Intestinal Dysbiosis Alters Seizure Burden and Antiseizure Medicine Activity Profile in the Theiler's Virus Model of Acute Encephalitis

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Abstract

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Background: Epilepsy is a neurological disorder characterized by recurring, unprovoked seizures and chronic cognitive comorbidities. Epilepsy can arise due to numerous factors, including genetics, brain injury, and central nervous system infection. Brain infection with Theiler's murine encephalomyelitis virus (TMEV) in C57BL/6J mice is thus a relevant animal model of infection-induced acute seizures and epileptogenesis. Acute seizures in this model are driven by brain infiltration of peripheral immune system macrophages, which secrete pro-inflammatory cytokines that further accelerate brain inflammation[1]. Diet formulation can dramatically modify the presentation of acute seizures in TMEV-infected mice and influence the diversity and composition of the intestinal microbiome (Zierath et al, Epilepsia 2024)[2] that critically shapes immune system reactivity. However, whether antibiotic-induced gut microbiome dysbiosis influences the phenotype of symptomatic seizures after TMEV infection is unclear. Further, no study has assessed whether antibiotic-induced intestinal dysbiosis influences antiseizure medicine activity (ASM) in the TMEV model. We thus sought to define the extent to

which antibiotic administration influenced acute seizure presentation, the anticonvulsant activity of the prototype ASM, carbamazepine (CBZ), and pharmacokinetics of this ASM in this mouse seizure model. We hypothesized that there would be an effect of intestinal dysbiosis on seizure burden and ASM efficacy in the TMEV model and that this change would be independent of pharmacokinetic differences.

Methods: Male C57BL/6J mice (4-5 weeks-old) received a broad-spectrum antibiotic cocktail (ABX) containing ampicillin, metronidazole, neomycin sulfate, and vancomycin (n=55) or vehicle (n=60) by oral gavage (p.o.) once daily beginning at arrival (Day -2) to Day 7 post-TMEV infection (p.i.) to induce intestinal dysbiosis. Mice were then infected with either intracerebral TMEV or PBS on Day 0. Mice received carbamazepine (CBZ; 20 mg/kg) or vehicle (VEH, 0.5% MC) intraperitoneally (i.p.) twice daily Days 3-7 p.i. and were assessed for handling-induced seizures 30 min after CBZ treatment. Plasma was collected on Day 7 p.i. at 15 and 60 min post-CBZ treatment to quantify the extent to which ABX-induced gut dysbiosis influences ASM pharmacokinetics.

Results: TMEV infection induced acute symptomatic seizures, regardless of pretreatment and CBZ history. There were 18/25 (72%) ABX-CBZ mice, 7/20 (35%) ABX-VEH mice, 7/20 (35%) SAL-CBZ mice, and 15/20 (75%) SAL-VEH mice that presented with seizures during the 7-day monitoring period. Average seizure burden was: 12.5 in ABX-CBZ, 4.7 in ABX-VEH, 5.7 in SAL-CBZ, and 16.1 in SAL-VEH mice. There was a significant pretreatment x ASM interaction ($F(1, 81) = 16.0$, $p=0.0001$), with post-hoc tests revealing marked differences in seizure burden in SAL- versus ABX-pretreated mice ($p=0.004$). Further, CBZ administration

significantly increased the latency to Stage 5 seizure during days 3-7 p.i.; an effect that was not present in ABX-treated mice similarly administered CBZ. In TMEV-infected mice, spleens were 0.32% of body weight in ABX-CBZ mice, 0.34% in ABX-VEH mice, 0.36% in SAL-CBZ mice, and 0.38% in SAL-VEH mice. In sham-infected mice, spleens were 0.45% of body weight in ABX-CBZ mice, 0.65% in SAL-CBZ mice, and 0.36% in SAL-VEH mice. Plasma CBZ concentrations did not differ between SAL and ABX pretreatment groups ($F(3, 37) = 0.3468$), suggesting that ABX history did not influence CBZ pharmacokinetics.

Conclusion: Antibiotic-induced gut dysbiosis markedly altered the presentation of symptomatic seizures and acute disease burden in the TMEV mouse model, reflecting a novel therapeutic target for seizure control: the gut microbiome. ABX-induced gut dysbiosis also significantly changed acute seizure control by CBZ, but did not significantly influence plasma concentrations of CBZ. The gut-brain axis is thus a relevant contributor to the clinical course of TMEV infection. This study altogether demonstrates that the gut-brain axis is an understudied therapeutic target in epilepsy that may benefit from greater investigation.

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Introduction:

Epilepsy is a neurological disorder characterized by recurrent, unprovoked seizures and cognitive comorbidities. Epilepsy affects more than 65 million people worldwide. It is especially common in pediatric and older adult populations[3], and is more prevalent in low- and middle-income countries[3]. There are many causes of epilepsy, including trauma, stroke, neoplasm, and infection[4]. Genetic causes are increasingly identified as contributing to a large portion of previously idiopathic epilepsies[5]. Regardless of etiology, the most common treatment options for epilepsy are antiseizure medications (ASMs). Over 20 effective ASMs are currently on the market. However, these drugs come with significant adverse effects such as cognitive impairment, teratogenicity, and hepatotoxicity. Further, approximately 30% of people with epilepsy do not achieve adequate seizure control with currently available ASMs[6], such that novel therapeutic targets and management strategies are necessary. Other treatment options include resective surgery, neurostimulation, and dietary modifications, although these strategies are only an option for some patients with specific disease parameters.

Epilepsy is a long-term complication of viral infection-induced encephalitis. Patients with viral encephalitis and seizures are at higher risk of developing epilepsy post-infection (up to 22 times)[7]. Viral infection-induced epilepsy accounts for a significant portion of the total worldwide epilepsy burden, especially in low- and middle-income countries such as Ecuador (14%) and Mali (47%)[8]. Infection-induced epilepsy is more likely to be pharmacoresistant. Therefore, preclinical studies of viral infection-induced epilepsy are useful to comprehensively address the clinical spectrum of acquired epilepsy.

There are few animal models of infection-induced acute seizures and epilepsy. However, one particularly useful model is evoked by brain infection with the Theiler's murine encephalomyelitis (TMEV). TMEV is a non-enveloped, positive-stranded RNA virus in the *Picornaviridiae* family[9,10,11]. When C57BL6/J mice are infected with TMEV delivered into the brain, they develop acute, symptomatic seizures around day 3 p.i., then clear the virus[12]. When monitored several months p.i., anywhere from 50-65% of mice that initially presented with acute symptomatic seizures will develop spontaneous recurrent epileptic seizures, i.e. epilepsy[13]. Further, TMEV-infected mice that initially presented with acute symptomatic seizures during the infection period demonstrate reduced seizure threshold long-term and exhibit cognitive and behavioral deficits, consistent with clinical comorbidities of epilepsy[12,14,15]. Finally, mice that develop acute seizures and epilepsy also present with extensive hippocampal damage, further demonstrating that the TMEV mouse model provides an important preclinical platform to study ictogenic mechanisms.

The gut microbiome has been increasingly recognized to be an important modifier of epilepsy pathology and treatment response. The gut microbiome plays a role in carbohydrate and amino acid metabolism, microglial and astrocytic function, and hippocampal neurotransmitter levels[16], all of which directly contribute to the progression and severity of epilepsy. Patients with drug-resistant epilepsy have an altered gut microbiome compared to patients with drug-sensitive epilepsy and healthy controls[17]. Microbiome changes have also been shown to influence animal models of epilepsy. Medel-Matus et. al used chronic stress as a microbiome modulator in an amygdala-kindled rat model of epilepsy[18], showing that the stress-induced gut dysbiosis led to more rapid epileptogenesis and increased severity of seizures. In humans, the ketogenic diet has

been used for over a century to treat a variety of epilepsy syndromes. It can reduce the seizure burden and improve cognitive and motor functions in children with drug-resistant epilepsy[19]. Further, the ketogenic diet alters the gut microbiome, and these alterations may be responsible for the anti-seizure effects of the diet[20]. Thus, environmental factors that modify the composition and diversity of the gut microbiome may directly modulate seizure susceptibility and burden.

Despite the high translational validity and etiological relevance to worldwide epilepsy prevalence, seizure burden and antiseizure medicine (ASM) efficacy is variable in the TMEV model. The diet that the mice are fed can modify the presentation of acute seizures in TMEV-infected mice[21]. Indeed, modulation of the formulation and sterilization of diet during acute TMEV infection is associated with marked differences in disease phenotype, and these dietary changes are associated with marked variability in the diversity and composition of the gut microbiome[2]. While less rigorously scrutinized, there is also evidence of variability in the efficacy of ASMs against TMEV induced seizures. Metcalf et al. demonstrated that levetiracetam (LEV) has an anti-convulsant effect[22], with 50% of TMEV-infected mice protected from handling-induced seizures. In contrast, Barker-Haliski et al. showed that a similar course of LEV administration was pro-convulsant in the TMEV model[23], with LEV increasing both cumulative seizure burden and total seizure burden. Therefore, we *hypothesized* that these differences in acute seizure presentation and ASM efficacy in the TMEV model are due to alterations in the gut microbiome.

To fill this knowledge gap, we thus sought to define the extent to which antibiotic (ABX) administration-induced depletion of the gut microbiome can influence acute seizure presentation,

seizure-induced neuropathology and clinical disease progression, the anticonvulsant activity of a prototype ASM, carbamazepine (CBZ), and the pharmacokinetic profile of this ASM in the TMEV infection-induced acute seizure model in mice. This study provides essential insight to definitively demonstrate that acute disease progression and ASM activity can be meaningfully influenced by the gut microbiome.

Methods:

Animal Handling and Diet Assignment. Male, wild type C57BL/6J, mice (n=115; 4–5 weeks old; Jackson Labs, Bar Harbor, ME) were group-housed throughout the infection and monitoring period (n=5/cage). Animals were given free access to autoclaved diet (ProLab RMH 3000) and filtered water except during periods of behavioral manipulation, as previously described[15]. Animals were maintained in standard housing chambers with corncob bedding in a temperature-controlled SPF vivarium on a 14:10 light/dark cycle (lights on: 6h00, lights off: 20h00) as previously detailed[24]. All studies were conducted between the hours of 9h00 and 17h00 during the animals' light phase. This study was not designed to assess the impact of sex as a biological variable or the impact of sex hormones on seizure severity, thus only male mice were used. All animal use was approved by the University of Washington Institutional Animal Care and Use Committee (protocol 4387-02), conformed to the ARRIVE Guidelines, and was conducted in accordance with the United States Public Health Service's Policy on Humane Care and Use of Laboratory Animals.

Chemical and Reagents. *In vivo drug administration studies.* Methylcellulose (VEH; Sigma Aldrich catalog #M0430), carbamazepine (CBZ; Sigma Aldrich catalog #C4024). CBZ

administered to mice was suspended in VEH. Ampicillin (Eugia US LLC, NDC 55150-114-20), Metronidazole (Azurity Pharmaceuticals, NDC 65628-0202), Neomycin Sulfate (Aspen Veterinary Resources Ltd., NDC 46066-211-07), and Vancomycin (Azurity Pharmaceuticals, NDC 65628-208-10) were dissolved in saline. *LC-MS/MS studies.* 7-chloro-1,3-dihydro-1-methyl-5-(phenyl-2,3,4,5,6-d_5)-2H-1,4-benzodiazepin-2-one (diazepam-d5) was purchased from Cayman Chemical (Ann Arbor, MI). MS-grade acetonitrile (ACN) and methanol (MeOH) were purchased from Fisher Scientific (Pittsburgh, PA). Formic Acid (FA) 88% ACS grade was purchased from Sigma Aldrich (St. Louis, MO). All stock drug solutions, buffers, and HPLC mobile phase were prepared using Milli-Q grade water (Millipore, Bedford, MA). All other consumables were purchased from Fisher Scientific (Pittsburgh, PA). *Histopathology studies.* Fluoro-Jade C (FJ-C; Histochem catalog #2FJC), Shandon FormalFixx, (Fisher catalog #9990234), Potassium Permanganate ($KMnO_4$; Sigma Aldrich catalog #223468), Acetic Acid (Sigma Aldrich catalog #695092), Hoechst 33342 nuclei acid stain (Invitrogen catalog #62249), phosphate buffered saline (PBS; VWR catalog #97062-948), DPX mounting media (Sigma Aldrich catalog #06522).

TMEV Infection. Mice were free-hand infected intracerebrally (i.c.) with either 20 µL Daniel's (DA) TMEV strain, titer concentration of 3.0×10^5 plaque-forming units (PFU) or sterile PBS under isoflurane anesthesia (Figure 1), as previously described[24]. TMEV titers were isolated from stock originally provided by Robert Fujinami at the University of Utah. All injection procedures were performed under sterile conditions. Following TMEV injection, the animals were monitored until they had recovered from anesthesia and were ambulatory.

Antibiotic Administration Prior to and During TMEV Infection: The oral ABX cocktail consisted of ampicillin (1 g/L), metronidazole (1 g/L), neomycin sulfate (1 g/L), and vancomycin (0.5 g/L) dissolved in saline[25]. Mice were pre-treated with ABX or saline (SAL) for 10 days beginning upon arrival at UW until conclusion of in-life testing 7 days p.i. (Figure 1). Mice were given 200 uL ABX or saline once daily, by gavage (Figure 1).

Carbamazepine Treatment During TMEV or SHAM Infection: Mice were administered CBZ or VEH (0.5% methylcellulose) twice per day on days 3-7 p.i. (Figure 1). ASM or VEH treatments were administered by the intraperitoneal (i.p.) route 30 minutes prior to each twice-daily behavioral seizure assessment (minimum 4 hours between sessions) during the acute TMEV infection period.

Assessment of Handling-Induced Seizures During TMEV Infection. TMEV and sham-infected mice were evaluated twice/day (minimum 4 hours between sessions), on days 3–7 p.i. for assessment of handling-evoked acute behavioral seizure severity (Figure 1). Seizure assessment involved brief cage agitation (<30 seconds), followed by individual handling of mice to visually assess righting reflex and gait. Typically, evoked secondarily generalized seizures occur in susceptible animals within this 5-10 minute observation period. The presence and severity of handling-induced seizures was scored according to the Racine scale (1- mouth and facial clonus; 2 – head bobbing; 3 – single forelimb clonus; 4 – bilateral forelimb clonus plus rearing; 5 – stage 4 plus repeated rearing and falling), as previously reported[26] (Figure 1).

Complete Blood Counts at 7 Days Post-TMV Infection: All TMEV- or sham-infected mice were euthanized via CO_2 asphyxiation using a gradual displacement method (10%–30% volume per minute) according to the 2013 AVMA Guidelines for the Euthanasia of Animals (https://www.avma.org/KB/Policies/Documents/euthanasia.pdf). Blood was collected via cardiocentesis from a subset of mice immediately following euthanasia and placed directly into blood tubes containing K_2EDTA (BD Microtainer Tubes with K_2EDTA, catalog 365974; BD Biosciences, San Jose, CA) and serum separator tubes (BD Microtainer Serum Separator Tubes, catalog 365978; BD Biosciences), as previously described (Meeker et al, JPET 2019)[27]. Serum was separated by centrifugation (9000 relative centrifugal force x 5 minutes) within 2 hours of collection, and serum and whole blood from a subset of animals were submitted to a veterinary diagnostic laboratory for complete blood count and clinical serum biochemistry profiling (Moichor, San Francisco, CA).

Fecal Sampling Prior to and During TMEV Infection. Fecal samples were collected from a subset of animals in the study (n=5/experimental group). Time points of collection were: baseline/prior to housing within the University of Washington (UW) SPF vivarium; TMEV or sham infection day (0 p.i.), and conclusion of acute infection period (7 p.i.; Figure 1). Fecal sampling occurred by placing each mouse into an individual sterilized container, and spontaneous fecal samples were collected with a sterile needle. Fecal pellets were transferred into individual, sterile tubes, flash frozen, and stored at -80°C until processing. Baseline fecal sampling occurred prior to dietary or infection group randomization and thus reflected the baseline intestinal microbiome diversity upon arrival from Jackson Labs and prior to housing in

the UW SPF vivarium. A total of 3 fecal pellets/mouse were obtained during the acute study period for bacterial 16S sequencing (Figure 1).

Fecal Sample 16S RNA Sequencing. Gut microbiome biodiversity was assessed by 16S rRNA amplicon sequencing of fecal samples. DNA was isolated from flash-frozen fecal pellets using a manual nucleic acid precipitation method at the University of Missouri Metagenomics Core Center (MUMC) as previously described[28]. Briefly, samples were placed in a 2 mL round-bottom tube containing 800 µL of lysis buffer and a sterile 0.5 cm diameter stainless steel bead and homogenized with a TissueLyser II.

Bacterial 16S rRNA amplicons were generated at the MUMC via amplification of the V4 hypervariable region of the 16S rRNA gene using single-indexed universal primers (U515F/806R) flanked by Illumina standard adapter sequences and the following parameters: 98°C(3:00) + [98°C(0:15)+50°C(0:30)+72°C(0:30)] × 25 cycles +72°C(7:00). Amplicons were then pooled for sequencing using the Illumina MiSeq platform and V2 chemistry with 2×250 bp paired-end reads, as previously described[28]. Samples returning greater than 10,000 reads were deemed to have successful amplification, consistent with established methods of the MUMC.

FluoroJade-C detection of cell death: Following in-life testing on 7 p.i., animals were euthanized by CO_2 asphyxiation and brains of all mice were removed, flash frozen, and maintained at −80°C until histopathological processing. Two consecutive 20 µm-thick sections/mouse from the dorsal hippocampus (anteroposterior [AP] from Bregma: −1.34 mm) and ventral hippocampus (AP from Bregma: −2.24 mm) were sectioned on a cryostat (Leica DM1860) and slide-mounted. Slides were fixed in 4% Formal Fixx (10 min) and rinsed in DI

water followed by FJ-C and Hoechst staining. Slides were imaged on an upright fluorescent microscope (Leica DM-4) with a 10x objective (40× final magnification) with constant acquisition settings, as previously conducted (Knox et al, Epilepsia 2021)[29]. Photomicrographs (n = 4 brain sections/mouse) were analyzed by an investigator who was blinded to treatment and visually scored for the presence of FJ-C positive cells in limbic structures (CA1, CA3, and dentate gyrus [DG] of dorsal hippocampus) and hypothalamic nucleus. If two or more of the consecutive sections from each mouse demonstrated FJ-C- positive staining in the designated brain region, the animal was considered to have FJ-C positive labeling.

Liquid Chromatography – Mass Spectrometry (LCMS) Quantitation of CBZ: Plasma was deproteinated prior to LCMS analysis. Subject plasma, calibration, or quality control samples (10 µL) were pipetted into a 1.2 mL Eppendorf microcentrifuge tube. Internal standard (10 µL) consisting of diazepam-d5 (1 mg/ mL in MeOH) was added to each microcentrifuge tube and vortexed for 5 seconds. ACN (100 µL) was then added to each microcentrifuge tube and samples were vortexed for 15 seconds and then centrifuged at room temperature for 5 min at 16 RCF. Supernatant (50 µL) was removed and pipetted into 1 mL autosampler vials (Thermo Sciences, catalog #6PRV11-TR1 and 6PRC11STS1X). MilliQ water (450 mL) was added to each vial. Sample (10 µL) was injected onto the LC–MS platform. Chromatographic separation was achieved using a Water's Acquity UPLC BEH C18 2.1x100 mm, 1.7 um (Waters Corporation, Milford, MA, USA) using a gradient consisting of 0.1% formic acid in H_2O (A) and 0.1% formic acid (FA) in acetonitrile (ACN) (B) at a flow rate 0.3 mL/min (See Table 2). A Water's Xevo-XS coupled to a Water's I-Class Ultra high-pressure liquid chromatography system was used (Waters Corporation, Milford, MA, USA). Analytes were monitored in electrospray positive

ionization mode (ESI+), using MRM mode. Instrument settings were as follows: Capillary (kV)—1.08, Source Temperature 150 °C, Desolvation Temperature 350 °C, Cone Gas Flow 150 L/hr, Desolvation Gas Flow 1000 L/hr, and Collision Gas Flow 0.16 mL/min. Two fragments for each analyte were used; one used for quantifying and the other as a qualifier. The m/z 237.1 > 194.0 and 178.9 transitions were used for carbamazepine. The m/z of 290.2 > 198.1 and 154.0 transitions were used for diazepam-d5 (see Table 3). Data was processed using MassLynx (Milford, MA) and linear equations were formulated by using peak area ratios (PAR's) allowing for 15% variability across calibrators and quality controls for acceptability. Calibration Curve and QC samples were prepared by making dilutions of stock solutions containing carbamazepine from 10 mg/mL stocks in MeOH and stored at −20 °C. Calibration curves were created by analyzing drug-free plasma samples fortified with carbamazepine at 0.312, 0.625, 1.25, 2.5, 5, 10, and 20 μg/mL. Quality control (QC) samples in plasma were prepared at 1 and 5 μg/mL from separate dilutions of stocks than those used for the calibration curves.

Statistical Analysis: For in-life studies, average seizure burden and cumulative seizure burden were analyzed by repeat-measures two-way analysis of variance (ANOVA). Latency to first Racine stage 5 seizure was determined with a Kaplan-Meier plot. Spleen weights and complete blood counts were analyzed by two-way ANOVA. Number of animals with and without FJ-C positive labeling by histology within each brain region was analyzed by X^2 test. Changes in microbiome commensal species were analyzed by two-way ANOVA. Changes in plasma CBZ concentrations were analyzed by two-way ANOVA. All statistical analysis was performed in GraphPad Prism version 10, with $p < 0.05$ considered significant.

Results:

Intestinal dysbiosis disrupts TMEV-induced seizure presentation. Intestinal dysbiosis in the TMEV model was experimentally induced because this mouse seizure model has been shown to be altered by dietary modification, potentially due to changes in the gut microbiome (Ref Zierath et al 2024). Sham-infected mice maintained or gained weight, whereas TMEV infection was associated with acute body weight loss (Figure 2A). Sham-infected mice did not have any seizures. TMEV infection induced acute symptomatic seizures, regardless of pretreatment and ASM history. In TMEV-infected mice pretreated with saline, CBZ treatment reduced the cumulative seizure burden compared to vehicle treatment. The average seizure burden of SAL-VEH mice was 16.1, while the average seizure burden of SAL-CBZ mice was 5.7, demonstrating that CBZ has an anticonvulsant effect.

In TMEV-infected mice with ABX-induced intestinal dysbiosis, VEH treated mice had a lower cumulative seizure burden relative to SAL-VEH mice. ABX-mediated intestinal dysbiosis invoked a lower seizure burden. CBZ treated mice with intestinal dysbiosis had a significantly higher cumulative seizure burden. Additionally, the average seizure burden of ABX-VEH mice was 4.7, while the average seizure burden of ABX-CBZ mice was 12.5, demonstrating that CBZ may be proconvulsant in the setting of experimentally induced intestinal dysbiosis (Figure 2B). In mice with an intact gut microbiome, VEH-treated mice had a higher number of seizures compared with CBZ-treated mice. In mice with intestinal dysbiosis, VEH-treated mice had a lower number of seizures compared with CBZ-treated mice (Figure 2C); 15/20 (75%) of SAL-VEH mice, 7/20 (35%) of SAL-CBZ mice, 7/20 (35%) of ABX-VEH mice, and 18/25 (72%) of ABX-CBZ mice presented with seizures during the 7-day monitoring period.

In mice with an intact microbiome, CBZ reduced the number of mice that developed a stage 5 seizure. In mice with intestinal dysbiosis, CBZ increased the number of mice that developed a stage 5 seizure. Further, the latency to Stage 5 seizure was substantially increased by repeated CBZ administration during days 3-7 p.i.; an effect absent in ABX-treated mice similarly administered CBZ (Figure 2D). There was a significant pretreatment x ASM interaction ($F(1, 81) = 16.0$, $p=0.0001$), with post-hoc tests revealing marked differences in seizure burden in SAL- versus ABX-pretreated mice ($p=0.004$). Overall, intestinal dysbiosis disrupted TMEV infection-induced acute seizure presentation and acute seizure control by CBZ.

Markers of overall health were not impacted by antibiotic administration. Markers of overall health were studied to rule out potential peripheral pathology. CBC analytes were similar across saline and ABX pretreatment groups (Figure 3) within both sham- and TMEV-infected groups, suggesting that a 10-day course of oral ABX administration did not adversely affect blood cell counts. However, CBZ administration was associated with a significant increase in % neutrophils, regardless of TMEV-infection history (Figure 3E).

TMEV infection was associated with reduced spleen weight, which was further impacted by repeated CBZ administration (Figure 4). In TMEV-infected mice, spleens were 0.32% of body weight in ABX-CBZ mice, 0.34% in ABX-VEH mice, 0.36% in SAL-CBZ mice, and 0.38% in SAL-VEH mice. In sham-infected mice, spleens were 0.45% of body weight in ABX-CBZ mice, 0.65% in SAL-CBZ mice, and 0.36% in SAL-VEH mice. These markers of overall health indicate that the mice were otherwise clinically normal, despite the brain TMEV infection.

A 10-day course of oral antibiotics significantly alters the makeup of the gut microbiome. The composition and diversity of the gut microbiome was studied at several points in the TMEV infection period to ensure that the ABX treatment induced gut dysbiosis. There were significant differences in the microbial composition between SAL pre-treated and ABX pre-treated animals across a diversity of Gram positive and Gram negative bacterial species, as well as differences in ABX pre-treated animals from Day -2 to Day 7 (Figure 5). This demonstrates that the ABX cocktail successfully induced intestinal dysbiosis.

Acute TMEV infection leads to hippocampal cell death. Hippocampal histopathology was performed to assess the degree of TMEV infection-induced neurodegeneration using FJ-C staining (Figure 6 and Table 1). Sham-infected mice did not experience hippocampal cell death. Conversely, mice infected with TMEV experienced hippocampal cell death in the CA1, CA3, and cortical regions. No mice experienced cell death in the dentate gyrus region of dorsal hippocampus (Figure 6 and Table 1). Altogether, these findings demonstrate that TMEV infection leads to hippocampal cell death that is reduced by CBZ use exclusively in mice with an intact intestinal microbiome (Table 1).

Antibiotic-induced intestinal dysbiosis does not alter plasma carbamazepine concentrations. Time-related plasma CBZ concentration was assessed at 7 days p.i. to determine if there is an interaction between 10-day ABX treatment and repeated CBZ administration from 3-7 days p.i. with TMEV. There was an effect of time post CBZ administration ($F(1.257, 23.26) = 9.479$). There was no effect of pretreatment on CBZ concentration ($F(3, 37) = 0.3468$). Further, there was no effect of time x pretreatment ($F(6, 37) = 0.4487$). This demonstrates that a

10-day course of oral ABX administration did not affect the plasma concentration of CBZ in mice.

Discussion:

This study establishes the significant variability in the behavioral phenotype of the TMEV model of infection-induced acute seizures. ABX-induced gut dysbiosis leads to alterations in the severity and onset of seizures in the TMEV model itself, as well as the anticonvulsant efficacy of CBZ. A 10-day course of oral ABX administration commencing prior to TMEV infection alone decreased the cumulative seizure burden relative to SAL pretreatment. ABX combined with CBZ increased the cumulative seizure burden relative to SAL pretreatment. Interestingly, ABX pre-treatment alone appears to act as an anticonvulsant. This trend also occurred across average seizure burden and latency to Stage 5 seizure. There were no clinically significant differences in markers of overall health. Histopathology as assessed at 7 days p.i. in the brains of mice with and without TMEV-infection was consistent with previous TMEV studies. Despite the lower seizure burden of ABX-VEH mice, there was no protection against hippocampal cell death. Despite these differences in behavioral seizure presentation across treatment groups, there were no significant differences between plasma CBZ concentrations in SAL and ABX pretreated animals. These results indicate that intestinal dysbiosis is responsible for the changes in acute seizure burden and ASM efficacy.

While the antibiotic pretreatment regimen successfully induced gut dysbiosis (Figure 5), it did not decrease the presence of all organisms. There were increases in some Gram-positive and anaerobic bacteria[30] in the antibiotic-treated mice, which may be a result of the antibiotic choice

or the extreme treatment. There were significant changes in many bacterial species. However, few of these organisms have been studied in the context of epilepsy or other neurological diseases. Some organisms that were notable in this study were *Akkermansiaceae*, *Lachnospiraceae*, *Lactobacillaceae*, *Erysipelotrichaceae*. An increase in *Akkermansia* is associated with the ketogenic diet administration to mice, and treatment with *Akkermansia* is able to confer seizure protection to mice fed a control diet[20]. *Lachnospiraceae* has been shown to be involved in depression (lower levels in patients with major depressive disorder)[31] and multiple sclerosis, specifically through effects on the immune system[32]. *Lactobacillaceae* has been shown to induce region-dependent alterations in GABA and may be associated with tight junction integrity and modulation of afferent sensory nerves[33]. An increase in *Erysipelotrichaceae* is associated with the ketogenic diet[20], which has been proven to be a very useful treatment strategy for drug-resistant epilepsy. With many studies finding relationships between these organisms and neurological function, it is clear that intestinal dysbiosis could play a role in epilepsy.

We studied CBZ because it has a known and consistent clinically-meaningful anticonvulsant effect and has been used in this model previously[15]. CBZ alone acts as an anticonvulsant. CBZ in combination with ABX-induced gut dysbiosis acts as a proconvulsant. Of note, CBZ prevented hippocampal cell death in SAL pre-treated mice, consistent with the reduced seizure burden in this population. CBZ in combination with ABX had no effect on hippocampal cell death. Plasma concentrations of CBZ demonstrated no difference between SAL- and ABX- pretreated mice. The variability in the efficacy of CBZ in this study is like the variability seen with LEV efficacy in Metcalf et. al and Barker-Haliski et. al[2223]. It is possible that difference between the husbandry condition and diets, previously shown to impact the gut microbiome in the TMEV model [2], also

had an impact on the ASM efficacy. Due to a known drug interaction between metronidazole and CBZ[34], future studies should assess the interplay of CBZ and ABX treatments, especially with regard to CYP metabolic enzymes. It would also be beneficial to quantify the concentration of CBZ metabolites, as well as brain CBZ concentrations.

The TMEV model is the only model of infection-induced epilepsy, and as such is used in the National Institute of Neurological Disorders and Stroke's Epilepsy Therapy Screening Program[22]. However, gut dysbiosis markedly alters the presentation of symptomatic seizures and acute disease burden in the TMEV model (Figure 2). The variability in this model may affect the efficacy of novel anti-seizure medicines, and care needs to be taken to ensure that the results are interpreted in context. The changes in the gut microbiome have a clear effect on seizure burden in untreated and ASM-treated mice in this acute seizure model. In fact, this study further illustrates that the gut microbiome may be a novel therapeutic target for seizure control. The gut-brain axis is an understudied target in epilepsy that may benefit from greater investigation.

It is well known that the gut microbiome has an impact in epilepsy, but there are still gaps in understanding. There is potential for modulation of the gut microbiome as a treatment for epilepsy. Some epilepsy patients experience seizure freedom, or a significant decrease in seizure burden, during a short course of ABX treatment[35]. Additionally, there is some evidence that probiotic supplementation can reduce seizure burden[36]. However, there is limited clinical and preclinical research on the impact of the gut microbiome on epileptogenesis. Our future studies will thus define the extent to which depletion of the intestinal microbiome modifies the long-term susceptibility to chronic epilepsy and behavioral comorbidities.

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Figures and Tables.

Figure 1. Overview of experimental design and study timeline to test the *hypothesis* that differences in acute behavioral seizure presentation and anticonvulsant efficacy in the TMEV model are due to antibiotics (ABX)-induced alterations in the gut microbiome.

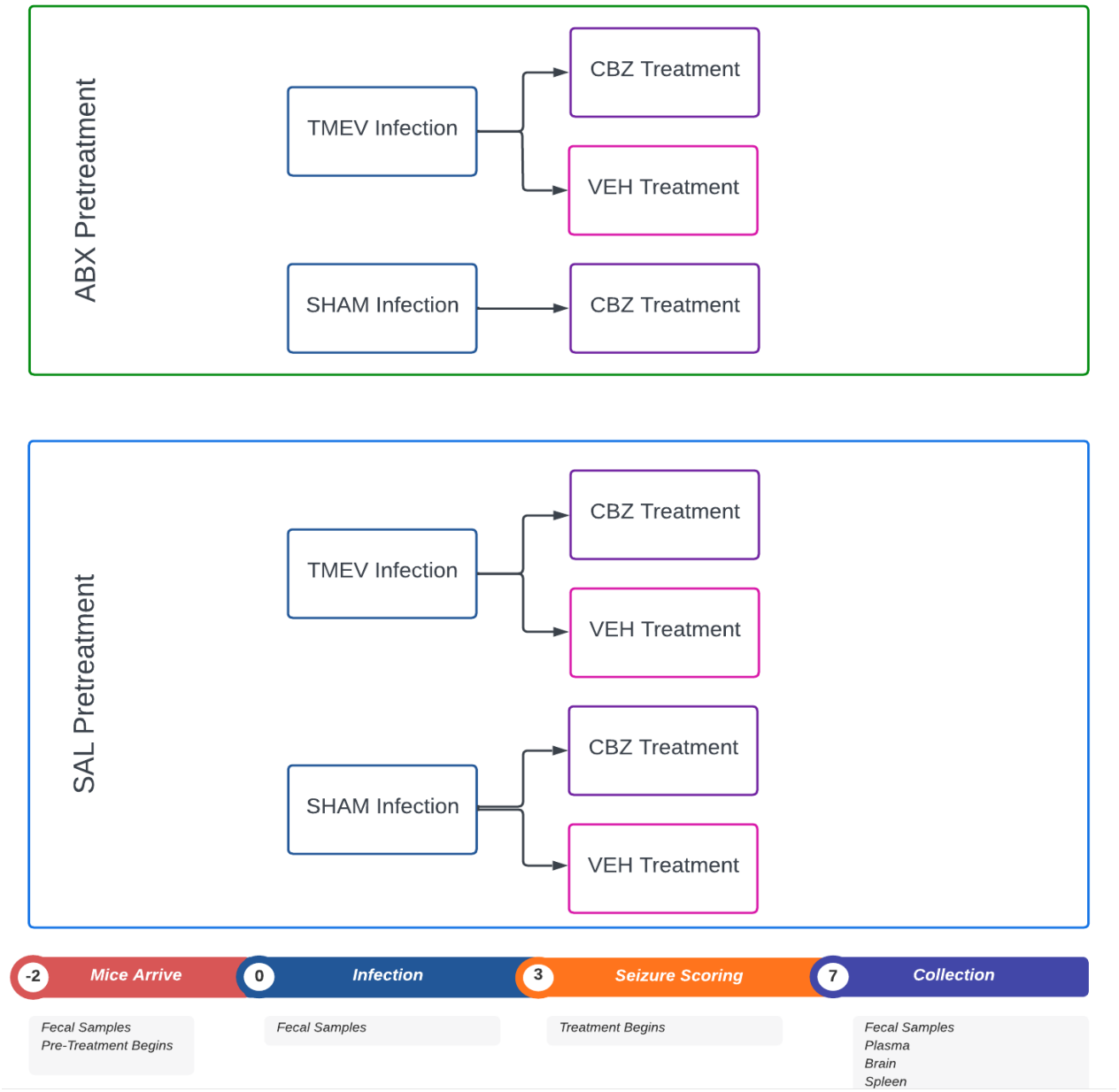

Figure 2. Intestinal dysbiosis disrupts TMEV-induced seizure presentation. a) TMEV infected mice lose weight during the acute infection period. b,c) In mice with an intact microbiome, carbamazepine (CBZ) significantly decreases seizure burden; in mice with antibiotic (ABX) induced gut dysbiosis, CBZ significantly increases seizure burden. d) In mice with an intact gut microbiome, CBZ increases the latency to a Stage 5 seizure; in mice with ABX-induced gut dysbiosis, CBZ decreases the latency to a Stage 5 seizure.

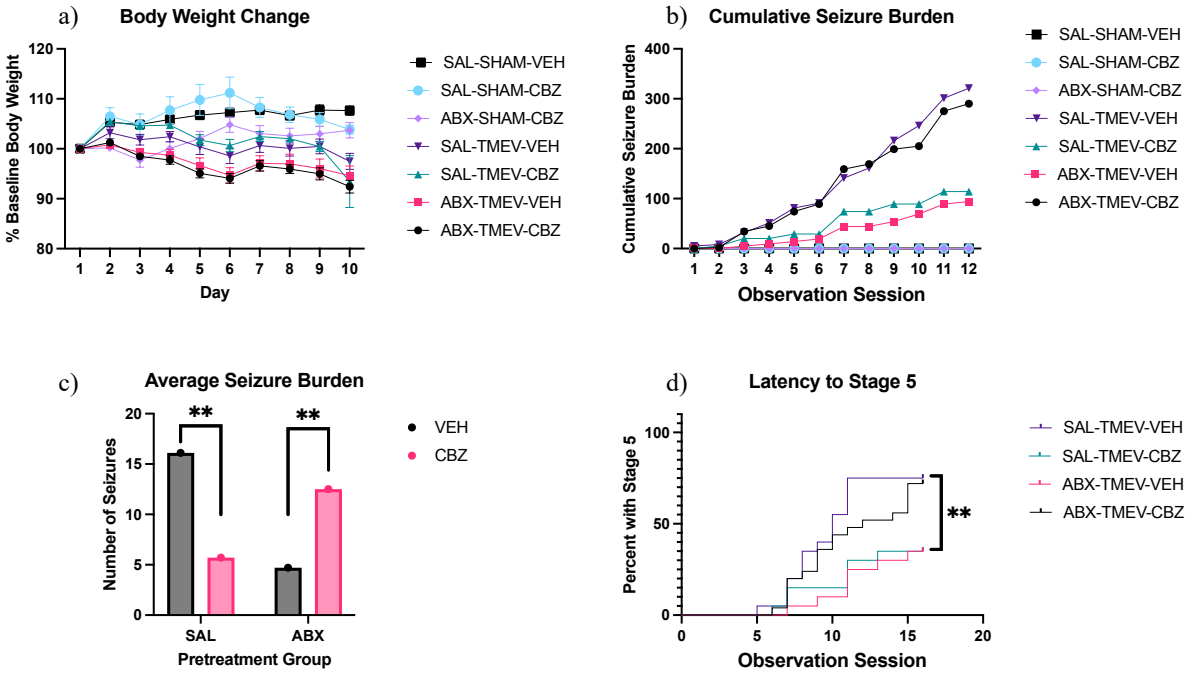

Figure 3. Markers of overall health were not impacted by a 10-day course of oral antibiotic (ABX) administration. Complete blood count (a, b) and differential (c-f) indicate that mice are clinically normal, other than brain TMEV infection. There is an effect of CBZ on the differential (d, e), but no effect of ABX.

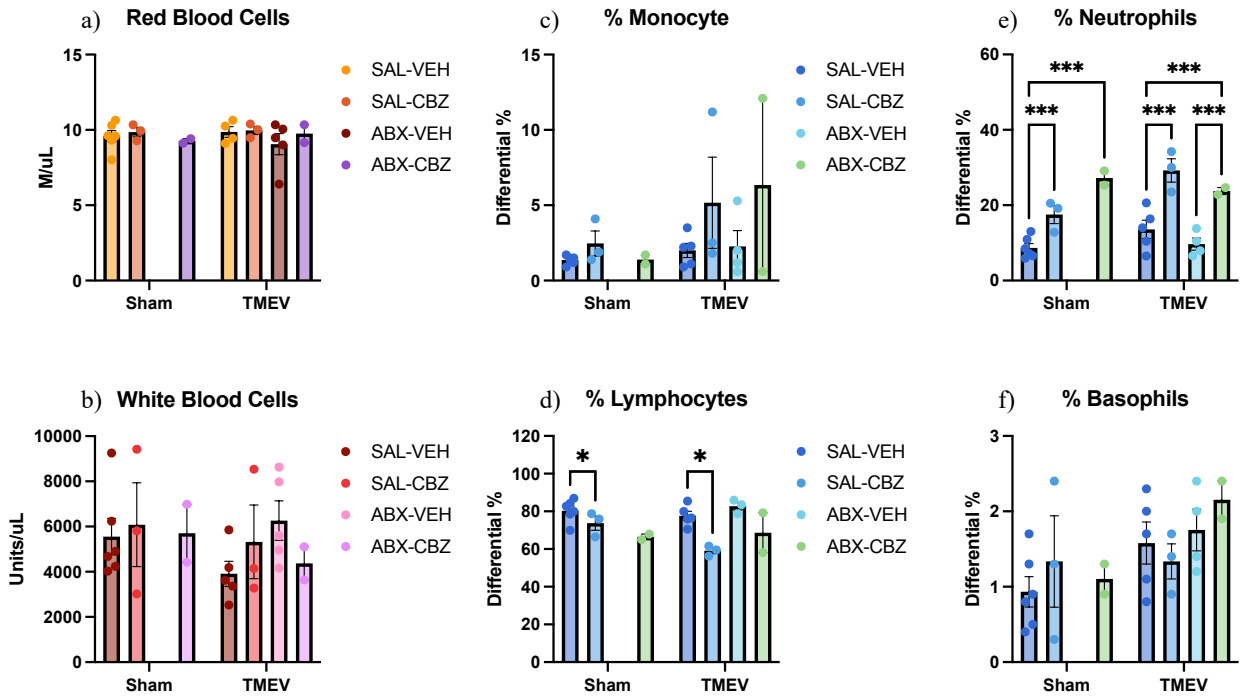

Figure 4. TMEV infection and carbamazepine (CBZ) treatment are associated with reduced spleen weight in mice with and without a history of 10-day antibiotics (ABX) administration to deplete the gut microbiome.

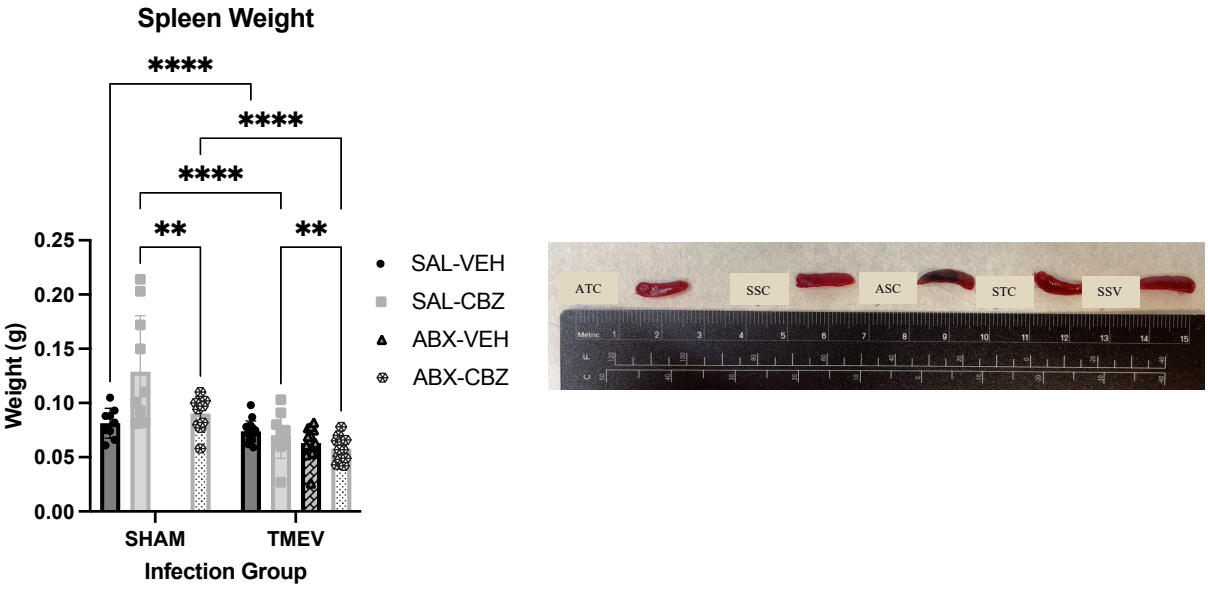

Figure 5. A 10-day course of oral antibiotics (ABX) administered to TMEV-infected or sham-infected mice significantly alters the composition of the gut microbiome with or without acute carbamazepine (CBZ) administration. The most significant differences were seen among Gram positive, anaerobic organisms (a), although some changes were seen in Gram negative organisms (b).

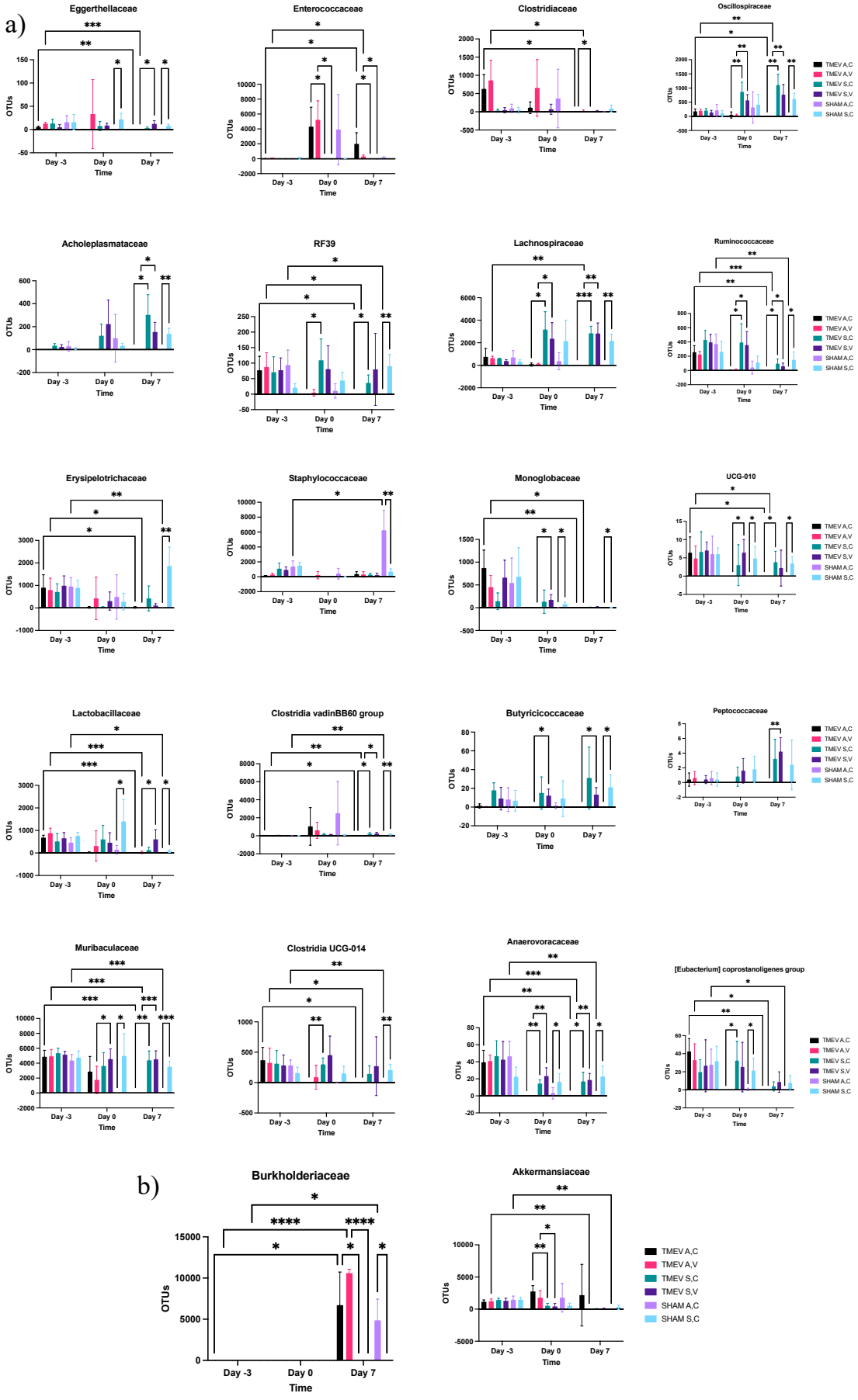
a)
Eggerthellaceae
Enterococcaceae
Clostridiaceae
Oscillospiraceae
Acholeplasmataceae
RF39
Lachnospiraceae
Ruminococcaceae
Erysipelotrichaceae
Staphylococcaceae
Monoglobaceae
UCG-010
Lactobacillaceae
Clostridia vadinBB60 group
Butyricicoccaceae
Peptococcaceae
Muribaculaceae
Clostridia UCG-014
Anaerovoracaceae
[Eubacterium] coprostanoligenes group
b)
Burkholderiaceae
Akkermansiaceae
OTUs
Time
Day -3
Day 0
Day 7
TMEV A,C
TMEV A,V
TMEV S,C
TMEV S,V
SHAM A,C
SHAM S,C

Figure 6. Hippocampal cell death, as assessed by FluoroJade-C staining (green) is seen in TMEV-infected mice, but not in sham-infected mice with and without a history of antibiotics (ABX) administration. Blue indicates DAPI, a nuclear counter-stain.

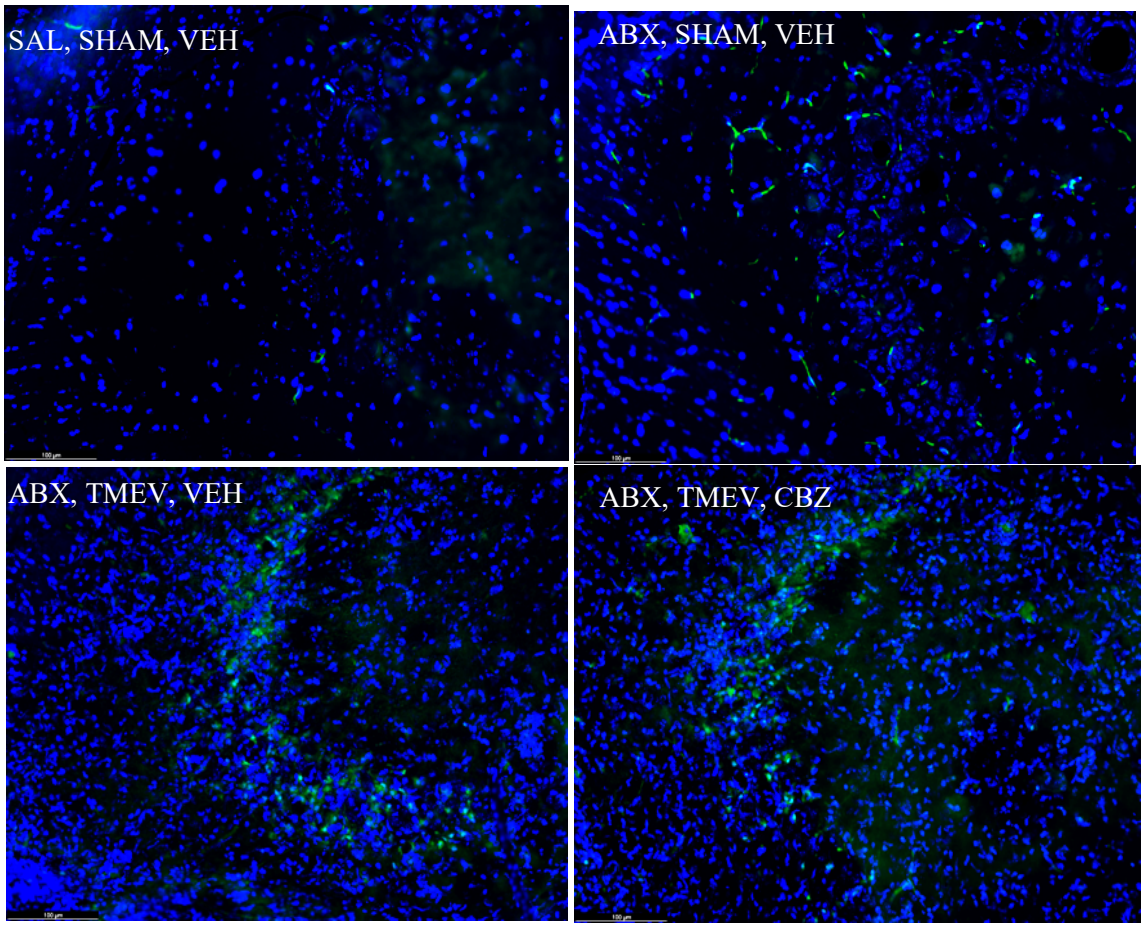

Figure 7. Antibiotics (ABX)-induced intestinal dysbiosis in mice does not alter plasma carbamazepine (CBZ) concentrations (n=3-9 mice/treatment group) in mice at 7 days post-infection with TMEV in the brain.

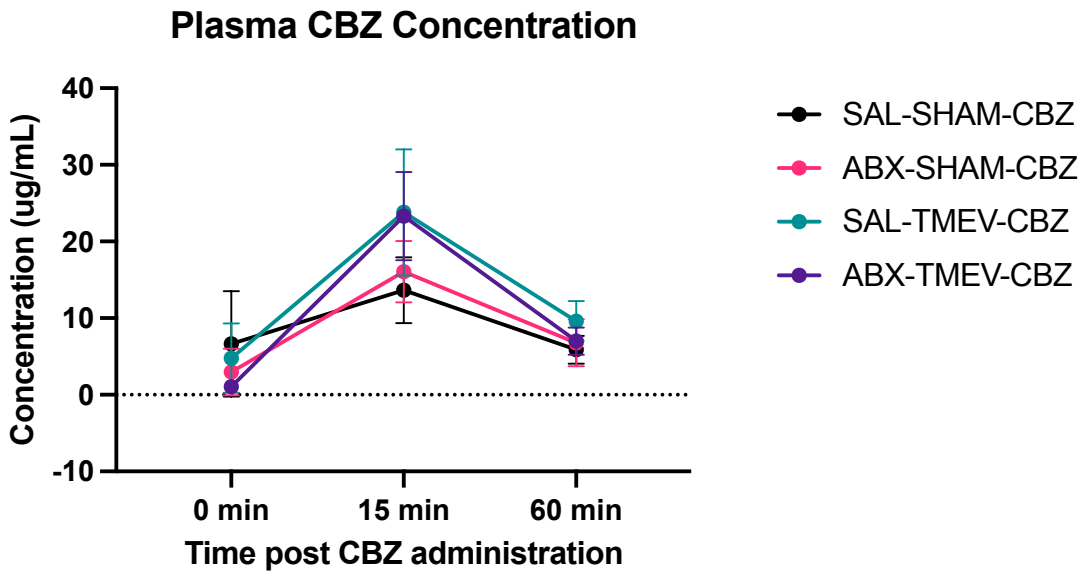

Table 1: Summary of FluoroJade-C Staining. Brain infection with TMEV leads to acute hippocampal neurodegeneration by 7 days post-infection, as assessed by FluoroJade-C histological staining (FJ-C)* indicates significantly different from ABX-TMEV-CBZ treatment group, $p<0.05$; #indicates significantly different from Saline-TMEV-CBZ treatment group, $p<0.05$

Treatment Condition			**FJ-C Positive Labeling in:**			
Gut Microbiome Depletion?	**Infection**	**Treatment**	**CA1 (N/F)**	**CA3 (N/F)**	**DG (N/F)**	**Cortex (N/F)**
No (Saline)	Sham	CBZ	0/5	0/5	0/5	0/5
Yes (ABX)	Sham	CBZ	0/5	0/5	0/5	0/5
No (Saline)	TMEV	VEH	7/7	7/7	0/7	5/7
No (Saline)	TMEV	CBZ	4/10*#	6/10	0/10	4/10
Yes (ABX)	TMEV	VEH	7/7	7/7	0/7	7/7
Yes (ABX)	TMEV	CBZ	7/7	7/7	0/7	7/7

Table 2: LC Gradient for LC separation of Carbamazepine

Time (min)	Flow Rate (mL/min)	Solvent A (%)	Solvent B (%)
Initial	0.3	80	20
2	0.3	0	100
4.5	0.3	0	100
4.6	0.3	80	20

Table 3: MS Transitions for Carbamazepine and Diazepam-d5 Quantification

Compound	**Parent (m/z)**	**Daughter (m/z)**	**Dwell time (s)**	**Cone (v)**	**Collision (eV)**
Carbamazepine	237.0957	194.0025	0.096	2	18
Carbamazepine	237.0957	178.9191	0.096	2	32
Diazepam-d5	290.1681	198.0652	0.096	100	36
Diazepam-d5	290.1681	154.049	0.096	100	28