (1,31). Permeability of the epithelial layer may increase interaction of antigens with immune cells, propagating a proinflammatory response (1). Microbial translocation from the intestinal lumen into the systemic circulation in the absence of acute infection is proposed as a mechanism behind MDD with chronic inflammation (32–34).

To assess the impact of stress exposure on gut barrier integrity and particularly the small intestine, which remains understudied, we used complementary mouse models of MDD and combined behavioral, molecular, morphological, and pharmacological experiments with blood-based assays. Our results provide characterization, in a sex-specific manner, of stress-induced changes in the jejunum following exposure to social or variable stressors. We developed tools and algorithms to analyze in detail tight junction morphological changes and identified circulating lipopolysaccharide (LPS)-binding protein (LBP) as a gut leakiness potential biomarker that could help better diagnose and inform treatment strategies for mood disorders.

METHODS AND MATERIALS

Animals

Male and female C57BL/6 mice were obtained from Charles River Laboratories along with male CD-1 mice used as residents for social defeat. Mice were maintained on a 12-hour light/dark cycle with temperature and humidity kept constant and provided free access to water and food (Supplemental Methods and Materials). All experimental procedures were approved by the Animal Care Committee of Université Laval and met the Canadian Council on Animal Care guidelines.

Experimental Paradigms

Chronic Social Defeat Stress. The CSDS paradigm was conducted as previously described (23). C57BL/6 mice were 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. The female CSDS protocol was performed according to Harris *et al.* (24). After the last day of defeat, mice were subjected to a social interaction (SI) test to establish behavioral phenotypes (Supplemental Methods and Materials) (23).

Chronic Variable Stress. In the CVS paradigm, mice were exposed to a series of alternating variable stressors (restraint stress, tail suspension, foot shocks) for 1 hour per day for 28 days (Supplemental Methods and Materials) (35).

Subchronic Variable Stress. The subchronic VS (SCVS) paradigm consisted of the same procedure as CVS but for a total duration of 6 days only (Supplemental Methods and Materials) (16,27).

Data Collection and Analysis

Quantitative Polymerase Chain Reaction of Mouse Tissue. After sacrifice, jejunum tissue samples were collected for evaluation of gene expression of targets related to intestinal permeability, such as tight junctions, associated proteins, and inflammatory markers (Supplemental Methods and Materials).

Immunohistochemistry of Claudin-3. Intestinal segments were prepared for protein analysis following a modified version of the Swiss-roll protocol (36,37). Tissue slices were double stained with CD326 for visualization of epithelial cells and tight junction claudin-3 (CLDN3) or for CLDN3 and actin filaments (F-actin) (Table S2). Images were acquired using an Axio Observer.M2 microscope (ZEISS) and processed with Imaris software (Oxford Instruments) for volume quantification and intensity colocalization (Supplemental Methods and Materials).

Machine Learning–Based Morphological Analysis. Ten acquired images were analyzed (6 from control mice and 4 from SCVS mice). Images were cropped and annotated using a custom software. An expert annotated each image crop based on 4 qualitative features: 1) ruffles quantity, 2) width, 3) fragmentation, and 4) CLDN3 diffusion. A clustering algorithm (38) was used to group the crops into clusters using the described four-dimensional feature space (39). The proportion of crops within each cluster was compared to reveal potential differences in tight junction populations between conditions (Supplemental Methods and Materials).

Microbiota Analysis. Fecal pellets were collected from each mouse, and fecal DNA was extracted for microbiota analysis (Supplemental Methods and Materials).

LPS Treatment. Male and female mice received an intraperitoneal injection of LPS or saline (vehicle group) administered at time zero and after 24-hour samples were collected for jejunum tight junction analysis (Supplemental Methods and Materials).

Murine Enzyme-Linked Immunosorbent Assays of Gut Leakiness. Blood samples were collected 72 hours before the start of the stress protocol and 48 hours after the last stressor. Level of serum LBP was assessed by a Mouse LBP ELISA Kit (Abcam), according to the manufacturer protocol (Supplemental Methods and Materials).

Serum Corticosterone Quantification. Blood samples were collected 48 hours after the last stressor. Level of serum corticosterone was assessed by enzyme-linked immunosorbent assay according to the manufacturer protocol (Supplemental Methods and Materials)

Human Serum Sample Collection. Human blood samples were provided by Signature Bank from the Centre de Recherche de l'Institut Universitaire en Santé Mentale de Montréal under approval of the institution's Ethics Committee. Samples were collected from depressed and healthy volunteers; all individuals were evaluated for depressive behaviors (Supplemental Methods and Materials).

Human Serum Enzyme-Linked Immunosorbent Assay of Gut Leakiness Marker. Human serum levels of LBP were assayed using the Human LBP ELISA Kit (Abcam) and following the manufacturer's protocol (Supplemental Methods and Materials).

Statistical Analysis

All data are presented as mean ± SEM. Comparisons between groups were performed using *t* test, one-way analysis of variance, and two-way analysis of variance with Bonferroni post hoc follow-up test when required (Microsoft Excel; Microsoft Corp.). Values of $p < .05$ were regarded as statistically significant. Graphs and statistics were generated using GraphPad Prism (GraphPad Software); further details are described in Supplemental Methods and Materials.

RESULTS

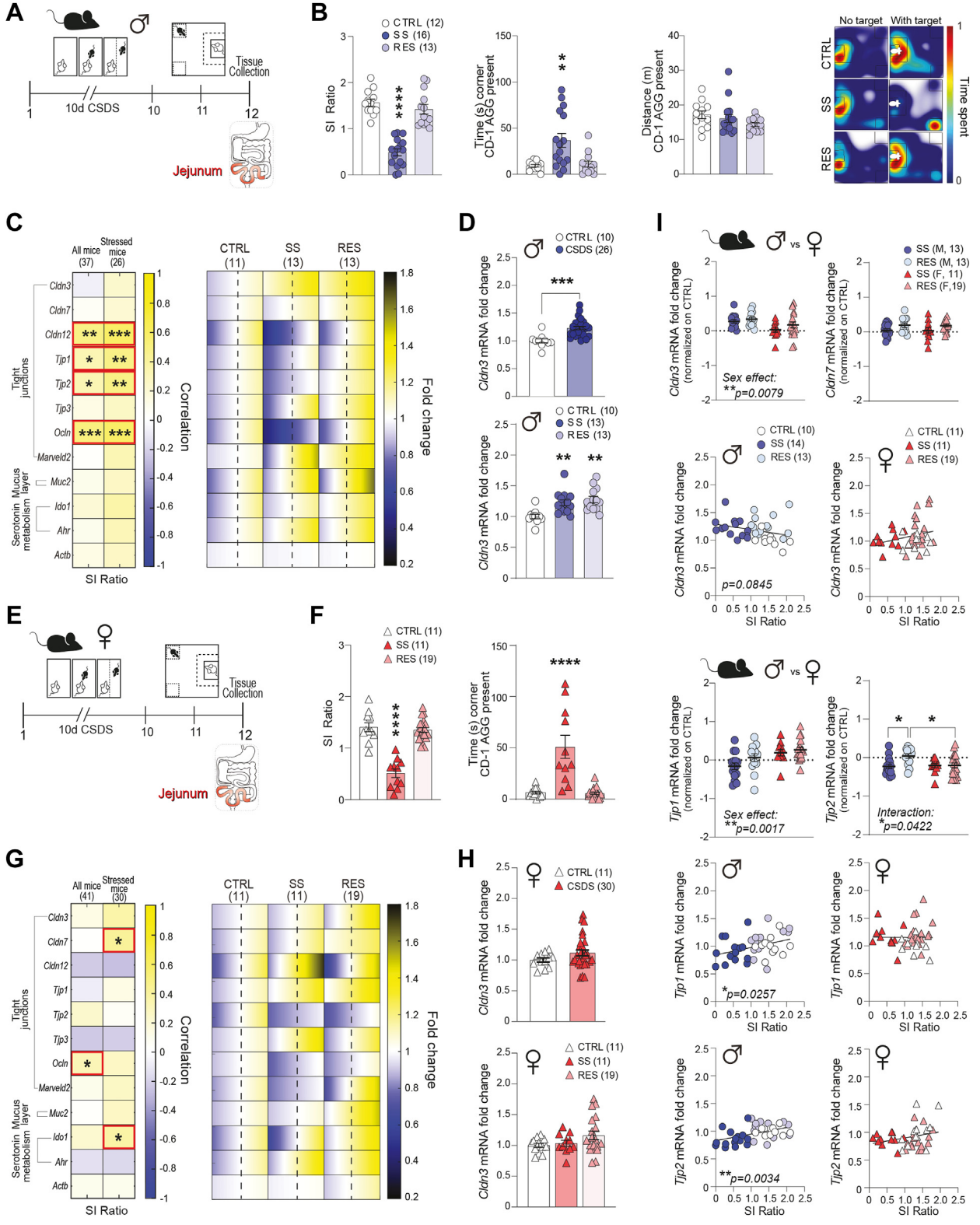
Chronic Social Stress Alters Intestinal Tight Junction Expression With Sex-Specific Effects

A 10-day CSDS exposure induced expression of SS (55.2%) and RES (44.8%) phenotypes based on the SI test (Figure 1A, B). Time spent in the corners when the CD-1 aggressor was present was increased for SS mice (Figure 1B), while total distance traveled was similar between groups (Figure 1B; Figure S1A, B). Tissue was collected 24 hours later, and quantitative polymerase chain reaction was performed for genes related to tight junctions (*Cldn3*, *Cldn7*, and *Cldn12*), tight junction–associated proteins (*Tjp1*, *Tjp2*, *Tjp3*, *Ocln*, MARVEL domain-containing protein 2 [*Marveld2*]), and proteins involved in serotonin metabolism (*Ido1* and *Ahr*) and mucus layer formation, mucin-2 (*Muc2*) on the jejunum of unstressed control, SS, and RES mice. Indeed, serotonin metabolism is altered in inflammatory bowel diseases, which are highly comorbid with MDD (7), and the mucus layer is essential to maintain gut barrier integrity. Fold changes were positively correlated with SI ratios for *Cldn12*, *Tjp1*, *Tjp2*, and *Ocln* (Figure 1C), suggesting that loss of intestinal barrier integrity may be linked to social avoidance. Intriguingly, *Cldn3*, an important intestinal tight junction, was upregulated after CSDS exposure (Figure 1D).

We reported that CSDS alters BBB integrity in a sex-specific manner (14,16). Exposure to CSDS (Figure 1E) also leads to two subpopulations of SS (36.7%) and RES (63.3%) female mice with SS mice displaying social avoidance (Figure 1F; Figure S1C, D); however, a correlation was noted only for *Ocln* jejunum expression with SI ratio (Figure 1G). Regarding stressed mice, a significant correlation was observed for *Cldn7* and *Ido1* (Figure 1G), highlighting stress-induced sex-specific patterns. In contrast to males, *Cldn3* was not elevated in female mice after CSDS (Figure 1H) leading us to explore further sex differences. In fact, *Cldn3* expression was higher in unstressed female mice compared with their male counterparts (Figure S1E) indicating baseline sex differences for the jejunum tight junctions. There was a main effect of sex on *Cldn3* and *Tjp1* expression after stress exposure, and an interaction between sex and behavioral phenotype was present for *Tjp2* expression (Figure 1I). Therefore, CSDS was associated with changes to expression of tight junction genes in jejunum of both male and female mice, but these changes were specific to each sex.

Changes in Jejunum Tight Junction Expression Are Dependent on Stress Type and Duration

Males and females were often the same at baseline, but when exposure to a stressor occurred, an effect could be observed in only one sex or had a greater effect in one sex. For example, female mice were more vulnerable to SCVS, with exposure to only 6 days of stressors being sufficient to induce anxiety- and depression-like behaviors in females, but not males (27). Considering the sex-specific effects of 10-day exposure to CSDS (Figure 1), we compared the impact of stress type and duration on *Cldn3* expression, one of the most abundant tight junction proteins of the jejunum (37). While social stress increased *Cldn3* in males (Figure 2A), it remained unchanged after 6 days of SCVS (Figure 2B, left) in line with unaltered behaviors (26). Conversely, *Cldn3* expression was reduced in the jejunum of female mice (Figure 2B), characterized by stress-induced anxiety- and depression-like behaviors (16). Exposure to 28 days of CVS is associated with the development of maladaptive behaviors in both sexes (35); nevertheless, *Cldn3* expression was reduced only in males (Figure 2C), suggesting that other alterations might be present. Thus, as for CSDS, quantitative polymerase chain reaction was performed for genes related to tight junctions, tight junction–associated proteins, mucus layer formation, and serotonin metabolism on the jejunum of unstressed control mice versus male and


A
Tissue Collection
1
10d CSDS
10
11
12
Jejunum
B
CTRL (12)
SS (16)
RES (13)
SI Ratio
Time (s) corner CD-1 AGG present
Distance (m) CD-1 AGG present
No target
With target
CTRL
SS
RES
Time spent
C
All mice (37)
Stressed mice (26)
CTRL (11)
SS (13)
RES (13)
Cldn3
Cldn7
Cldn12
Tjp1
Tjp2
Tjp3
Ocln
Marveld2
Muc2
Ido1
Ahr
Actb
Tight junctions
Mucus layer
Serotonin metabolism
Correlation
Fold change
SI Ratio
D
CTRL (10)
CSDS (26)
Cldn3 mRNA fold change
CTRL (10)
SS (13)
RES (13)
Cldn3 mRNA fold change
I
vs
Cldn3 mRNA fold change (normalized on CTRL)
Sex effect:
**p=0.0079
SS (M, 13)
RES (M, 13)
SS (F, 11)
RES (F,19)
Cldn7 mRNA fold change (normalized on CTRL)
CTRL (10)
SS (14)
RES (13)
p=0.0845
CTRL (11)
SS (11)
RES (19)
SI Ratio
E
Tissue Collection
10d CSDS
Jejunum
F
CTRL (11)
SS (11)
RES (19)
SI Ratio
Time (s) corner CD-1 AGG present
Tjp1 mRNA fold change (normalized on CTRL)
Sex effect:
**p=0.0017
Tjp2 mRNA fold change (normalized on CTRL)
Interaction:
*p=0.0422
G
All mice (41)
Stressed mice (30)
CTRL (11)
SS (11)
RES (19)
H
CTRL (11)
CSDS (30)
Cldn3 mRNA fold change
CTRL (11)
SS (11)
RES (19)
Tjp1 mRNA fold change
*p=0.0257
Tjp2 mRNA fold change
**p=0.0034

female mice subjected to 6 days or 28 days of variable stress, revealing specific sex, stress type, and duration patterns (Figure 2D). No difference was observed for the estrus cycle phase (Figure S2). In males, 10 days of CSDS increased *Cldn3* only, while 28 days of CVS decreased expression of several tight junctions and tight junction–associated proteins. No effect was observed for mucus layer formation, for serotonin metabolism, or after the 6-day SCVS paradigm. In contrast, changes were mostly observed after exposure to variable stress in females (Figure 2D). Overall, assessment of *Cldn3* expression across stress paradigms suggested a more profound impact after 28 days of CVS in males versus 6 days of SCVS in females (Figure 2E). Other genes differentially impacted in a sex-specific manner by 28 days of CVS included *Cldn7*, *Cldn12*, and *Ido1* (Figure 2F).

Detailed Morphological Assessments of Stress-Induced Changes in Jejunum CLDN3 Expression

Next, we aimed to confirm that stress-induced alterations in tight junction gene expression are also reflected at the protein level. Female mice were subjected to the 6-day SCVS paradigm, then jejunum tissue was collected 24 hours later (Figure 3A). Thin 6-μm slices were double stained with CLDN3 and F-actin, and morphological analysis was performed with Imaris (Figure 3B–F). Structural and organizational properties of tight junctions play a major role in their function and maintenance of the gut barrier integrity (1). Functional tight junctions are formed when claudins interact with other transmembrane proteins, junction-associated scaffold proteins, and the actin cytoskeleton (40). Exposure to 6 days of SCVS reduced CLDN3 protein level (Figure 3C), in line with the changes observed at gene expression (Figure 2B), while no difference was measured for F-actin (Figure 3C). Next, overlap between CLDN3 and F-actin was assessed as shown on Figure 3D. The Pearson correlation coefficient revealed a significant relationship between loss in CLDN3/F-actin overlap and 6-day SCVS exposure, while no change was measured with the Manders correlation coefficient (Figure 3E). Nonetheless, a trend toward lower colocalization volume was observed after stress (Figure 3F).

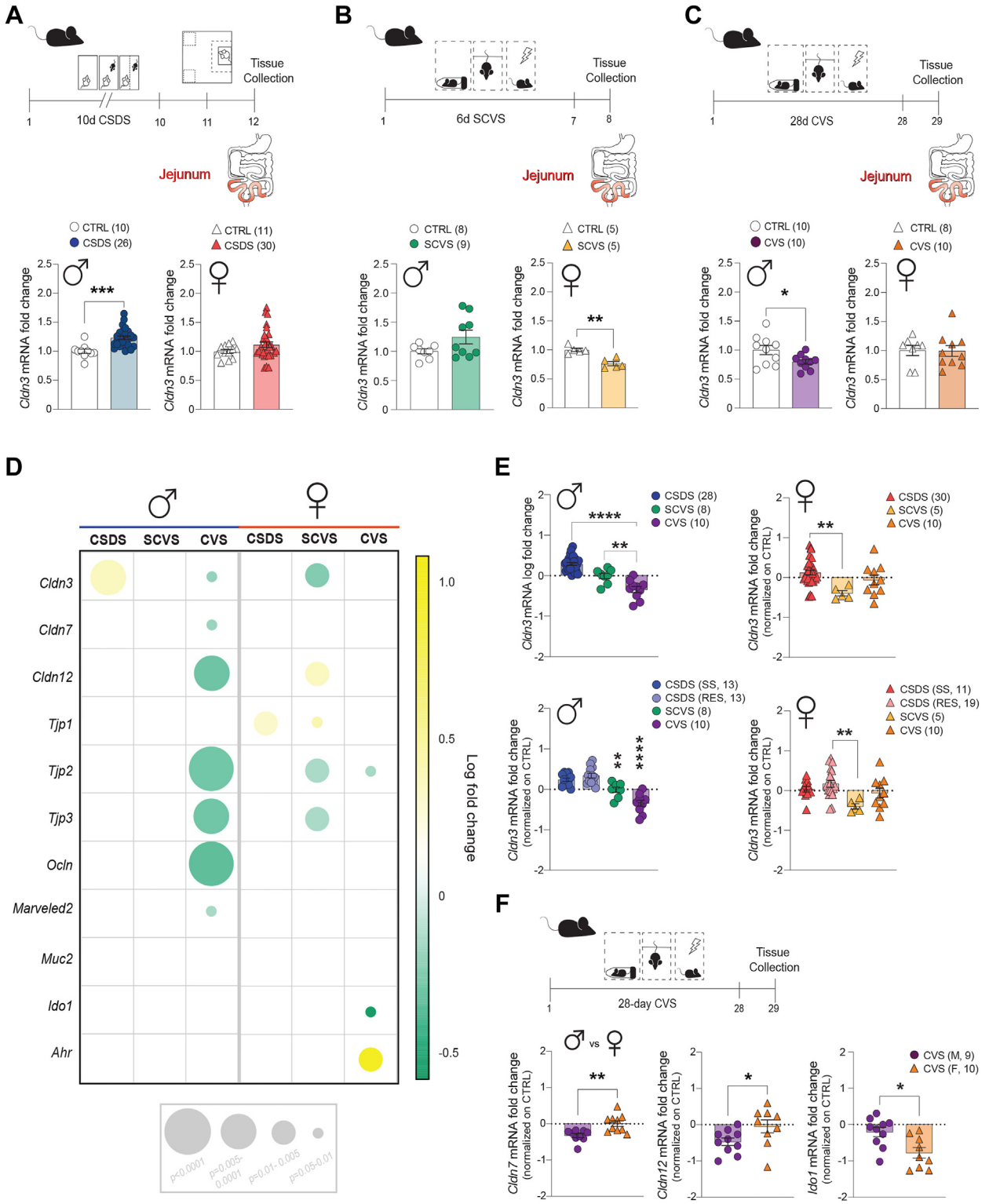
Nanoscale architecture of the BBB tight junctions could help in better understanding their functions and contribution in disease pathology (41). Here, we used machine learning–based algorithms to characterize a further 4 features of the jejunum tight junctions: ruffles, width, fragmentation, and diffusion. Images of jejunum samples from female mice exposed to 6 days of SCVS (Figure 4A) and double stained for CLDN3 and F-actin were acquired (Figure 4B) and analyzed using custom software. According to the criteria defined in Figure 4C, 1426 image crops (control mice: 452; SCVS mice: 974) were annotated with a value ranging from 0 to 1. Unsupervised k-means clustering allowed grouping, in a blinded manner, of the crops into 7 different clusters (Figure 4D) providing an overview and comparison of the CLDN3 tight junction properties between conditions (Figure 4E). Representative images are provided for each cluster (Figure 4F) along with their properties for the 4 feature values (Figure 4G). Cluster 7 (pink), which is associated with a high number of ruffles, was of particular interest, as it was absent in the control mice but observed in all stressed animals (Figure 4E). Indeed, ruffled tight junctions are associated with increased paracellular permeability and thus a loss of barrier integrity (40). To our knowledge, this is the first detailed morphological characterization of the impact of stress on the gut barrier integrity. An increase in ruffled tight junctions suggests that stress-induced jejunum leakiness may contribute to the neuroimmune mechanisms of MDD by allowing gut-related inflammatory mediators to leak into the bloodstream.

LPS-Induced Inflammation Promotes Loss of Jejunum Tight Junctions in Male Mice Only

To confirm a causal role of stress-associated inflammation in the alterations observed at the jejunum tight junctions, mice were pharmacologically treated with LPS. This endotoxin is a product of the outer membrane of gram-negative bacteria commonly used to study inflammation-induced behavioral changes in rodents (42). Importantly, it has translational value, as LPS is elevated in the plasma of individuals with MDD (43). Male and female mice received an intraperitoneal injection of LPS, and jejunum samples were collected for tight junction analysis (Figure 5A, D). Treatment with LPS led to profound

Figure 1. Chronic social stress alters jejunum tight junction expression with sex-specific effects. **(A)** Timeline of the male CSDS paradigm. **(B)** (Left panel) Ratio of time spent interacting with novel social target decreased in SS vs. unstressed CTRL and RES male mice (SI ratio: $p < .0001$). (Middle panels) Cumulative time (in seconds) spent in corners with social target present was increased in SS males ($p = .0004$) and cumulative distance traveled (meters) in arena with social target present was unchanged between groups. (Right panel) Representative heatmaps of normalized time spent in the arena during SI test in males. **(C)** (Left panel) Effects of social stress 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: *Cldn12* ($p = .006$, $r = 0.44$), *Tjp1* ($p = .03$, $r = 0.36$), *Tjp2* ($p = .004$, $r = .46$), and *Ocln* ($p < .001$, $r = 0.55$). (Right panel) Quantitative polymerase chain reaction revealed significant changes in jejunum of SS and RES mice compared with CTRL mice for gene expression of targets related to tight junctions. The range of color indicates individual differences within a group. SEM from the average represented by dashed line. **(D)** Significant increase in *Cldn3* for male mice was independent of phenotype group ($p = .0002$). **(E)** Time line of the female CSDS paradigm. **(F)** Ratio of time spent interacting with novel social target is decreased in SS female mice ($p < .0001$). Cumulative time (in seconds) spent in corners with social target present was increased in SS females ($p < .0001$). **(G)** (Left panel) *Ocln* ($p = .04$, $r = 0.31$), *Cldn7* ($p = .02$, $r = 0.41$), and *Ido1* ($p = .04$, $r = 0.24$) expression correlated with social avoidance behaviors. Red boxes highlight genes with significant correlation with social avoidance. (Right panel) Tight junction changes as a function of phenotype in female mice. **(H)** *Cldn3* expression is unchanged in female mice following social stress. **(I)** There is an effect of sex as a factor on *Cldn3* ($p = .008$) and *Tjp1* ($p = .002$) gene expression. Post hoc tests confirmed that stressed female mice had lower *Cldn3* expression than males in both SS and RES groups ($p = .0499$). Similarly, SS males had lower *Tjp1* expression than SS females ($p = .01$), and an interaction between sex and behavioral phenotype was present for *Tjp2* expression ($p = .04$). Data assessed by *t* tests and one-way analysis of variance followed by Bonferroni's multiple comparison test for changes between groups and two-way analysis of variance followed by Bonferroni's multiple comparison test for comparison between sexes; correlations were evaluated with Pearson's correlation coefficient; $^{*}p < .05$, $^{**}p < .01$, $^{***}p < .001$, $^{****}p < .0001$. AGG, aggressor; CSDS, chronic social defeat stress; CTRL, control; F, female; M, male; mRNA, messenger RNA; RES, resilient; SI, social interaction; SS, stress-susceptible.

changes in jejunum gene expression in males with a loss of tight junctions, tight junction–associated proteins, mucus-associated *Muc2*, and finally, *Ido1* and *Ahr* (Figure 5B, C; Figure S3B). Conversely, no significant change was observed in females except for serotonin-related *Ido1* and *Ahr* (Figure 5E, F; Figure S3C, D), suggesting that other biological



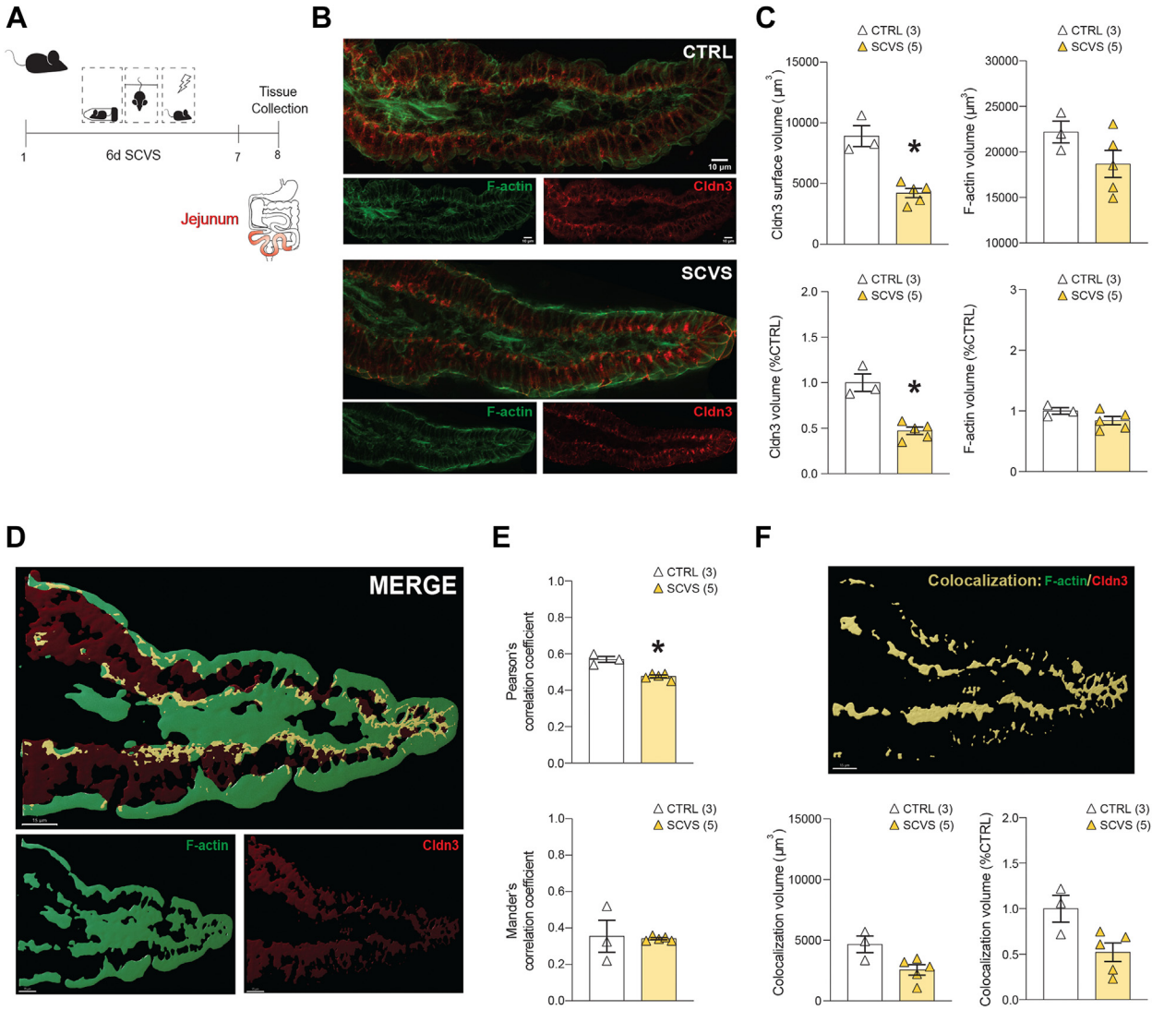


Figure 3. Morphological assessments of stress-induced changes in jejunum CLDN3 expression. **(A)** Experimental time line of female SCVS paradigm and tissue collection. **(B, C)** CLDN3 protein level was lower in SCVS-exposed mice, while no difference was measured for F-actin ($p = .0357$). **(D)** Representative image of Imaris volume rendering for surface volume determination. **(E)** Pearson's correlation coefficient revealed decreased colocalization of F-actin and CLDN3 signal intensities. However, no significant differences in co-occurrence of F-actin and CLDN3 were detected with Manders' colocalization coefficient. **(F)** Surface volume of colocalized regions of F-actin and CLDN3 extracted from the image in panel **(D)** was lower in SCVS mice without reaching significance ($p = .0714$). *$p < .05$. CTRL, control; SCVS, subchronic chronic variable stress.

Figure 2. Changes in jejunum tight junction expression are dependent on stress type and duration. **(A)** Experimental time line of the CSDS paradigm with graphs of *Cldn3* gene expression comparing CTRL and stressed groups of male ($p = .0002$) and female mice. **(B)** SCVS experimental time line with comparison of *Cldn3* gene expression results of male and female mice ($p = .008$). **(C)** Experimental time line of CVS with *Cldn3* gene expression results between CTRL and stressed groups of male ($p = .03$) and female mice from this paradigm. **(D)** Representation of gene expression changes in the jejunum of stressed mice across stress models in both males and females. Circle diameter represents statistical significance of the gene expression change. Circle color represents directionality of change vs. unstressed CTRL with green as a downregulated gene and yellow as an upregulated gene. **(E)** Direct comparison of *Cldn3* gene expression changes in (left panels) male ($p < .01$; $p < .0001$) and (right panels) female ($p = .0071$; $p = .0118$) stressed mice exposed to different stress types, (top panels) CSDS, SCVS, and CVS, and (bottom panels) with CSDS phenotypes separated to SS and RES. **(F)** Direct sex comparison of *Cldn7* ($p = .002$), *Cldn12* ($p = .035$), and *Ido1* ($p = .013$) gene expression changes in mice exposed to 28-day CVS. Data assessed by *t* tests and one-way analysis of variance followed by Bonferroni's multiple comparison test for changes between groups; *$p < .05$, **$p < .01$, ***$p < .001$, ****$p < .0001$. CSDS, chronic social defeat stress; CTRL, control; CVS, chronic variable stress; F, female; M, male; mRNA, messenger RNA; RES, resilient; SCVS, subchronic chronic variable stress; SS, stress-susceptible.

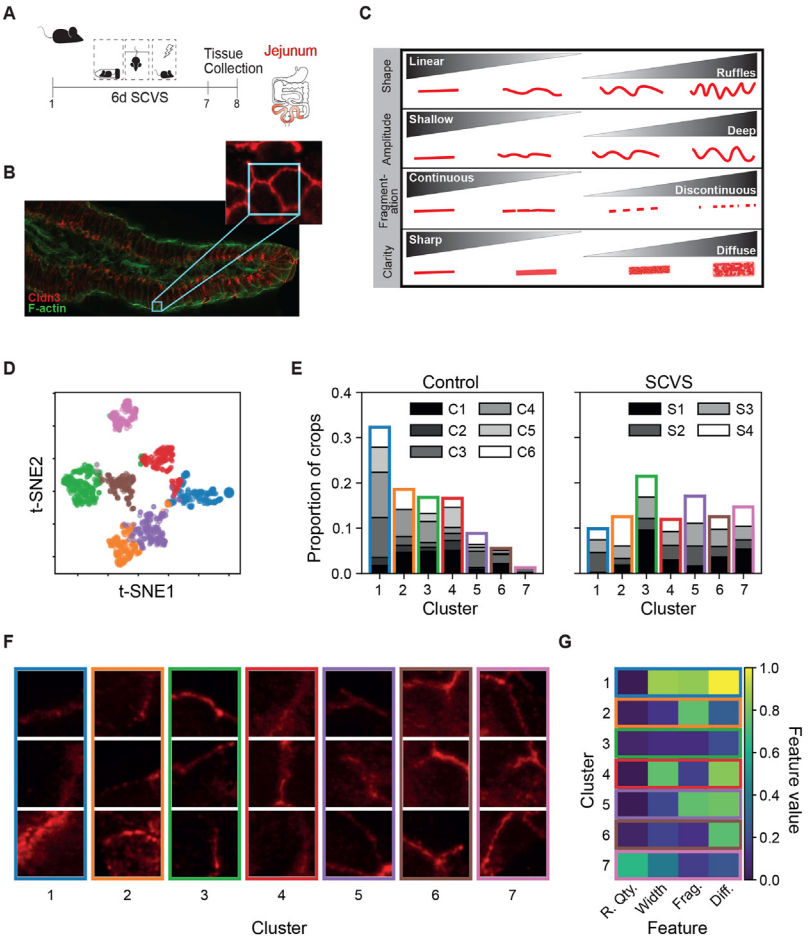


Figure 4. Detailed morphological assessments and k-means clustering analysis of stress-induced changes in jejunum CLDN3 protein expression. **(A)** Experimental time line of female SCVS paradigm. **(B)** Representative immunofluorescent image of CLDN3 and F-actin. **(C)** Chart describing the jejunum tight junction features and parameters analyzed. **(D)** *t*-SNE visualization of the k-means clustering. The 4 features (ruffle quantity, width, fragmentation, and diffusion) are projected in 2 dimensions using the *t*-SNE algorithm. Each color corresponds to a different cluster identified with k-means clustering. Tight junction crops (5.52 × 5.52 μm) with similar feature values are clustered together and are closer together in the *t*-SNE projection. **(E)** Proportion of tight junction crops from each image in each cluster for control and stressed animals. The different shades of gray correspond to different images to show that the distribution was not skewed by the overwhelming presence of a cluster in a single image. In the control condition, cluster 1 contains more than half of the tight junction crops, while very few are in cluster 7. For the chronic stress condition, there is an increase in crops belonging to clusters 5, 6, and 7. **(F)** Examples of CLDN3 tight junction crops associated with each cluster. These were selected from the 20 crops with features closest to the median point of each cluster using cosine distance. **(G)** Feature "barcode" of the clusters identified with k-means clustering. Each entry corresponds to the median value of a feature in the given cluster. Diff., diffusion; Frag., fragmentation; R. Qty., ruffle quantity; SCVS, subchronic chronic variable stress; *t*-SNE, *t*-distributed stochastic neighbor embedding.

mechanisms underlie stress-induced alterations in jejunum tight junctions in females.

Sex-Specific Effects of Stress Exposure on Fecal Microbiota of Male and Female Mice

Due to the relationship between intestinal microbiota and tight junctions (44–46), we analyzed bacterial populations in our various stress models, for both sexes, to determine if dysbiosis may be linked to sex-specific changes in jejunum tight junctions. Male and female mice were exposed to 28 days of CVS, then fecal microbiota were compared (Figure 6A). No difference in the alpha-diversity Shannon and Chao1 indices was observed in either sex (Figure S4A–C). In contrast, beta-diversity measures of dissimilarity of whole microbiota communities between groups revealed 2 distinct clusters for CVS male (Figure 6B), but not female (Figure 6C), mice versus unstressed control mice. In line with the changes reported for males at the jejunum tight junctions after 28 days of CVS (Figure 2), a loss of *Bacteroidetes* and a rise of *Firmicutes*, the 2 most abundant phyla composing our fecal microbiota samples, were observed (Figure 6D). In contrast, no difference was noted for females (Figure 6E), again in line with the limited number of alterations observed for tight junction expression after exposure to this stress paradigm (Figure 2). CVS-exposed males also had a decrease of the S24-7, Lactobacillaceae, and Bacteroidaceae families, along with a rise in Lachnospiraceae and in Ruminococcaceae (Figure 6F).

After 6 days of SCVS (Figure 6G), alpha- and beta-diversity metrics did not differ between groups (Figure S4D–H), but the phylum Proteobacteria was decreased (Figure 6H) in male mice, a change potentially driven by reductions of Alphaproteobacteria and Burkholderiales. SCVS-exposed males also had fewer Clostridiales and Lactobacillaceae (Figure 6J). Regarding females, no differences were detected in relative abundances of the various phyla (Figure 6I), although a few changes at lower levels were apparent in this group, including an enrichment of the family Ruminococcaceae and lower levels of the genus *Alistipes* (Figure S4I).

The gut microbiota have been implicated in vulnerability of male mice to CSDS (47), but, to our knowledge, it has not been explored in females. Thus, female mice were subjected to 10 days of CSDS (16,24) with feces collected after stress exposure (Figure S5A). The Shannon and Chao1 indices and beta-diversity did not differ between groups (Figure S5B, C), but the examination of relative abundances throughout taxonomic

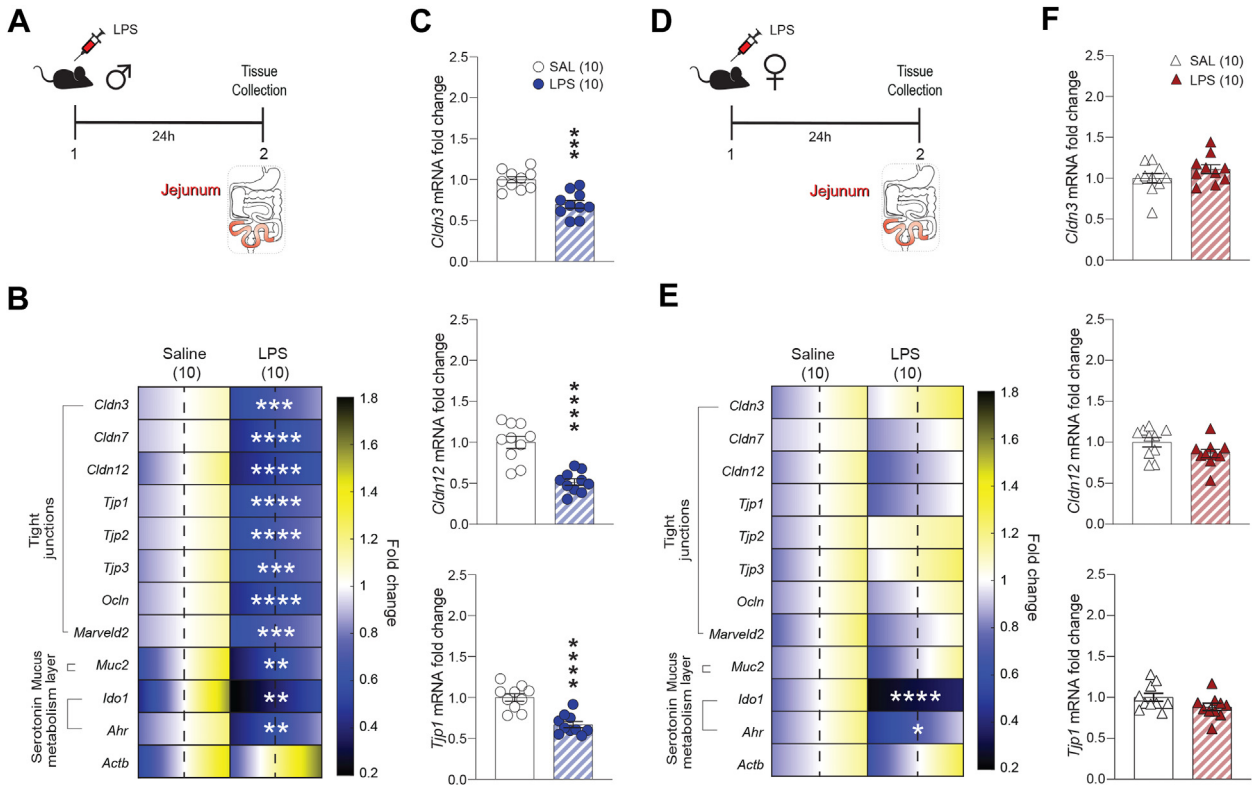


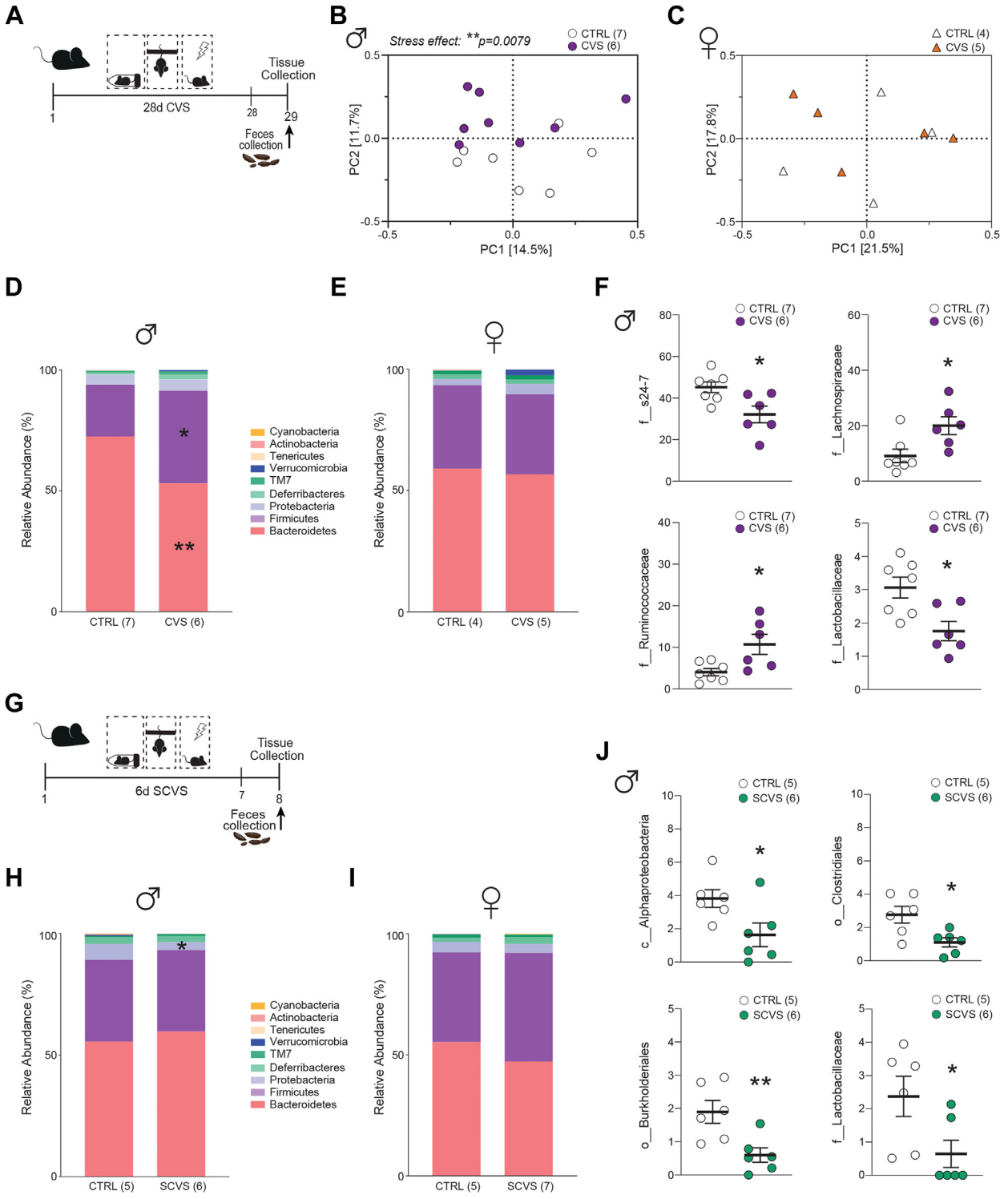
Figure 5. LPS-induced inflammation promotes loss of jejunum tight junction expression in males only. **(A)** Experimental time line of LPS injection and tissue collection in males. **(B)** Quantitative polymerase chain reaction revealed significant changes in jejunum of LPS-treated mice compared with control mice (saline) for gene expression of targets related to tight junctions, the mucus layer, or serotonin metabolism. The range of color indicates individual differences within a group; SEM from the average represented by the dashed line. *Cldn3* ($p = .0001$), *Cldn7* ($p < .0001$), *Cldn12* ($p < .0001$), *Ocln* ($p < .0001$), *Marveld2* ($p < .0001$), *Tjp1* ($p < .0001$), *Tjp2* ($p < .0001$), *Tjp3* ($p = .0001$), *Muc2* ($p = .0076$), *Ido1* ($p = .0052$), and *Ahr* ($p = .0016$) expression was reduced in males after LPS. **(C)** Graphs are provided for *Cldn3*, *Cldn12*, and *Tjp1*. **(D)** Experimental time line of LPS injection and tissue collection in females. **(E)** Quantitative polymerase chain reaction revealed no significant changes in jejunum of LPS-treated female mice compared with control mice (saline injection) for gene expression of targets related to tight junctions. **(F)** Graphs are provided for *Cldn3*, *Cldn12*, and *Tjp1*. However, a significant loss was noted for serotonin-related *Ido1* ($p < .0001$) and *Ahr* ($p = .0115$). Data assessed with Mann-Whitney *U* test; $^{*}p < .05$, $^{**}p < .01$, $^{***}p < .001$, $^{****}p < .0001$. LPS, lipopolysaccharide; mRNA, messenger RNA; SAL, saline.

ranks highlighted elevations of unassigned members of the Bacteroidales order in female mice exposed to social stress (Figure S5D, E). Post hoc analysis revealed that SS, but not RES, mice had higher unassigned Bacteroidales compared with unstressed control mice (Figure S5E), suggesting that this subpopulation might play a role in social stress vulnerability in females.

Blood Biomarkers Are Associated With Loss of Gut Barrier Integrity

We identified blood-based vascular biomarkers, associated with BBB inflammation, in SS mice and women with MDD (16). Identification of MDD-related biomarkers is greatly needed to help guide diagnosis (1). Here, we explored the potential of gut-related circulating markers as indicators of stress vulnerability versus resilience. Blood was collected before and after exposure to 10 days of CSDS, and the serum of male mice was analyzed for LBP, a marker of gut leakiness (Figure 7A; Figure S6C–E) (1). Social stress exposure induced an increase in LBP in the blood serum of SS mice, but not RES or unstressed mice. Moreover, LBP level was negatively correlated with SIs (Figure 7B). Subtracted pre-CSDS blood LBP level was used to dampen individual differences and confirm causality with stress exposure. Next, we evaluated if this biomarker could be relevant for females. Blood LBP was evaluated in the serum of female mice before and after exposure to the SCVS 6-day paradigm (Figure 7C). Indeed, it is in this context that we observed the most significant changes in jejunum CLDN3 tight junctions (Figure 2D). Similar to males, stress exposure was associated with elevated circulating LBP compared with unstressed control mice (Figure 7D). The mean intra-assay variability was 5.68% and 7.80%, and the mean interassay variability was 4.07% and 6.21% for females and males, respectively. Three data points were removed due to high percentage coefficient of variation between replicates. Baseline LBP was not different between unstressed control males and females (Figure 7E). We also evaluated serum corticosterone levels in male mice following CSDS and in females after SCVS. Stress exposure was not associated with changes in circulating corticosterone, possibly due to the 24-

to 48-hour time point of blood collection after stress (47) or its chronic nature (48) (Figure S7). The corticosterone assay was conducted in a single run to prevent interassay variability, and the intra-assay variability was less than 10%. Baseline corticosterone was not different between unstressed control males and females (Figure S7).



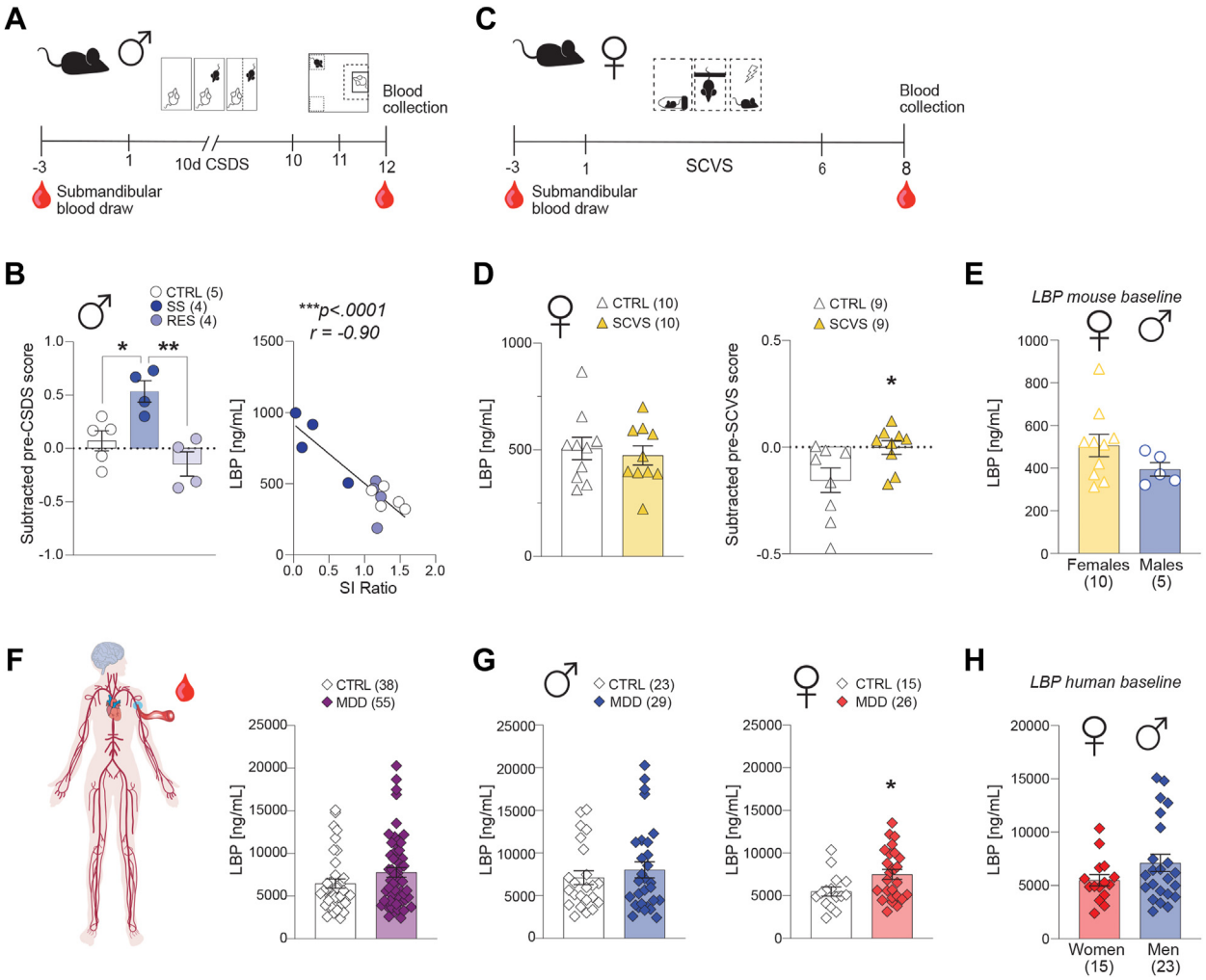


Figure 7. Blood biomarkers are associated with loss of gut barrier integrity in stressed mice and individuals with MDD. **(A)** Experimental time line of 10-day CSDS and blood collection before and after stress exposure. **(B)** LBP is increased in SS, but not RES, male mice compared with unstressed CTRL mice after CSDS ($p = .0033$) and negatively correlated with SI ratio ($p < .0001$). **(C)** Experimental time line of 6-day SCVS and blood collection before and after stress exposure. **(D)** Circulating LBP appears similar between unstressed and stressed groups of female mice, but is in fact increased after 6-day SCVS when LBP level is compared after vs. before stress ($p = .0188$). **(E)** Baseline blood LBP is higher in unstressed CTRL female mice compared with males of the same group without reaching significance. **(F)** Circulating LBP is upregulated in individuals with MDD. **(G)** The effect is driven by women ($p = .0434$). **(H)** Baseline blood LBP in healthy CTRL women and men is similar. Data assessed by *t* tests and one-way analysis of variance followed by Bonferroni's multiple comparison test for changes between groups. Human data assessed with two-tailed Mann-Whitney *U* test; $^{*}p < .05$, $^{**}p < .01$, $^{***}p < .001$. CSDS, chronic social defeat stress; CTRL, control; LBP, lipopolysaccharide-binding protein; MDD, major depressive disorder; RES, resilient; SCVS, subchronic variable stress; SI, social interaction; SS, stress-susceptible.

Finally, we evaluated translational value of LBP as a potential biomarker by measuring it in blood serum samples from individuals with MDD. High circulating LBP was observed for men and women with MDD versus control participants, without reaching significance (Figure 7F). However, consideration of sex revealed an increase in women, but not in men, with MDD

Figure 6. Sex-specific effects of stress exposure on fecal microbiota of male and female mice. **(A)** Experimental time line of 28-day CVS exposure and feces collection. **(B)** Analysis of beta-diversity revealed that CVS males significantly differed from unstressed CTRL mice ($p = .042$). **(C)** No difference was noted for females. **(D)** Relative abundance of phylum communities showed decreased *Bacteroidetes* ($p = .013$) and increased *Firmicutes* ($p = .033$) following CVS in males. **(E)** Again no change was noted for females. **(F)** At the family level, CVS-exposed males had significant changes in the S24-7 ($p = .016$), Lachnospiraceae ($p = .022$), Ruminococcaceae ($p = .039$), and Lactobacillaceae ($p = .011$) families. **(G)** Experimental time line of 6-day SCVS exposure and feces collection. **(H)** A reduction in the Proteobacteria phylum was observed in males after 6-day SCVS compared with unstressed CTRL mice ($p = .041$). **(I)** No significant change was noted for females despite exposure to the same stress paradigm. **(J)** Alphaproteobacteria (unassigned; $p = .035$), Clostridiales (unassigned; $p = .015$), Burkholderiales ($p = .009$), and Lactobacillaceae ($p = .026$) abundances all were decreased in 6-day SCVS males. Unpaired *t* tests were used for 2-group comparisons; $^{*}p < .05$, $^{**}p < .01$. CTRL, control; CVS, chronic variable stress; PC, principal component; SCVS, subchronic chronic variable stress.

(Figure 7G). Within plates, mean intra-assay variability was 5.68%, and mean interassay variability was 6.93%. Seven data points were removed due to high percentage coefficient of variance between replicates. Similar to mice, no difference was noted for men and women in the control groups (Figure 7H). These findings support an increase in gut barrier permeability following stress exposure in mice and, possibly, in individuals with MDD.

DISCUSSION

Growing evidence supports aberrant gut-brain axis signaling in MDD; nevertheless, a direct link between gut permeability and BBB leakiness facilitating passage of circulating inflammatory mediators into the brain in this context is still debated (1). The BBB and gut barrier had not been compared directly until a demonstration of shared stress-induced modifications to *Ocln*, *Tjp1*, and *Cldn5* in the prefrontal cortex and ileum of female rats (49). Considering our work identifying sex-specific BBB tight junction changes in response to social (14,16) and variable stress (15), we examined how such stress influences gut integrity. Our results indicate altered expression of jejunum tight junctions following stress, depending on the type and duration of exposure with sex-specific patterns, consistent with the knowledge of disparate behavioral and biological response to stress in female and male humans and mice (27,50). Compared with the male CSDS paradigm, confounding variables affect validity in females (51) with modifications necessary to induce aggressive bouts leading to altered behaviors. Slightly lower aggression occurs, and overnight sensory exposure does not enhance susceptibility, and it may even enhance resilience (25). LPS treatment decreased jejunum tight junction proteins in males exclusively, highlighting the potential role of inflammatory pathways in the male gut. Serum LBP provided indirect evidence of increased intestinal permeability in both sexes. Microbiota sequencing revealed sex-specific altered microbial populations following stress, which may be linked to different changes in intestinal permeability along with depressive-like behaviors. Stool samples were collected and sequenced before stress exposure to possibly identify individual differences leading to susceptibility versus resilience; however, no difference was observed (data not shown). Similarly, no group housing effect was noted for mice subjected to CVS or SCVS (data not shown).

Alterations in *Cldn3* expression, part of the "tightening" group of tight junction proteins (37,52), suggest that chronic stress exposure affected jejunum permeability. In rodents, Cldn3 expression relates to functional measurements of permeability (53), and in vitro overexpression reduces paracellular ion and large-molecule permeability (54). Cldn3 alterations are implicated in inflammatory bowel and celiac diseases (55), though pathological roles are unclear. Our finding that CSDS elevated *Cldn3* expression aligns with evidence of a repair initiative by the host in response to epithelial barrier injury (56). Acute stress transiently alters tight junction morphology in the rat ileum with changes being restored after 24 hours (57). On the contrary, the CVS 28-day paradigm caused a *Cldn3* reduction, which implies that the reparative strategy is not sufficient to overcome longer stress exposure. Given that shifts in jejunal *Cldn1*, *Cldn5*, *Cldn8*, *Ocln*, and *Tjp1* do not persist in rats after chronic crowding stress, though increased permeability remains (13), other barrier components such as *Cldn3* may be implicated. Shifts in claudin expression are not simply restricted to protein or RNA quantity, as tight junction structures and functions are highly dynamic. Borders are typically displayed as linear structures between cells; however, adjustments in junctional assembly and interactions with scaffolding and cytoskeletal proteins alter these morphologies (40,58). Ruffles presenting as a zigzag-shaped border may indicate enhanced paracellular permeability (40). Increased border ruffling supports *Cldn3* alterations as indicative of disrupted barrier function. Bacterial toxins produced by enteric pathogens can disturb ion flux and barrier function through tight junctions (44,46). Spikes at tight junction borders, emblematic of LPS-induced barrier dysfunction, can be rescued by treatment with commensal microbial metabolites (59).

Most stress or intestinal permeability studies focus on the colon. Mice subjected to water avoidance stress display enhanced ion secretion and permeability to the jejunum, ileum, and colon (60), though effects in each region were distinct. Heightened bacteria adherence and/or penetration occurred (60), aligning with our hypothesis of bacterial translocation. This type of stress also escalates colonic permeability in rats with prolonged effects, attributed to modulation of tight junction expression and heightened inflammation (61). Similarly, restraint stress induces ileal permeability via proinflammatory tumor necrosis factor α signaling (62). Acute early life stress predisposes rats to intestinal dysfunction later in life by priming the stress hormone pathway (63), hinting that consequences of stress exposure can be long-lasting or sensitize the intestinal barrier.

Neuroimaging of individuals with inflammatory bowel diseases demonstrates aberrant brain function in emotion processing and regulation regions (64,65). In addition, increases in colonic transcellular permeability is linked to connectivity changes in brain regions implicated in MDD (66,67). These relationships have prompted clinical investigations into biomarkers of intestinal dysfunction in the context of depression (32,33,43,68). However, limited investigations exist for chronic stress rodent models. Here, elevated serum LBP predominated in SS, and not RES, males following CSDS, highlighting its potential as a biomarker for vulnerability to this form of stress. LBP elevation provides indirect evidence of microbial translocation, due to its specificity to LPS endotoxin. Our findings align with reports of bacterial translocation in mice following CSDS (69) and rats subjected to chronic water avoidance stress (70). To our knowledge, this is the first report of this intestinal-related biomarker in mouse models of depression such as chronic social defeat or variable stress. In accordance with clinical studies (32), serum LBP levels were elevated in MDD. Here, this effect was specific to women only, and we did not find reports comparing sexes.

Regarding MDD, women have a higher risk of developing inflammatory bowel diseases (71,72); therefore, tight junction alterations were expected in female mice following LPS-induced inflammatory challenge. Surprisingly, tight junction gene expression was unaltered, while considerable decrease occurred in male mice. Gene markers implicated in gastrointestinal disorders or claudins with still undetermined functions

could be changing in a sex-specific manner. Peripheral serotonin is implicated in inflammatory, immune, and metabolic signaling pathways (73,74), prompting inclusion of *Ido-1* and *Ahr* in our analysis. LPS treatment reduced levels of both serotonin-related molecules in female mice without affecting tight junctions as for males, highlighting a potentially female-specific pathway. Indeed, progesterone modulates the colonic serotonin system, and in women with inflammatory bowel diseases, both the serotonin transporter and the serotonin levels are diminished (75). IDO-1 is highly upregulated in the human gut epithelium during inflammation (76) and linked to MDD symptoms (77,78). Thus, reduction following LPS implies another mechanism at play. AHR responds to microbial metabolites in an attempt by the host to resist colonization by opportunistic pathogens (79); therefore, these changes may reflect microbiota population changes.

LPS can interact with tight junctions directly (46) with implications for both the gut barrier and the BBB (59). Indeed, in mice, the absence of gut microbes is associated with increased BBB permeability (80). Traumatic stress induces shifts in microbiota diversity and intestinal inflammation with effects on hippocampal CLDN5 (81). Throughout the examined stress paradigms, only the CVS-exposed males differed from control mice in terms of beta-diversity. This group also had microbiota shifts at the phylum level similar to those seen in individuals with irritable bowel syndrome (82), for whom CLDN3 and OCLN reductions were reported (83). Compositional changes in CVS-exposed males coincides with chronic unpredictable mild stress exposure in rats, especially within the family Lachnospiraceae (84). Although Lachnospiraceae, along with Lactobacillaceae and Ruminococcaceae, can produce butyrate and other short-chain fatty acids able to strengthen the intestinal barrier through upregulation of tight junctions (85,86), genera of Lachnospiraceae are implicated in intraintestinal and extraintestinal diseases (85). As for SCVS, decreases in taxa with proinflammatory (Proteobacteria) as well as anti-inflammatory (Lactobacillaceae) properties were apparent in male mice. Proteobacteria elevations have been reported in male mice exposed to longer or more severe stress (47), and thus reductions after the SCVS 6-day paradigm could represent a temporary stress-induced compensatory change. Intriguingly, enteral administration of *Lactobacillus* can modulate intestinal CLDN3 expression (56). After CVS exposure, limited modifications were accounted for in females, though many were expected. We speculate that adaptive mechanisms may bolster the gut after this type of stress. Males and females have different behavioral strategies in reactions to and coping with stress (87); therefore, physiological differences in peripheral responses may occur. Sex differences in microbiota composition exist in healthy adult populations, which are mostly attributed to the interactions and modulation of sex hormones (88).

Limitations of this study include investigating only the jejunum and feces instead of gut content, eliminating the potential of region-specific identifications. Reports of stress effects on jejunal permeability are scarce with conflicting evidence for the small intestine or the colon only (13,89), while others indicate heightened effects on the jejunum compared with other regions (60). Intestinal permeability in MDD has not been associated with a single region of the gastrointestinal tract, and considering its functional roles, the jejunum needs further investigation (29,30). Isolating and sequencing the jejunum contents and assessing morphology of the mucosal layer would aid in gaining mechanistic insights. Furthermore, due to the plethora of comparisons across models and between sexes, we deemed functional measures of permeability beyond the scope of this study and acknowledge this caveat. Another potential drawback involves performing the CSDS paradigm, in which physical injuries are a concern for potential stimulation of inflammation. Physical examinations were performed to consider this confounder, including for peripheral measurements of LBP. Still, repeated social disruption and restraint stress induced bacterial translocation to the mesenteric lymph nodes independently of wounding condition (90).

In summary, our study shows that social or variable stress can induce gut barrier alterations in male and female mice. Changes in jejunum tight junctions along with microbiota populations are dependent on sex, type, and duration, which might be associated with the differences reported in MDD. We provide the first to our knowledge detailed morphological characterization of the jejunum tight junctions following stress exposure by applying novel tools and algorithms that will be freely available and could be applied to various conditions including inflammatory bowel diseases. Finally, by focusing on the small intestine, this study brings novel insights into the biology underlying stress responses in mice and informs on potential biomarkers of MDD such as circulating products related to gut barrier leakiness.

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ED and CM designed the research. ED, LD-A, FC-R, SEJP, FNK, JLS, RG, KAD, and ML performed the research including behavioral experiments; pharmacological treatments; and molecular, biochemical, and morphological analysis. RB, ROA, ADu, and FL-C developed the detailed morphological analysis algorithms and pipeline. Fecal sample collection and microbiota sequencing and analysis were performed by ED with NO and JKS and supervised by M-CA. The Signature Consortium contributed the human blood samples and related demographic data. ED and CM analyzed the data and wrote the manuscript, which was edited by all authors.

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ARTICLE INFORMATION

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