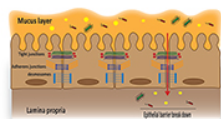


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RESEARCH PAPER



The Zonulin-transgenic mouse displays behavioral alterations ameliorated via depletion of the gut microbiota

Alba Miranda-Ribera^{a,b}, Gloria Serena^{a,b,±}, Jundi Liu^{c,±}, Alessio Fasano^{a,b}, Marcy A. Kingsbury^{b,d}, and Maria R. Fiorentino^{a,b,±}

^aDepartment of Pediatric Gastroenterology and Nutrition, Mucosal Immunology and Biology Research Center, Massachusetts General Hospital, Boston, MA, USA; ^bDepartment of Pediatrics, Harvard Medical School, Harvard University, Boston, MA, USA; ^cDepartment of Poultry Science, University of Georgia, Athens, GA, USA; ^dLurie Center for Autism, Boston, MA, USA

ABSTRACT

The gut-brain axis hypothesis suggests that interactions in the intestinal milieu are critically involved in regulating brain function. Several studies point to a gut-microbiota-brain connection linking an impaired intestinal barrier and altered gut microbiota composition to neurological disorders involving neuroinflammation. Increased gut permeability allows luminal antigens to cross the gut epithelium, and via the blood stream and an impaired blood-brain barrier (BBB) enters the brain impacting its function. Pre-haptoglobin 2 (pHP2), the precursor protein to mature HP2, is the first characterized member of the zonulin family of structurally related proteins. pHP 2 has been identified in humans as the thus far only endogenous regulator of epithelial and endothelial tight junctions (TJs). We have leveraged the Zonulin-transgenic mouse (Ztm) that expresses a murine pHP2 (zonulin) to determine the role of increased gut permeability and its synergy with a dysbiotic intestinal microbiota on brain function and behavior. Here we show that Ztm mice display sex-dependent behavioral abnormalities accompanied by altered gene expression of BBB TJs and increased expression of brain inflammatory genes. Antibiotic depletion of the gut microbiota in Ztm mice downregulated brain inflammatory markers ameliorating some anxiety-like behavior. Overall, we show that zonulin-dependent alterations in gut permeability and dysbiosis of the gut microbiota are associated with an altered BBB integrity, neuroinflammation, and behavioral changes that are partially ameliorated by microbiota depletion. Our results suggest the Ztm model as a tool for the study of the cross-talk between the microbiome/gut and the brain in the context of neurobehavioral/neuroinflammatory disorders.

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Gut permeability; zonulin transgenic mouse; microbiota; dysbiosis; blood-brain barrier; neuroinflammation; behavior

Introduction

Over the past decade, research into the gut-brain axis (GBA) has grown exponentially. It has become evident that other elements besides genetic susceptibility and immune system dysfunction, such as the gut microbiota and changes in gut permeability, play a role in the development of inflammatory diseases in the intestine and the brain.^{1–17} These changes in the gut environment appear to have a profound influence on brain function and behavior.¹⁸ In the pediatric population, attention has been focused on neurodevelopmental and neurobehavioral disorders in children and adolescents, such as autism spectrum disorder (ASD), attention deficit hyperactivity disorder (ADHD), schizophrenia, anxiety and depression, and their frequent

gastrointestinal comorbidities.^{19–27} Paracellular antigen trafficking is strictly controlled by intercellular tight junctions (TJs) that play a critical role in immune tolerance and whose mechanisms of action are still incompletely understood. Zonulin peptides are members of a family of structurally and functionally related proteins that reversibly regulate intestinal permeability by modulating intercellular TJs.^{28–30} The first member of this family, pre-haptoglobin 2 (pHP2) (referred in this paper as zonulin, for simplicity) is the precursor of the mature haptoglobin 2 and has been identified in humans.³¹ Elevated zonulin serum levels positively correlate with loss of gut barrier function³² and have been reported in ASD³³ and associated with hyperactivity and social dysfunctions in children

CONTACT Alba Miranda-Ribera ✉ amiranda-ribera@mgh.harvard.edu Department of Pediatric Gastroenterology and Nutrition, Mucosal Immunology and Biology Research Center, Massachusetts General Hospital, Boston Ma, USA

[±]Current addresses Maria R. Fiorentino, Takeda Pharmaceuticals, Cambridge, Massachusetts Gloria Serena, Sqz Biotech, Watertown, Massachusetts Jundi Liu, Eastman Chemical Company, Animal Nutrition BU, Kingsport TN 37660

with ADHD.³⁴ In neurobehavioral disorders, including ASD and schizophrenia clinical observations of gut hyperpermeability have also been described.^{35–38}

Zonulin release is triggered by several environmental stimuli, including gluten and gut dysbiosis,^{39–41} both of which are interestingly implicated in ASD.^{42–49}

Several species of commensal gut bacteria have the ability to strengthen or weaken gut barrier function,^{50–57} thereby altering intestinal permeability.⁵⁸ Intestinal dysbiosis and inflammation are associated with a dysfunctional epithelial barrier.^{59–65} Increased antigen trafficking through an impaired gut barrier due to a shift in microbiota composition allows harmful substances from the intestinal lumen into the bloodstream,^{52,66,67} leading to the activation of immune cells and subsequent systemic inflammation that can, in turn, affect the integrity of the blood–brain barrier (BBB) and promote neuroinflammation and disease.^{68–70} Defects in gut barrier function and/or gut microbiota dysbiosis, often associated with defects in BBB integrity, have been reported in several inflammatory neurological diseases,^{9,71–73} including ASD and schizophrenia, epilepsy, multiple sclerosis, Parkinson's and Alzheimer's disease.^{9,17,74–100} Furthermore, it has been shown that zonulin increases the permeability of the BBB *in vitro*¹⁰¹ and seems to be involved in the degradation of the BBB in the context of brain tumors.^{102,103} We and others have reported a dysfunctional gut–brain axis associated with neuroinflammation in ASD, adding to the evidence that the BBB is breached in this group of patients.^{100,104–107} Overall, our previous results and data from the literature suggest a role of gut microbiota dysbiosis and hyperpermeability of gut and BBBs in influencing brain function. However, despite accumulating pieces of evidence, the role that increased gut permeability plays in behavioral alterations that characterize neurobehavioral disorders is still unknown. In this study, we used the Zonulin transgenic mouse (Ztm) model of increased gut permeability to assess the role of a zonulin-dependent dysfunctional intestinal barrier and increased trafficking of bacteria and/or

their products from a dysbiotic microbiota on BBB integrity, brain inflammation, and behavior. To this aim, we characterized the composition of the gut microbiota of Ztm male and female mice and assessed their brain function and behavior at baseline and after antibiotic depletion of gut commensal bacteria. We also looked at changes in the small intestinal TJs gene expression profiles in antibiotic-treated mice to assess the impact of bacteria depletion on the integrity of the intestinal barrier.

Materials and Methods

Animals

To work on a murine model expressing the pHP2/zonulin gene, we acquired a mouse (C57Bl/6 background) in which the native mouse haptoglobin 1 (HP1) allele was substituted with a murine HP2 allele by targeted insertion to generate mice with the HP2-2 genotype under the control of its own promoter.¹⁰⁸ A breeding pair was donated by Andrew Levy and kept in our Massachusetts General Hospital (MGH) facility. A colony of C57Bl/6 WT mice was housed in our animal facility as well. Both colonies were reared and maintained under standard conditions (12 h light: 12 h dark cycle, standard humidity and temperature). WT and Ztm male and female mice 8–12 weeks of age were used in this study. Animal studies were approved by the Institutional Animal Care and Use committee at MGH (2013N000013).

Experiment 1: Previously, we have shown that Ztm mice have high small intestinal permeability that is paralleled by a reduction in intestinal TJs gene expression, the presence of pro-inflammatory gut microbiota dysbiosis and an immune system prone to inflammation when exposed to environmental inflammatory stimuli.^{109,110} In Experiment 1, we sought to extend our previous findings and assess whether the observed gut and immune system alterations in the Ztm mouse affected BBB function, brain inflammation, and behavior. We also collected stool samples from male and female WT and ZTM mice to examine the gut microbiota and confirm that gut dysbiosis accompanies brain changes in both sexes. For Experiment 1 we used

13–15 WT males and 13–14 WT females, 16–17 Ztm males and 16–17 females for behavioral tests. Of these, we collected stools from 7 WT males and 9 WT females, 6 Ztm males and 10 females for microbiota analysis.

Experiment 2: We then sought to examine whether the BBB, brain, and behavioral alterations observed in Ztm mice could be ameliorated by depletion of gut bacteria. To this aim, we administered a cocktail of antibiotics in the mice drinking water starting at 3–4 weeks of age to deplete gut bacteria¹¹¹ and eliminate pro-inflammatory gut microbiota, followed by BBB, brain and behavior assays. For this set of experiments, we used 13 WT males and 8 WT females, 8 Ztm males and 8 Ztm females for behavioral assays and microbiota analysis with the exception of Ztm males for which 7 males were used for behavior. All mice in experiment 2 were between 9–11 weeks.

Behavioral assays

To assess if there were behavioral differences between our experimental groups, we used three standard behavioral tests that included marble burying, open field, and elevated zero maze. Mice were tested during their light cycle, over three consecutive days, at the same time of the day (in the mornings within the same 3-hour window) and participated in one behavioral test per day. Tests were run in sequential order from the least stressful to the most stressful assay [marble burying (day 1), open field (day 2), elevated zero maze (day 3)]. Ethovision XT, an automated software for detection and quantification, was used for behavioral scoring of the open field and elevated zero maze assays. Mice were moved to the testing room at least 30 minutes prior to start the testing to allow for acclimation to the animal behavioral room. Males and females were always tested and acclimated separately.

Marble burying

The Marble Burying test measures obsessive/compulsive/repetitive behavior, as a paradigm of the ethologically natural behavior of defensive burying.^{112–114} Regular housing cages (28x20x12 cm) were filled

with 7 cm of autoclaved, and evenly distributed bedding material (Sani-chips®). Twenty Marbles (Fisher Science Education TM Glass Marbles #S04581, 1.42 cm) were placed equidistant in 4 parallel lines with alternating colors of black and blue. Mice were placed in the center of the cage and free to interact with the marbles. After 20 minutes, animals were removed carefully so as not to disturb the bedding and/or the marbles and the number of buried marbles was counted. Marbles were considered buried when at least 2/3 of their volume was covered by bedding material.

Open field

The Open Field test measures anxiety and hyperactivity in a low anxiety setting^{115–117} based on the idea that mice naturally prefer to be near a protective wall rather than in an open field where they are exposed to predators (in nature). Each animal was placed in a square (28x28x28 cm) white opaque plexiglass box for 10 minutes, and the animal's movements were tracked with an infrared video camera. Time spent in the center of the box (defined as a central square that covered 20% of the field) versus closer to the walls was tracked and total distance traveled was calculated using the EthoVision XT Software. Males and females were never present in the behavioral testing room at the same time and the testing apparatus was thoroughly cleaned between test animals.

Elevated zero maze

The Elevated Zero Maze measures anxiety. It is a modification of the elevated plus maze first described by Shepherd et al.¹¹⁸ and consists of an elevated annular arena with two open areas and two closed areas (both of equal area). During the test the animal was placed in the open arm and allowed to explore freely for 5 minutes. While the open areas are, by design, anxiogenic due to the increased exposure and height of the maze, the animal is free to move into the closed areas. Time spent in the open arm vs. closed arm was measured. Both parameters were automatically tracked by an overhead infrared camera and analyzed by EthoVision XT Software.

Antibiotic treatment

We adopted antibiotic depletion protocols widely used in the literature, showing depletion of commensal bacteria.^{111,119,120} Mice at weaning (3–4 weeks of age) were treated with a mix of antibiotics in their drinking water (*ad libitum*) containing Ampicillin (1 gr/L), vancomycin (500 mg/L), metronidazole (1 g/L), and neomycin (1 g/L). All antibiotics were purchased from Sigma. Since mice were not drinking when the full dose was administered because of the taste given by metronidazole, to ensure mice would drink regularly and consistently, the antibiotic cocktail was introduced at 1/8 of the final concentration and increased to 1/4, 1/2, and 3/4 every 5 days, until reaching full concentration. Mice were treated with a full antibiotic dose for 2 weeks. At the end of the 2 weeks, DNA was extracted from stools and bacterial load was assessed by 16S qPCR. Both antibiotic-treated WT and Ztm mice showed a 70% reduction in bacterial load (data not shown).

Total RNA extraction and quantitative PCR (real-time PCR)

Mice were anesthetized using Forane (isoflurane, USP, Baxter, US) and euthanized by cervical dislocation. Tissues were immediately collected, fresh frozen and kept at –80 C until processed. Total RNA was extracted from frozen tissues using TRIzol™ reagent (Thermo Fisher, US) and the Direct-zol RNA mini prep plus Kit (ZymoResearch, US)

following the manufacturer's protocol. To ensure full removal of genomic DNA, extracted RNA from each sample was treated with the DNA free™ Kit (Thermo Fisher, US). Total RNA was quantified and A260/280 and A260/230 ratios measured using a Nanodrop 2000 (Thermo Fisher, US). cDNA was made using the Maxima H⁺ First Strand cDNA Synthesis Kit (Thermo Fisher, US) according to manufacturer's instructions. Quantitative real-time PCR was performed using PerfeCTa SYBR® Green SuperMix (Quanta, US) and a Bio Rad CFX connect thermocycler. All primers were designed in-house, obtained from Integrated DNA Technologies (US) and listed in Table 1.

Zonulin qPCR gene expression in the small intestine and the brain

Only for the zonulin gene expression profile, qPCR data from Experiment 2 (Abx-treated Ztm mice) were compared to Experiment 1 (Ztm mice) to assess whether depletion of the gut microbiota affected the expression of zonulin in the gut and/or brain.

Microbiota analysis

Sample collection and DNA extraction

Collected fecal pellets were flash frozen and kept at –80 C until further processing. Genomic DNA for each sample was extracted using the DNeasy powersoil extraction kit (Qiagen, US) following

Table 1. List of primers and FASTA accession numbers of the genes analyzed by real time qPCR analysis. All primers were designed in house using NIH's Primer Blast and acquired from Integrated DNA Technologies (USA).

Gene	Accession number	5' OLIGO	3' OLIGO	Gene Function
CD11b	NM_008401.2	GCAACCATGGAATTCAGCCC	GAGCGGACAGGGTCTAAAGC	Microglia marker
CLDN 1	NM_016674.4	GGCTTCTCTGGGATGGATCG	CTTTGCCGAAACGCAGGACAT	Barrier-forming
CLDN 10	NM_001160096.1	CCCAGAATGGGCTACACATA	CCTTCTCCGCTTGATACTT	Pore-forming
CLDN 15	NM_021719.4	AGGCACACCTTATCTGGCAC	TGCCCCCTGAACAATCACAA	Pore-forming
CLDN 3	NM_009902.4	CCTAGGAACTGTCCAAGCCG	CCCGTTTCATGGTTTGCTG	Barrier-forming
CLDN 4	NM_009903.2	CGTAGCAACGACAAGCCCTA	TGTCCCCAGCAAGCAGTTAG	Barrier-forming
CLDN 5	NM_013805.4	GTTAAGGCACGGGTAGCACT	TACTTCTGTGACACCGGCAC	Barrier-forming
CLDN 7	NM_016887.6	GCATACTTTCTGGGGGCCA	TGAAGCGACACTCTCACAGC	Pore-forming
CLDN 2	NM_016675.4	CCGTGTCTGCGGAGATTCTC	AGGAACGACGCGGAGTAG	Pore-forming
GABA	NM_019439.3	TGGGAAGGTTTCTCTGACGG	TGAGTGTGGCGTTCGATTCA	Brain neurotransmitter
IL1β	NM_008361.4	GCCACCTTTTGACAGTGATGAG	AGCTTCTCCACAGCCACAAT	Proinflammatory cytokine.
JAM3	NM_023277.4	GCTGTGAGGTCTGTGCTCTA	AGTGGACATCATTGCGGTA	Barrier-forming
Myo1C	NM_008659.3	CCGATCACCCGAAGAACCAC	CGCCGGAGGTTCTCAATGAA	Scaffolding
OCCLN	XM_011244634.2	CTGACTATGCGGAAAGAGTTGAC	CCAGAGGTGTTGACTATAGAAAGAC	Barrier-forming
veCad	XM_006530630.1	AATTTGCCAGCCCTACGAA	ACTGCCCATCTTGACCGTG	Endothelial barrier-forming.
ZO1	NM_009386.2	AAGAAAAAGAATGCACAGAGTTGT	GAAATCGTGCTGATGTGCCA	Scaffolding
ZO2	NM_001198985.1	AGTTGTAGTTCTGAGCCCG	CCGACACGGCAATTCCAAAT	Scaffolding
Zonulin	from Sturgeon et al, 2017 (24)	GAATGTGAGGCAGATGACAG	GTGTTCAACCATGCTTCTC	TJ modulator
18S	X03205	AGAAACGGCTACCACATCCA	CCCTCAATGGATCCTCGTT	Ribosomal RNA

Qiagen's instructions. DNA was quantified using Nanodrop. In order to carry out the phylogenetic profiling, the hypervariable V4 region of the 16S rRNA gene was amplified by PCR using 5X prime master mix (Prime, US), reverse 806 primers were barcoded and a unique forward 515 primer (Integrated DNA Technologies, US) was used. To confirm correct amplification of the V4 regions, a regular gel electrophoresis was run. PCR products were purified using the QIAquick PCR purification kit (Qiagen, US) and their concentration was measured by Quant-iT Picogreen dsDNA kit following manufacturer's instructions. Sequencing of the samples was done at the MGH NextGen Sequencing Core facility (Boston, US), on the Illumina system using the MiSeq v2 500 cycles reagent kit as per manufacturer's instructions. To allow maximum coverage of the amplicon, the system sequenced a total of 250 paired-end cycles. The following primers were used for the sequencing:

read 1 (TATGGTAATT GT GTGYCAGCM GCCGCGGTAA)
 read 2 (AGTCAGCCAGCCGGACTACNVG GGTWTCTAAT)
 index (AATGATACGGCGACCACCGAGAT CTACACGCT).

Microbiota data analysis

Sequencing data were processed and analyzed with QIIME2 software package v. 2018.2.0.¹²² The sequencing reads with low-quality score (average Q < 25) were truncated to 240 bp followed by filtering using *deblur* algorithm with default settings.¹²³ The remaining high-quality reads were aligned to the reference library using *mafft*.¹²⁴ Next, the aligned reads were masked to remove highly variable positions, and a phylogenetic tree was generated from the masked alignment using the FastTree method.¹²⁵ Alpha and beta diversity metrics and Principal Component Analysis plots based on Jaccard distance were generated using default QIIME2 plugins.¹²² Taxonomy assignment was performed using *feature-classifier* method and naïve Bayes classifier trained on the Greengenes 13_8 99% operational taxonomic units (OTUs). Differential abundance analysis of OTUs was performed using ANCOM.¹²⁶

Statistical analysis

Statistical analyses for behavioral experiments and real-time PCR were performed in Graph Pad Prism 9. Behavioral experiments are expressed as means \pm SEM using unpaired t-test to assess statistical significance. Comparisons between 2 groups for the qPCR analysis are expressed as means \pm SEM and analyzed with the non-parametrical Mann–Whitney *U* test for statistical significance. $p < .05$ was considered statistically significant in both tests. Statistical analysis of the microbiome was performed using the Kruskal–Wallis test to assess the statistical significance of abundance differences, with multiple testing corrections performed using Benjamini–Hochberg false discovery rate (FDR). The FDR cutoff was set at 0.05.

Results

Ztm male and female mice show altered repetitive and anxiety-like behavior compared to WT mice

No differences were observed in the number of marbles buried in Ztm males and WT males (Figure 1a). In contrast, Ztm females buried significantly more marbles than WT females (Figure 1b). In the open field assay, both Ztm and WT males traveled similar distances (Figure 1e), yet Ztm males spent significantly more time in the center of the open field compared to WT males (Figure 1c). Within females, no significant differences were observed for total distanced traveled or time in the center of the open field between Ztm and WT mice (Figure 1d, f). For the elevated zero maze behavioral assay, Ztm males spent significantly less time in the open arms of the maze than WT males (Figure 1g) while there was no difference in open arm time between Ztm females versus WT females (Figure 1h).

Ztm male and female mice show altered gene expression profiles for BBB TJs and markers of neuroinflammation compared to WT mice

To determine whether the behavioral changes observed in the Ztm mice were linked to molecular changes in the BBB and the brain, we examined TJs and neuroinflammation-associated genes in the prefrontal cortex of mice. We observed significant

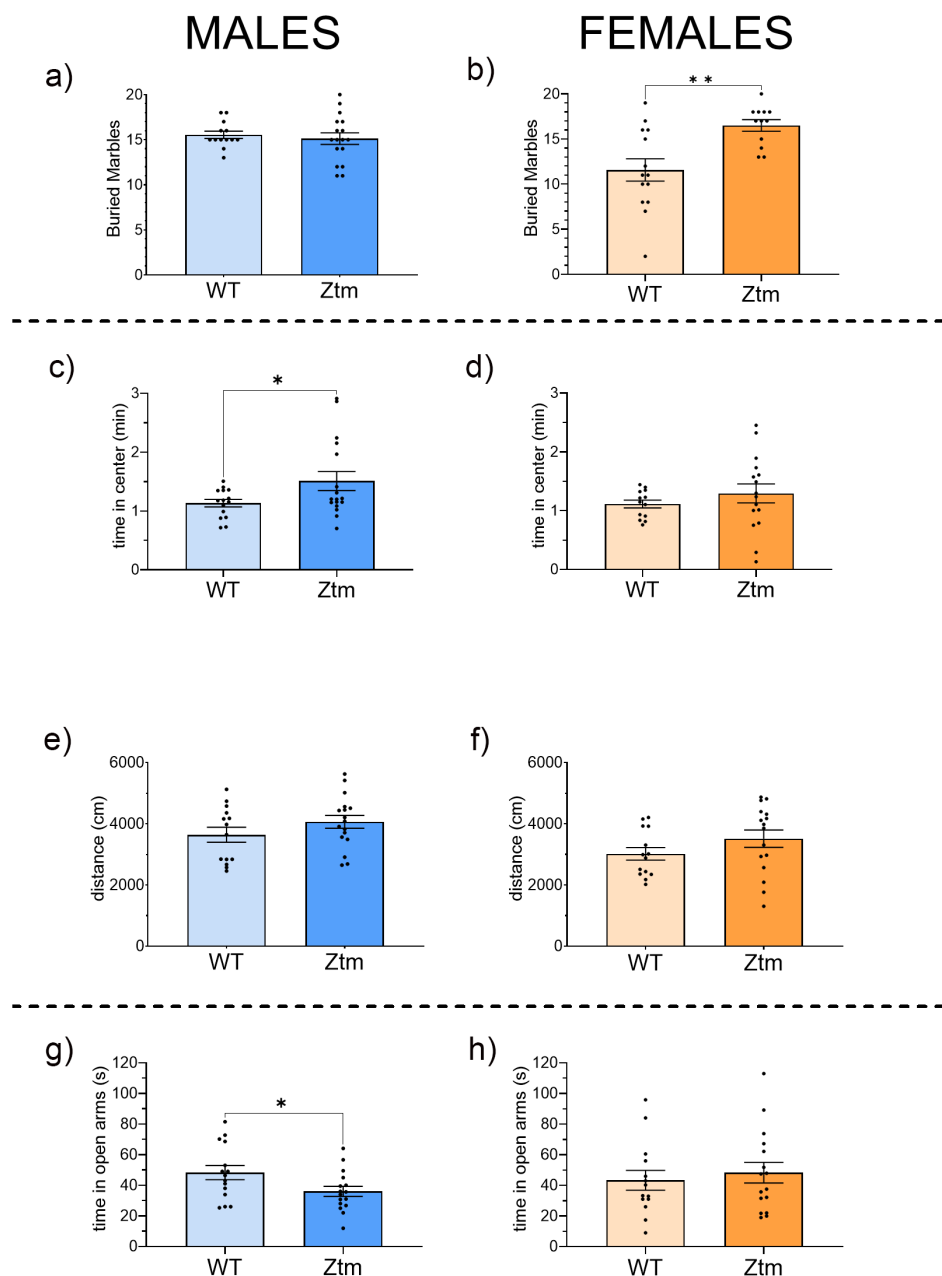


Figure 1. Behavior profile of Ztm mice compared to WT mice. Marble Burying (a, b): Total number of marbles buried is significantly higher in Ztm females than wt females (b), no differences seen in males (a). Open field (C, D, E, F): Time spent in the center of the field is significantly higher in Ztm males than wt males (c). No significant difference was seen on females (d). Distance traveled was similar for both males (e) and females (f). Track images are representative of each group. Elevated Zero Maze (g, h): Ztm mice spent significant less time in the open arm than WT mice (g), no difference seen in the female group (h). N = 13–17.

differences in BBB-associated TJs gene expression in Ztm mice compared to WT mice, with each sex displaying a unique pattern of changes within the Ztm mouse model (Figure 2). Ztm males exhibited a significant increase in the expression of TJs *occludin* (*OCCLN*) and *ve-Cadherin* (*veCad*) that was accompanied by significantly higher levels of pro-inflammatory markers *Interleukin* (*IL*)1 β and

CD11b (Figure 2a, B; Table 2). Ztm males were also characterized by a significant increase in *GABA* gene expression (Figure 2b, Table 2). Unlike males, Ztm females showed a significant decrease in key TJs components of the BBB, such as *claudin* (*CLDN*)-5, 3, and 1 and no change in the neuroinflammatory genes *IL*-1 β and *CD11b* (Figure 2c, D; Table 2).

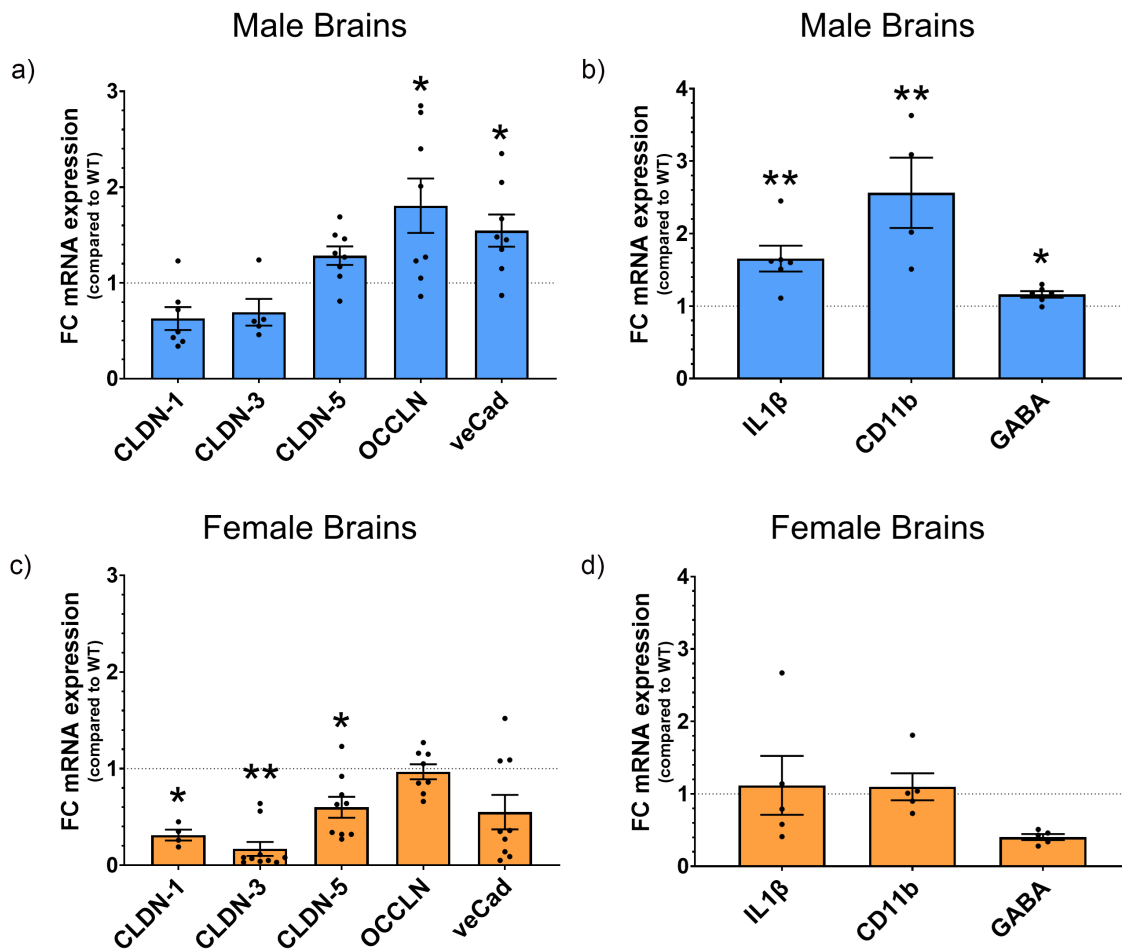


Figure 2. Gene expression profile for BBB TJs and neuroinflammation markers. mRNA expression was analyzed by qPCR normalized to WT and expressed as fold change \pm SEM. (a) Increased *OCCLN* and *veCad* in Ztm males compared to WT males in the BBB. (b) Altered neuroinflammatory profile in the Ztm male brain. (c) Significant downregulation of BBB TJs in the Ztm female brain. (d) No change in the neuroinflammatory profile of the Ztm females.

Table 2. List of genes analyzed by real time PCR in the brain of adult mice ($n = 4-10$). Expressed as Fold Change (FC), statistics calculated with the non-parametric Mann-Whitney test (p).

Brain	Males		Females	
	FC	p	FC	p
CD11b	2.56	0.006	1.1	>0.9999
CLDN 1	0.63	0.274	0.31	0.038
CLDN 3	0.7	0.662	0.17	0.002
CLDN 5	1.29	0.081	0.6	0.038
GABA	1.16	0.024	0.41	0.571
IL1 β	1.65	0.004	1.12	0.556
OCCLN	1.8	0.043	0.97	0.852
veCad	1.55	0.022	0.55	0.145

The gut microbiota of Ztm male and female mice is shifted toward a pro-inflammatory phenotype

Analysis of the 16s rRNA sequencing of the gut microbiota showed a high degree of beta diversity (between groups) in PCoA plot between Ztm and WT males (Figure 3a). The Shannon index of alpha

diversity showed that Ztm males harbored a less diverse and less uniform gut microbiota compared to WT males (Figure 3c). Beta diversity analysis of the microbiota from female mice also revealed that Ztm females clustered separately from WT females in the PCoA plot (Figure 3b), indicating a different microbiota composition compared to WT. However, the Shannon index was not different between female Ztm compared to female WT mice (Figure 3d). At the phylum level, Ztm males had lower Proteobacteria (class Alphaproteobacteria) and Verrucomicrobiota (Figure 4a) with a drastic decreased *Akkermansia* (sp. *muciniphila*) sp. On the other hand, they showed an increased abundance of the inflammatory-associated *Rikenella* sp. (class Bacteroidia)(Figure 5a). Similarly, Ztm females also harbored dramatically reduced Verrucomicrobiota and decreased abundance of Proteobacteria

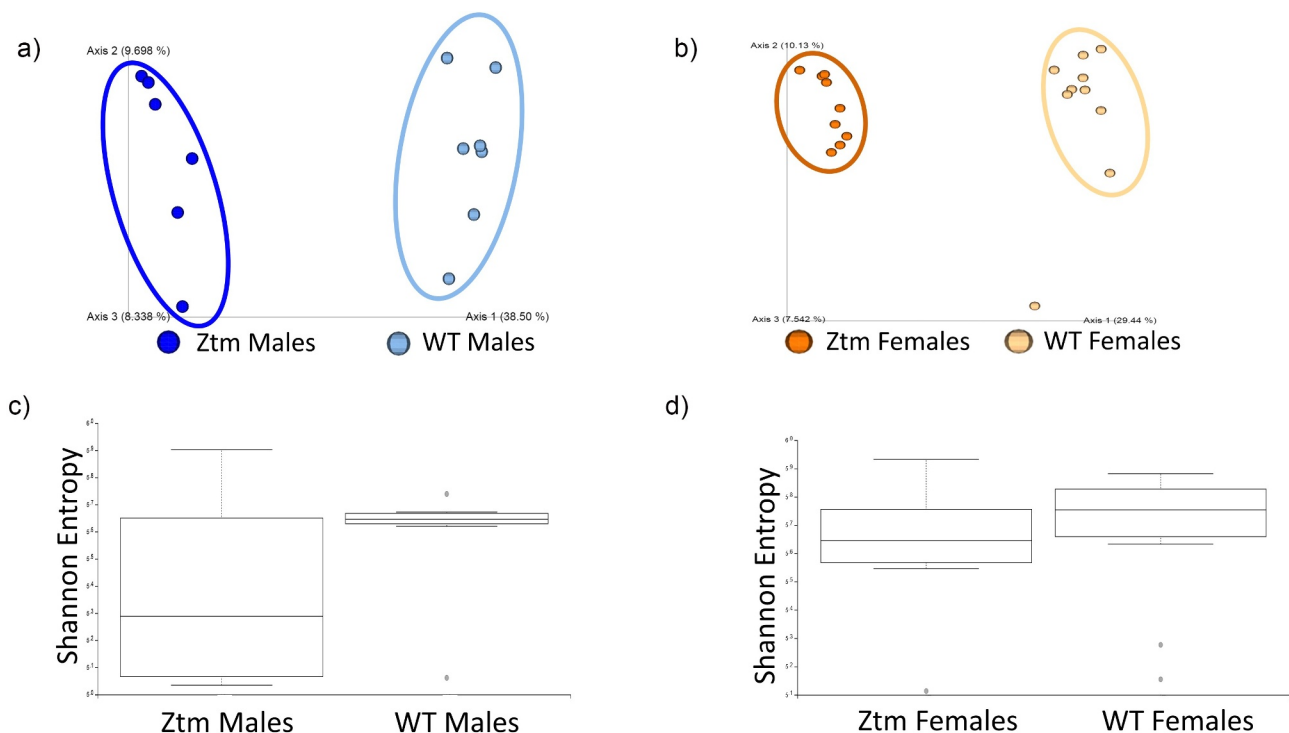


Figure 3. Gut microbiota dysbiosis in males and females Ztm mice. Stools from WT (males $n = 7$, females $n = 9$) and Ztm mice (males $n = 6$, females $n = 10$) were analyzed. (a, b): Principal Component Analysis (PCA) of Jaccard distances for Ztm and WT mice show clustering between Ztm and WT males (a) and females (b). The closer the spatial distance of the sample, the more similar the species composition of the sample. (c, d): Shannon Index expression of alpha diversity (variation/complexity of the microbiome within the group). WT males show a less diverse microbiota than Ztm males (c). Similar alpha diversity observed in the Ztm and WT females (d).

(Alphaproteobacteria) and Cyanobacteria in their gut (Figure 4b) with a dramatic reduction in the relative abundance of *A. muciniphila* sp. and significantly greater abundance of *Rikenella* sp. compared to WT females, as shown in the ANCOM volcano plot analysis (Figure 5b).

Abx-treatment shifts gut microbiota composition

As expected, Abx-treatment drastically reduced bacterial load (70% reduction; data not shown) and shifted the gut microbiota composition in all groups from baseline conditions (Figure 7 compared to Figure 4). PCoA showed that Abx-treated Ztm mice did not cluster separately from Abx-treated WT animals (Figure 6a,b). Alpha-diversity in Abx-treated mice was considerably reduced and no significant differences were observed between Abx-treated Ztm and Abx-treated WT (Figure 6c,d).

Abx-treated WT and Abx-treated Ztm males showed a drastic reduction of Bacteroidota versus a higher prevalence of Firmicutes (Bacilli) and

Proteobacteria (Gammaproteobacteria) in Abx-treated Ztm and Proteobacteria (Gamma- and Alphaproteobacteria) and Cyanobacteria in Abx-treated WT (Figure 7a).

In females, we also observed a drastic reduction of Bacteroidota in both Abx-treated Ztm and Abx-treated WT concurrent with an expansion of Firmicutes (Bacilli). While Abx-treated Ztm females also showed a prevalence of Proteobacteria of the class Gammaproteobacteria, Abx-treated WT females had a prevalence of Proteobacteria of the class Alphaproteobacteria as well as Cyanobacteria (Figure 7b). Analysis of the gut microbiota at the genus level showed that almost 80% of the microbiota of Ztm mice treated with Abx was predominantly composed of *Ureaplasma* (Firmicutes, Bacilli; 42.5% in females and 52.1% in males) and *Escherichia-Shigella* (Proteobacteria, Gamma proteobacteria; 35.9% in females and 23.2% in males) with a drastic reduction of Bacteroidota (including the genus *Rikenella*) that was noticeably more pronounced in females (retained only 0.3%) than in males (retained only

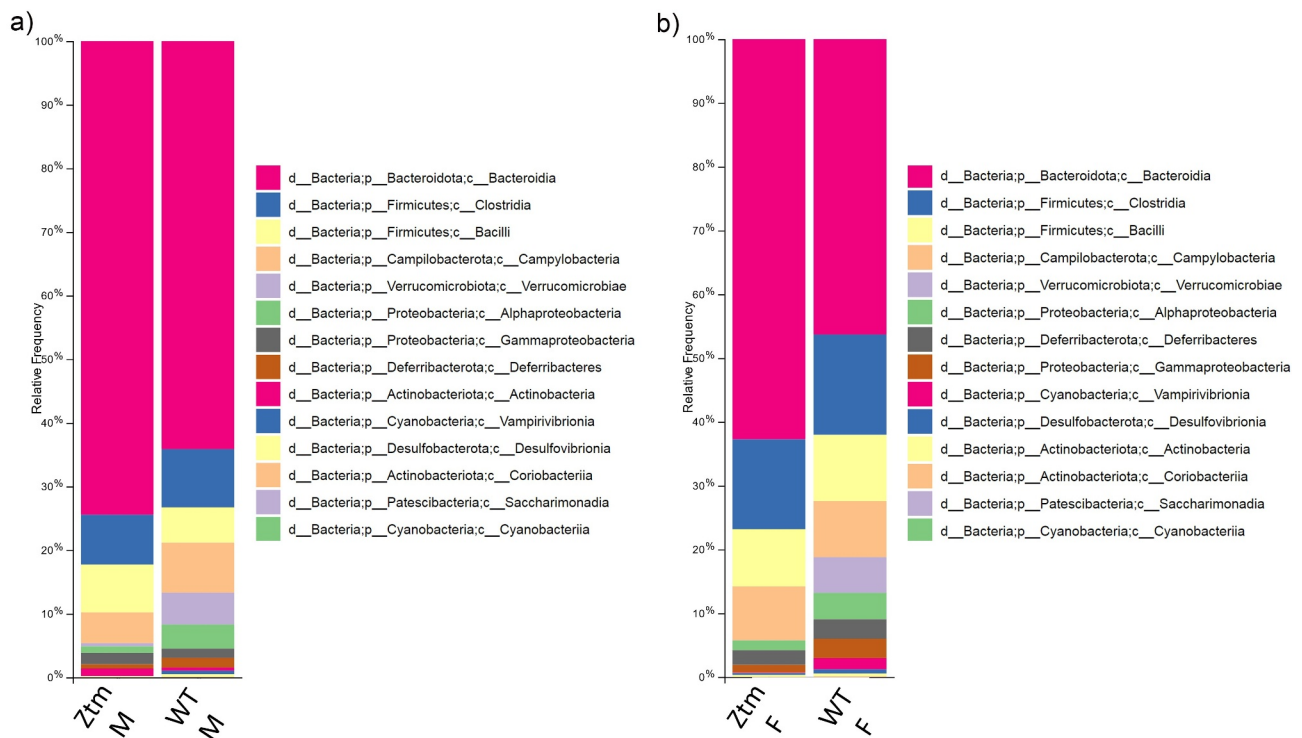


Figure 4. Histogram of the community composition of gut microbiota at the class level. The abscissa represents the group, and the ordinate represents the relative abundance. The figures show species with a relative abundance of 1% or more. (a): Lower abundance of Alphaproteobacteria and Verrucomicrobiae in Ztm males when compared to WT males. (b): Ztm females show lower abundance of Proteobacteria, Verrucomicrobiae and Cyanobacteriia in their stools.

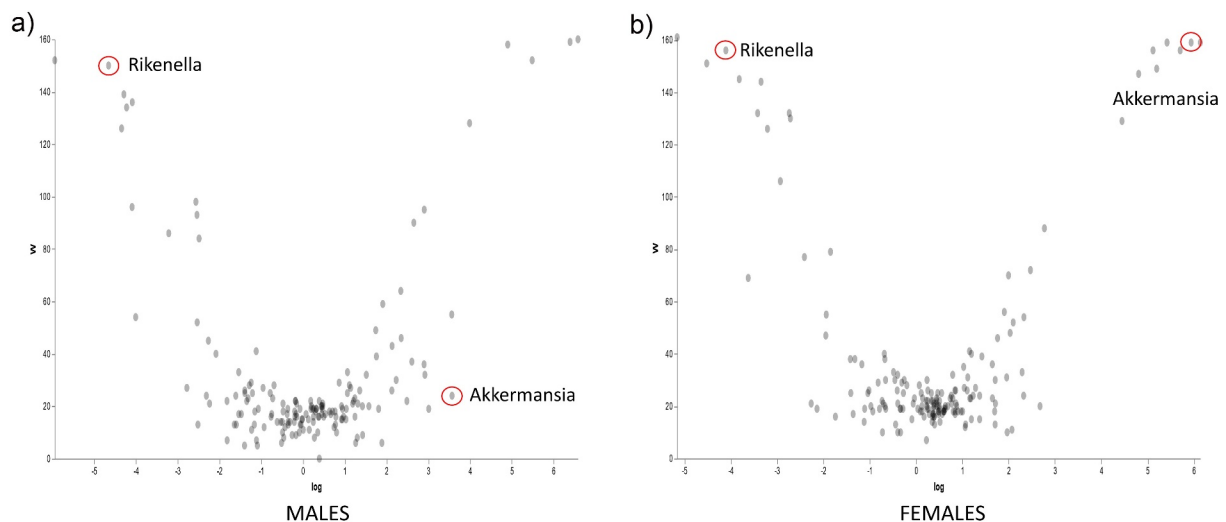


Figure 5. ANCOM volcano plot of statistical differences between Ztm and WT at genus level. (a): Ztm males harbor a significant increased abundance of *Rikenella* sp. vs. an abundance of *A. muciniphila* in WT males. (b): Ztm females show an abundance of *Rikenella* sp. and a reduction of *A. muciniphila* when compared to WT females.

12.3%). Ztm females also retained 18.7% of the genus *Parasutterella* while it was reduced to a 4.6% in males.

In Abx-treated WT males, Proteobacteria were represented by the genus *Ralstonia* and *Mitochondria* that contributed 64.4% of the total

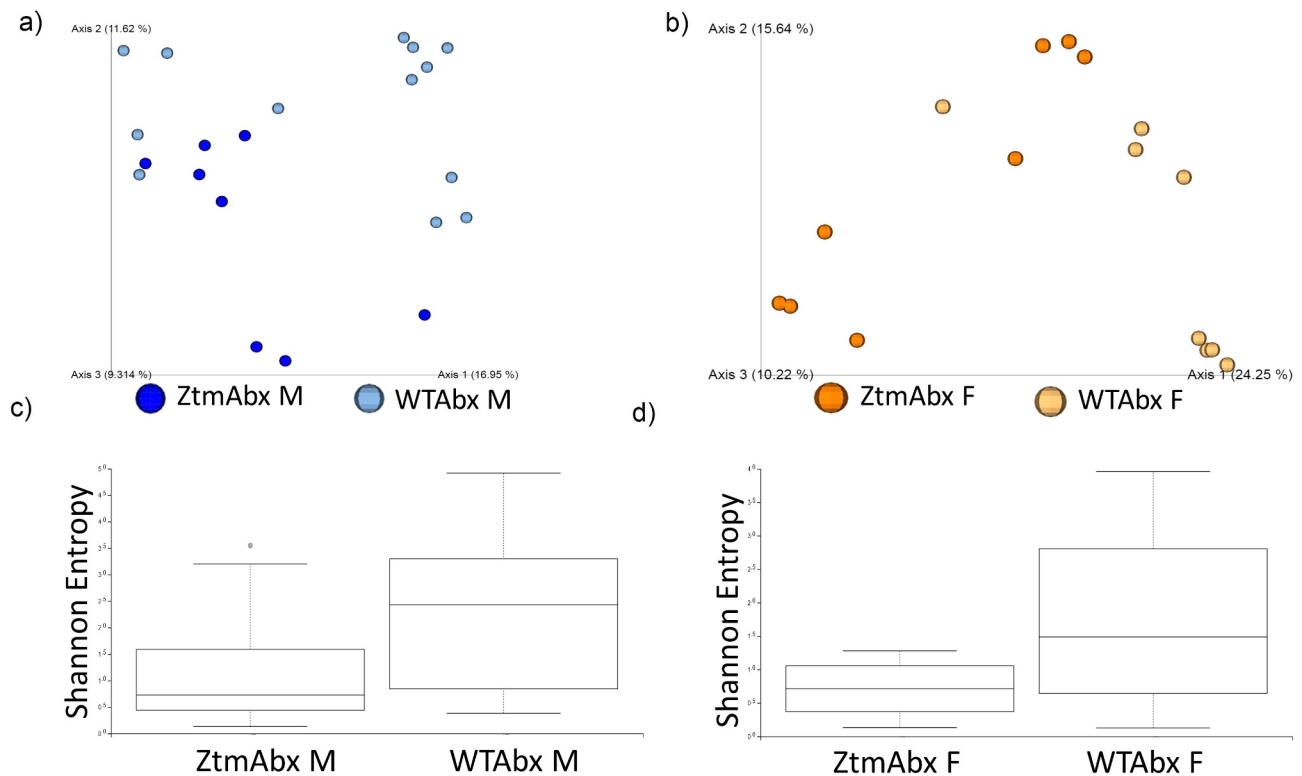


Figure 6. Effect of Abx treatment on gut microbiota composition in Abx-treated Ztm and WT mice. Stools from 21 WT mice (males $n = 13$, females $n = 8$) and 16 Ztm (males $n = 8$, females $n = 8$) were analyzed. (a, b): In Principal Component Analysis (PCA) of Jaccard distances for Abx-treated Ztm (ZtmAbx) and Abx-treated WT (WTAbx) mice revealed no distinct microbiota clustering between Abx-treated Ztm and WT males (a) or females (b). (c, d): Shannon Index, expression of alpha diversity (variation/complexity of the microbiome within the group). Alpha diversity was reduced in Abx-treated Ztm males when compared to Abx-treated WT males (c), and in Abx-treated Ztm females when compared to Abx-treated WT females.

microbiota composition. Another 21.3% was made of Cyanobacteria of the genus *Chloroplast*. In Abx-treated WT females we observed a considerable expansion of Firmicutes of the genus *Ureaplasma* (58.1%), while 18.3% were Proteobacteria (genus *Mitochondria*) and 15.5% were Cyanobacteria (genus *Chloroplast*). Of note, Abx treatment severely depleted the genus *Akkermansia* from the Abx-treated WT microbiota. Volcano plot analysis did not show significant differences between Abx-treated Ztm and Abx-treated WT mice except for the *Ureaplasma* genus that was significantly more abundant in Abx-treated Ztm males and the *Erwiniaceae* family of the class Gammaproteobacteria, which was more abundant in Abx-treated WT females (Figure 8a, b).

Abx treatment restores small intestinal gut barrier integrity

We previously reported decreased gene expression of several TJs components in Ztm males compared to WT male mice.¹¹⁰ In contrast, Abx-treated Ztm

male mice did not show differences in TJs gene expression compared to Abx-treated WT male mice (Figure 9a). In Abx-treated females, we only detected a significantly decreased *CLDN-4* in Ztm mice compared to WT females (Fig. B9, Table 3). Noteworthy, in both Abx-treated Ztm males and females (from Experiment 2), we detected reduced levels of zonulin expression compared to Ztm males and females (from Experiment 1) (Figure 10).

Abx-treatment affects gene expression profiles of BBB TJs and markers of neuroinflammation

We analyzed the gene expression profile of BBB TJs as well as neuroinflammatory markers in the prefrontal cortex, as we had done at baseline, to assess the role of gut bacteria in brain function in Abx-treated mice. In Abx-treated Ztm male mice we observed a significant higher expression of *CLDN-1*, *-3* and *-5*, while *ve-Cad*, *Cd11b* and *GABA* expression were significantly decreased (Figure 11A, B, Table 3), as compared to

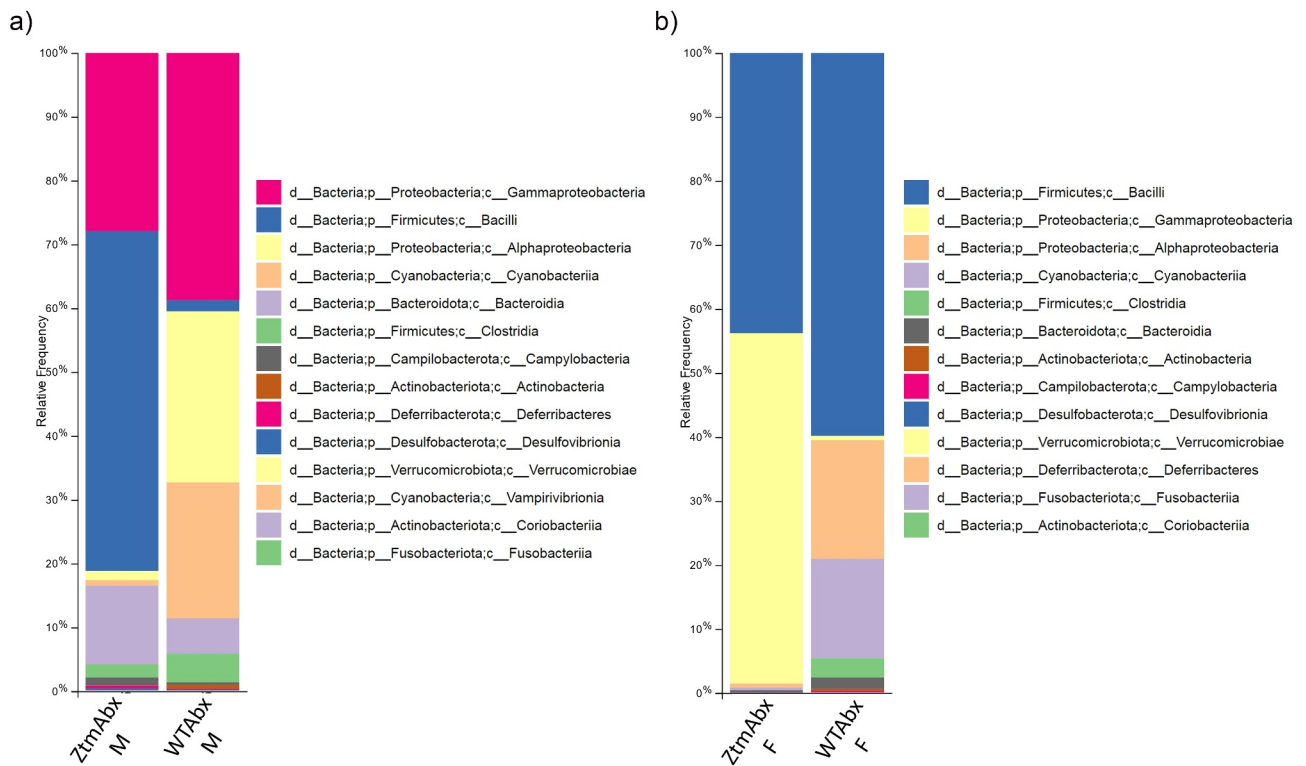


Figure 7. Histogram of the community composition of gut microbiota at the class level after Abx treatment. Abx-treated Ztm (ZtmAbx) males have a higher abundance of Firmicutes (Bacilli), and severely decreased Alphaproteobacteria and Cyanobacteriia composition compared to Abx-treated WT (WTAbx) mice. (b): Abx-treated Ztm females show a dramatic increase in Gammaproteobacteria and a drastic reduction of Alphaproteobacteria and Cyanobacteriia when compared to WTAbx females.

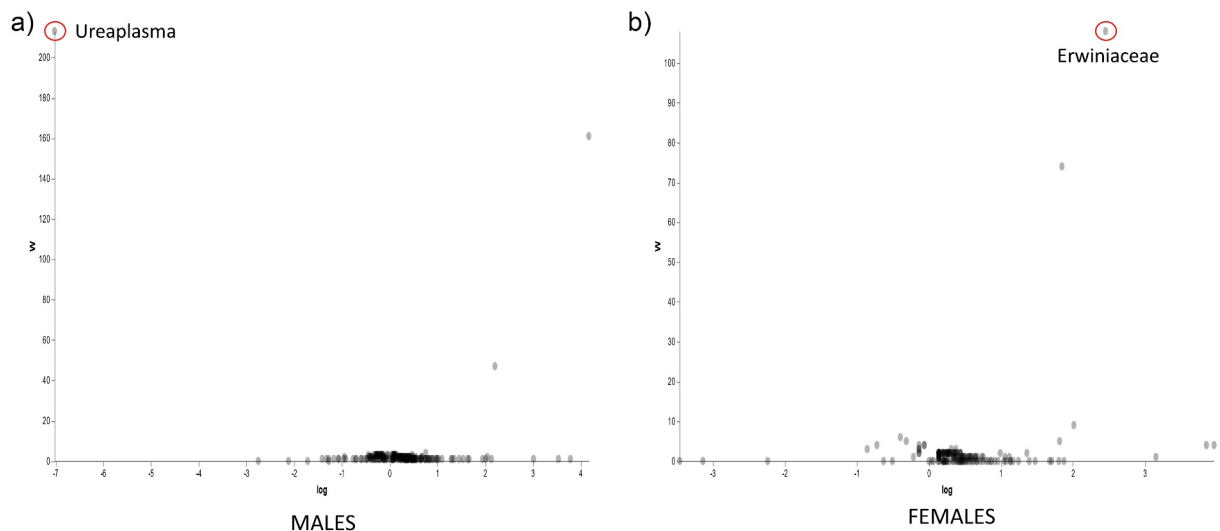


Figure 8. ANCOM volcano plot of statistical differences between Abx-treated Ztm and Abx-treated WT mice at genus level. (a): Abx-treated Ztm (ZtmAbx) males harbor a significantly higher abundance of *Ureaplasma* sp. compared to Abx-treated WT (WTAbx) males. (b): Abx-treated WT (WTABX) females show significantly more abundant *Erwiniaceae* sp. when compared to Abx-treated Ztm (ZtmAbx) females.

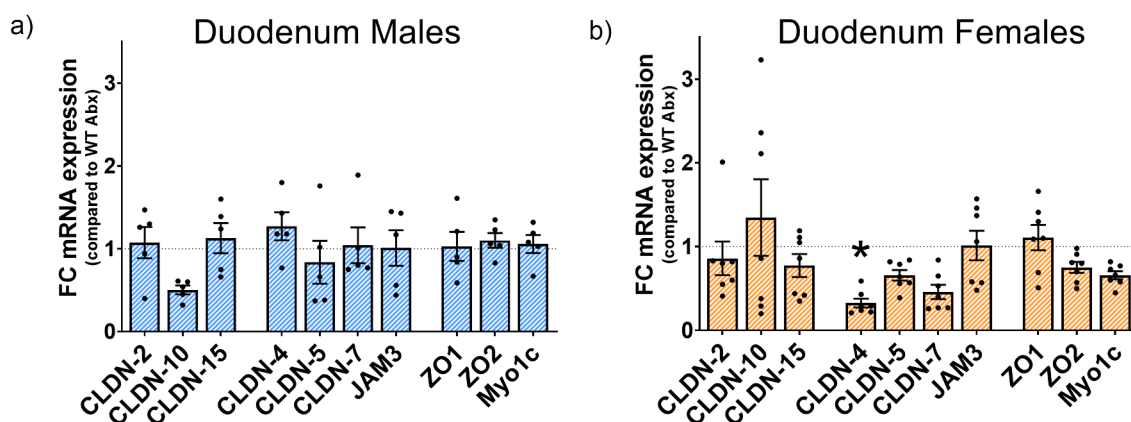


Figure 9. Effect of Abx gut microbiota depletion on the gene expression profile of TJs in the duodenum of Abx-treated mice. mRNA expression was analyzed in ZtmAbx treated mice by qPCR, normalized to WTAbx, and expressed as fold change \pm SEM. (a): No differences were seen in the Abx-treated Ztm male mice when compared to Abx-treated WT males. (b): Abx-treated Ztm females show a significantly decreased CLDN-4 mRNA expression when compared with Abx-treated WT females.

Table 3. List of genes analyzed by real time PCR in the brain of Abx-treated Ztm mice ($n = 8$ per group), normalized to Abx-treated WT mice. Expressed as Fold Change (FC), statistics calculated with the non-parametric Mann-Whitney test (p).

Brain	Males		Females	
	FC	p	FC	p
CD11b	0.25	0.000	0.27	0.000
CLND 1	2.14	0.038	2.60	0.002
CLND 3	9.30	0.000	6.14	0.001
CLDN 5	4.68	0.000	2.46	0.001
GABA	0.23	0.000	0.19	0.000
IL1 β	1.45	0.130	1.15	0.195
OCCLN	1.53	0.105	1.57	0.022
veCad	0.28	0.000	0.24	0.000

Abx-treated WT male mice. No significant changes were observed for *IL-1 β* and *OCCLN* between Abx-treated Ztm and Abx-treated WT male mice (Figure 11A, B; Table 3). The exact same changes in gene expression were observed in Abx-treated Ztm

female mice compared to Abx-treated WT females but unlike males, *OCCLN* expression was significantly increased (Figure 11c,d Table 3). Notably, we detected reduced expression levels of zonulin in the prefrontal cortex of Abx-treated Ztm (from Experiment 2) compared to not treated mice (from Experiment 1) (Figure 10).

Gut microbiota depletion affects behavior and ameliorates some anxiety-like traits in the Ztm mouse

To assess whether the behavioral patterns we observed in the Ztm mouse were dependent on the composition of the gut microbiota, we administered antibiotics (Abx) to deplete intestinal bacteria within Ztm and WT mice and tested for repetitive and anxiety-like behavior. We did not observe any

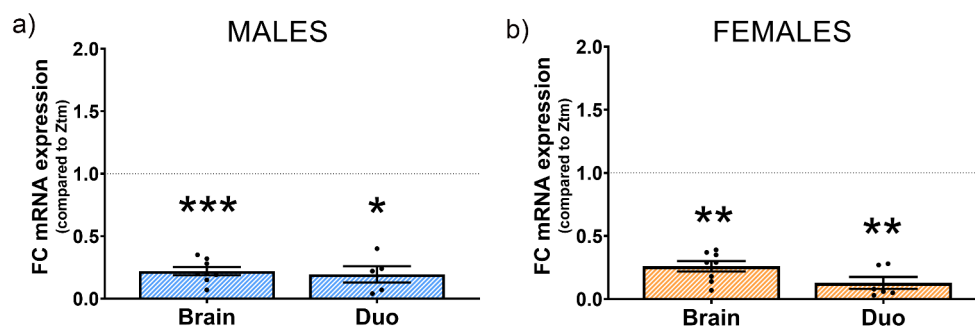


Figure 10. Effect of Abx gut microbiota depletion on Zonulin mRNA expression in the brain prefrontal cortex and the duodenum of Abx-treated Ztm mice. mRNA expression was analyzed by qPCR in Abx-treated Ztm mice, normalized to Ztm mice (non Abx-treated), and expressed as fold change \pm SEM. Microbiota depletion significantly decreased mRNA zonulin expression in the brain and duodenum of (a): males and (b): female Abx-treated Ztm mice when compared to Ztm.

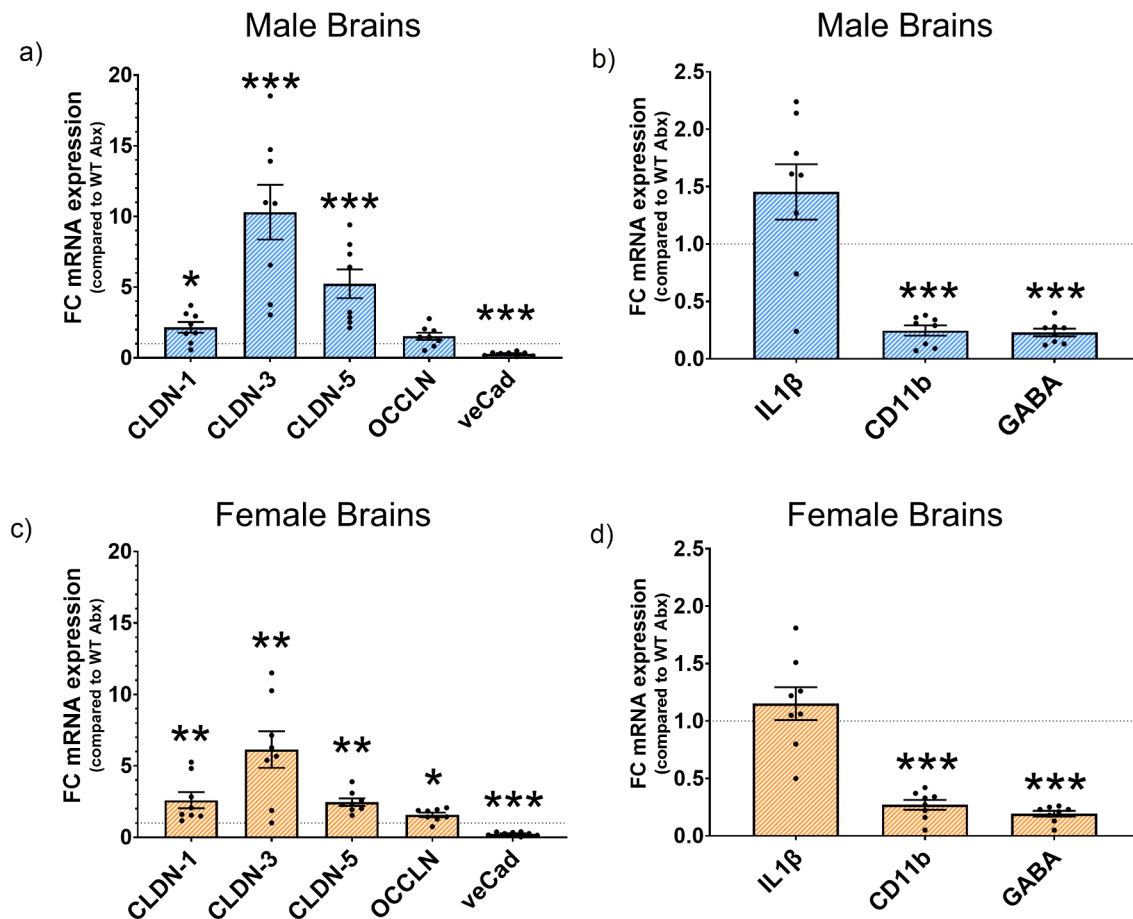


Figure 11. Effect of Abx gut microbiota depletion on the gene expression profile for BBB TJs and neuroinflammation markers on Abx-treated Ztm. mRNA expression was analyzed in Abx-treated Ztm (ZtmAbx) mice by qPCR, normalized to Abx-treated WT (WTAbx) mice, and expressed as fold change \pm SEM. (a): Abx-treated Ztm males show an increased mRNA expression of *CLDN-1*, *CLDN-3*, and *CLDN-5* in the BBB, whereas *veCad* is significantly decreased when compared to Abx-treated WT males. (b): Altered neuroinflammatory profile in the Abx-treated Ztm male brain prefrontal cortex, showing a significant decrease in *CD11b* and *GABA*. (c): mRNA expression in Abx-treated Ztm females shows a generalized increase, except for *veCad* that is significantly decreased when compared to Abx-treated WT females. (d): *CD11b* and *GABA* are decreased in Abx-treated Ztm females when normalized against Abx-treated WT females.

difference between WT and Ztm mice treated with Abx in the number of buried marbles (Figure 12A, B). Within the open field test, we did not observe significant differences between Abx-treated Ztm males and Abx-treated WT males in terms of time spent in the center of the field or total distance traveled (Figure 12c,e). Conversely, Abx-treated Ztm females showed a significant increase in time spent in the center of the open field compared to Abx-treated WT females (Figure 12d), with no difference in the distance traveled (Figure 12f). In the elevated zero maze, Abx-treated Ztm mice spent significantly less time in the open arms compared to Abx-treated WT males (Figure 12g) while no significant differences were observed in either Abx-treated Ztm female mice compared to Abx-treated WT females (Figure 12h).

Discussion

We have previously reported that the zonulin-expressing mouse (Ztm) has an intrinsic increased gut permeability in the small intestine at baseline that influences the development of an immune system and a gut microbiota composition predisposed to inflammation.^{109,110}

In the present study, we sought to understand whether a primary defect in small intestinal gut permeability caused by increased zonulin expression in the Ztm mouse synergizes with a dysbiotic, pro-inflammatory microbiota to affect brain function and behavior and whether these changes are sex dependent. We found that Ztm mice exhibited behavioral abnormalities and that these alterations were often different in males versus females.

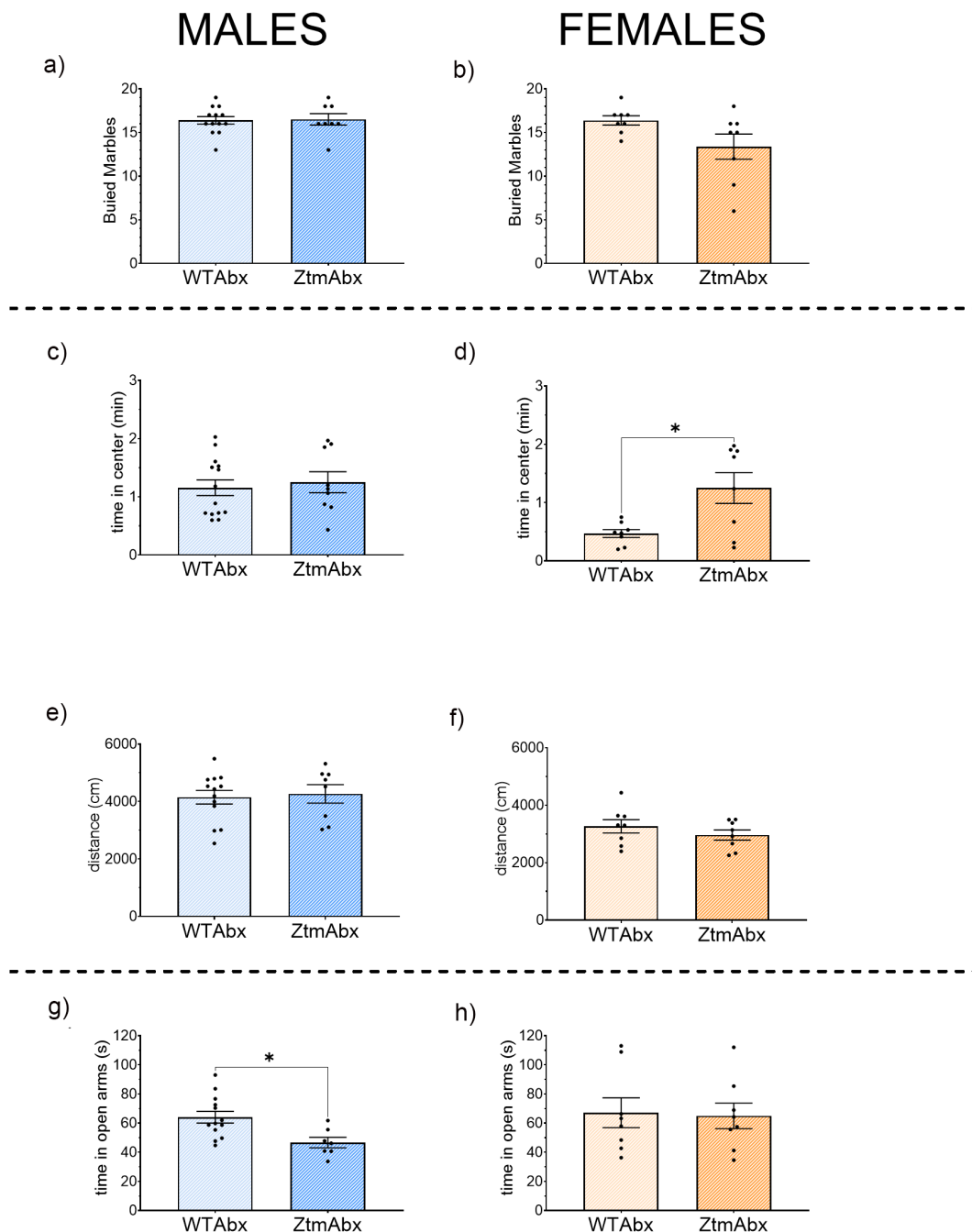


Figure 12. Effect of Abx gut microbiota depletion on the behavior profile in Abx-treated Ztm (ZtmAbx) mice compared to Abx-treated WT (WTAbx) mice. Marble Burying (a, b): No differences in the number of marbles buried were observed in Abx-treated males (a) and Abx treated females (b). Open field (C, D, E, F): No significant difference in the time spent in the center of the field was observed in Abx-treated Ztm males compared to Abx-treated WT mice (c). Abx treated Ztm females spend significantly more time in the center of the field than Abx-treated WT females (d). Distance traveled was similar for both males (e) and females (f). Track images are representative of each group. Elevated Zero Maze (g, h): Abx-treated Ztm male mice spend significantly less time in the open arm than Abx-treated WT mice (g). No differences were detected in the female group (h). N = 8–13.

Compared to WT mice, female Ztm mice buried more marbles, suggesting an increase in obsessive/compulsive/repetitive behavior, while male Ztm mice exhibited lower or higher anxiety dependent on the test performed. In the open field, male Ztm

mice showed lower anxiety by spending more time in the center of the field compared to WT males, yet they exhibited an increased level of anxiety when introduced to the elevated zero maze, suggesting a differential response associated with the

anxiogenic level of the stimulus. However, the increased time spent by Ztm males in the center of the open field can be the result of the slightly increased hyperactivity showed by these mice, that might be interpreted as an overall increased anxiety-like behavior.

These sex-dependent behavioral anomalies observed in Ztm mice were accompanied by differential expression of critical BBB TJ associated and neuroinflammatory genes. Ztm females showed decreased expression of essential BBB claudins such as *CLDN-5* and *CLDN-3*, pointing to defects in BBB integrity, yet they did not display increased levels of the inflammatory markers *IL-1 β* and *CD11b* or changes in *GABA* within the prefrontal cortex. In contrast, Ztm males showed increased gene expression of some barrier forming BBB components (*veCad* and *OCCLN*), as well as neuroinflammation-associated markers (*IL1b*, *CD11b* and *GABA*) in the prefrontal cortex. Increased gene expression of some of the TJs might suggest a more competent BBB or be explained as compensatory mechanism for post-translational destruction/degradation of the protein leading to a sustained gene expression and protein accumulation. A role of zonulin has been suggested in the regulation of vascular endothelial cells and/or the BBB and in neuroinflammation,^{101–103,127–131} so zonulin expression might be responsible for the altered BBB TJs gene expression profile in Ztm mice. Increased *CD11b*, the β -integrin marker of microglia and elevated *IL-1 β* in the prefrontal cortex of Ztm male mice suggest microglia activation. Interleukin-1 β is a major microglial pro-inflammatory cytokine that acts on endothelial cells and studies have shown that by modulating TJ or by driving leukocyte recruitment IL-1 β increases BBB permeability.^{132–134} *GABA* is the major inhibitory neurotransmitter of the central nervous system and is involved in behavioral disorders, such as anxiety and depression.^{135–138} Several studies indicate that increased production and/or synaptic availability of *GABA* can have anti-inflammatory effects.^{139–146} The elevated *GABA* expression in the Ztm male mice at baseline might act as a compensatory mechanism to neuroinflammation.

We also found that the gut microbiota in both male and female Ztm mice harbor an abundance of pro-inflammatory species and is skewed toward a more maladaptive and pathogenic profile.

Expansion of Rikenellaceae has been reported in obesity and diabetes and is associated with the pathological progression of inflammatory bowel disease.^{147–150} Moreover, low levels of *A. muciniphila* are positively correlated with several human diseases and this species has been shown to be a critical contributor to epithelial barrier integrity and strength.^{46,151–156} It is conceivable that the intrinsic defect in gut permeability in the Ztm mice allows the passage of pathobionts (commensal organisms with pathological potential) or their products into the bloodstream and across a compromised BBB, thereby contributing to the onset of a neuroinflammatory status that leads to behavioral abnormalities. Furthermore, because microbiota dysbiosis triggers zonulin secretion,^{39–41} the abundance of pathobionts in the gut of Ztm mice might further induce expression of zonulin in a vicious cycle.

To assess synergy between the intestinal microbiota and a zonulin-dependent increase in gut permeability in triggering behavioral alterations, we used antibiotics to drastically reduce intestinal bacteria within mice. We hypothesized that a reduction of microbial products within the intestinal lumen following antibiotic treatment could decrease their trafficking through a compromised intestinal barrier and eliminate and/or ameliorate the observed behavioral differences between WT and Ztm mice. In addition, the behavioral differences may likely be attributed to other pathways of communication between the gut microbiota and the brain and we cannot exclude cytokine regulation in the brain or other antibiotic treatment effects on neuroinflammation that are not related to gut barrier permeability.

When we assessed behavior in Abx-treated mice, we did not observe significant differences in the marble burying test between Abx-treated Ztm and Abx-treated WT females in contrast to baseline conditions where untreated Ztm females buried more marbles. These results suggest that Abx treatment of intestinal bacteria ameliorated obsessive/compulsive/repetitive behavior in Ztm females. Similarly, no differences in open field behavior were observed between Abx-treated Ztm and Abx-treated WT males in contrast to baseline behavior where Ztm males spent more time in the center of the open field than WT males, indicating that Abx

depletion of gut microbiota affected the level of anxiety in Ztm males. On the other hand, in the elevated zero maze, in a higher anxiety setting, Abx-treated Ztm males still spent significantly less time in the open arms than Abx-treated WT males, consistent with their behavior at baseline, suggesting that Abx treatment did not restore their anxiety-like behavior to WT levels for this test. Interestingly, whereas there was no difference between Ztm and WT female mice in open field behavior at baseline, Abx depletion of the gut microbiota resulted in Abx-treated Ztm females spending significantly more time in the center of the open field than Abx-treated WT females.

While some of these differences might be attributable directly to the reduced bacterial translocation from the gut into the bloodstream of Ztm mice, other differences may be due to the absence/reduction of bacteria and/or their metabolites affecting specific pathways of communication between the gut and the brain. For instance, microorganisms can communicate with the brain via neurotransmitters and neuromodulators like serotonin, dopamine, and bacterial metabolites like short-chain fatty acids (SCFAs).^{157–161} The gut microbiota can also induce the secretion of a variety of gut peptides, such as leptin and neuropeptide Y, by enteroendocrine cells located in the gut epithelium. Several of these peptides are known to affect anxiety levels and behavior via the host nervous system.^{162–164}

It has been reported that the absence of microbiota in the gut of germ-free mice reduces anxiety^{165–167} while other studies have reported increased anxiety in germ-free mice,^{168,169} together suggesting that the absence of gut bacteria affects brain function and behavior. Our data suggest that while zonulin and increased trafficking through an impaired gut barrier might be involved in some of the behavioral traits we observed in the Ztm mouse, such as obsessive/compulsive/repetitive behavior in females (marble burying) and some of the anxiety traits in males (open field), other behaviors might be influenced by alternative pathways of communication between the gut and the brain.

It is interesting to note that in both male and female Abx-treated Ztm mice there was an increased expression of key TJ genes of the BBB and decreased expression of neuroinflammation-

associated markers *CD11b* and *GABA*, as compared to Abx-treated WT, suggesting that the interaction of a dysbiotic gut microbiota with zonulin-dependent trafficking of bacterial products might influence BBB integrity and the inflammatory state of the brain. Of note, Abx microbiota depletion reduces the expression level of zonulin in the prefrontal cortex and the small intestine of Ztm mice, further stressing the effect of dysbiosis on gut and BBBs' integrity.

Taken together, these data demonstrated that microbiota depletion in the gut of Ztm mice ameliorated some behavioral traits that characterize Ztm mice, likely via a reduction in BBB impairment and neuroinflammation, likely secondary to reduced zonulin expression.

Abx treatment drastically reduced Bacteroidota in both WT and Ztm mice against an expansion of Firmicutes that we observed in all Abx-treated groups except for AbxWT males that showed a growth of Alphaproteobacteria and Cyanobacteria, instead. While *Ureaplasma* spp. are commensals isolated from the human male and female urogenital tracts¹⁷⁰ and have been associated with urethritis, cervicitis or bacterial vaginosis, fetal chorioamnionitis and adverse pregnancy outcome,^{171–174} not much is known about the Erwiniaceae family and we cannot associate either of these species with a pathogenic role in mice.

Furthermore, Abx treatment had a similar effect on the gene expression profile of TJs in the small intestine of males and females Abx-treated Ztm and Abx-treated WT mice. Differently from our published data showing reduced gene expression of several TJs in the small intestine of Ztm male mice, in Abx-treated Ztm mice we did not observe differences in the duodenum compared to Abx-treated WT animals, suggesting that Abx treatment depleted the Ztm gut of pathobionts responsible of defects in small intestinal gut permeability. This is further corroborated by the reduced expression of zonulin in the duodenum of Abx treated Ztm mice compared to Ztm animals.

The interaction of gut bacteria with the brain is bidirectional and involves multiple routes of communications. Gut bacteria can influence brain function via several pathways including the enteric nervous system (ENS), the vagus nerve, the immune system and cytokines release,

and the metabolic products of gut bacteria (SCFAs).^{157,162,164,175–182} Our data strongly suggest that in the Ztm animal model, increased trafficking of bacteria through an impaired gut barrier affect BBB/brain function and behavior and that by decreasing the antigen trafficking in the small intestine (or by shifting the microbiota composition), the differences between the WT and Ztm phenotypes are reduced.

It is clear from this study that the gut-microbiota-brain crosstalk happens via several pathways, and that the microbiota dysbiosis-zonulin pathway might contribute to neuroinflammatory disorders. Overall, we have shown that in the Ztm mouse, a zonulin dependent increased gut permeability synergizes with a pro-inflammatory dysbiotic gut microbiota contributing to brain function and behavioral alterations. More studies are needed to dissect the specific role of zonulin and of distinct microbial species/products in the gut-brain crosstalk in Ztm mice.

Disclosure statement

AF is a stockholder at Alba Therapeutics, serves as a consultant for Inova Diagnostics and Innovate Biopharmaceuticals, is an advisory board member for Axial Biotherapeutics and Ubiome, and has a speaker agreement with Mead Johnson Nutrition. The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Ethics Statement

This study was carried out in accordance with the recommendations and guidelines of the Institutional Animal Care and Use Committee at the MGH. The protocol was approved by the Institutional Animal Care and Use Committee at the MGH (2013N000013).

Author Contributions

MRF and AM-R conceived and designed the experiments. AM-R, GS, and JL carried out the experiments. MRF, AM-R, MAK, and AF contributed to the interpretation of the results.

AM-R, MRF, MAK, and AF wrote the manuscript. All authors provided critical feedback and helped shape the research, analysis, and manuscript.

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