Impact of Dysbiosis and Antiseizure Medication on Seizure Pathophysiology in a Viral Infection-Induced Epilepsy Model

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Abstract

Viral infections are associated with the development of seizures and epilepsy. Inflammation in the brain, particularly in the hippocampus, is thought to play a key role in orchestrating this process. We have previously demonstrated in mice that the gut microbiome influences seizure susceptibility following brain infection with Theiler's virus (TMEV). Further, gut dysbiosis significantly alters the antiseizure activity of carbamazepine (CBZ) in TMEV-infected mice with acute seizures. Thus, we herein sought to define how experimentally evoked intestinal dysbiosis influences hippocampal pathophysiology and neuroinflammation following acute symptomatic seizures in TMEV-infected mice, and whether acute CBZ treatment could further influence this neuropathology. Mice were pretreated with antibiotics or saline, followed by TMEV infection, and CBZ or vehicle administration (days 3-7 post-infection). Histological analyses were then performed on hippocampal tissue to quantify neuronal death, microgliosis, astrogliosis, and cellular proliferation after acute infection. While overall microglial reactivity did not markedly differ between groups, microglial proliferation was significantly increased in CA1 of CBZ-treated versus VEH-treated intact mice. Dysbiosis appeared to blunt this CBZ-induced microglial proliferation. Further, dysbiosis contributed to neuroprotection while also increasing neural proliferation within CA3, which may worsen disease outcomes due to aberrant migration and integration. The gut microbiome may thus prime CNS-specific immune responses in viral infection-induced seizures, opening the opportunity for potential GI-specific therapeutic interventions for seizures.

Keywords: Carbamazepine, microglia, proliferation, microbiome, neurogenesis

Introduction

Viral encephalitis and meningitis are common and understudied drivers of acute symptomatic seizure (ASyS) risk and subsequent epilepsy development (1). Viral infection leads to epileptogenesis due to the inflammatory cytokine cascade that subsequently induces neuronal hyperexcitability (2). Since neuroinflammation has been implicated as a key contributor to acute neuropathology and epileptogenesis, better understanding of its role in virus-induced seizure models would aid in better treatment and mitigation of disease outcomes.

The Theiler's murine encephalomyelitis virus (TMEV) is a relatively new and lesser integrated mouse model of naturally acquired temporal lobe epilepsy (TLE) that closely mimics clinically observed ASyS, neuropathology, and later epilepsy development following brain infection (2). Moreover, the pathological features of the TMEV model include neuroinflammation (3–5), neurodegeneration (3,4,6), and hippocampal sclerosis (5), further recapitulating clinical pathology of infection-induced epilepsy. Interestingly, inflammation both within and outside of the CNS contributes to ASyS onset (7) via increased circulation of inflammatory cytokines (7,8). However, how the peripheral immune system is primed and how extrinsic factors drive an individual's seizure susceptibility and pathological burden in the TMEV model of infection-induced acute seizures is understudied.

Peripheral inflammation can promote epilepsy risk. For example, patients with irritable bowel syndrome (IBS) and Crohn's disease have a higher risk of developing epilepsy (7,8). IBS increases intestinal inflammation, which contributes to systematic inflammation and peripheral immune activation, thereby increasing neuroimmune responses through infiltrating macrophages and enhancing microglial activation (9). Additionally, people with epilepsy also tend to have gastrointestinal symptoms and altered gut-microbiome composition (7,8), further implicating a significant role of the microbiome in inflammation and epileptogenesis. For example, short course antibiotic administration to people with

drug-resistant TLE may alleviate seizure frequency (10). Further, reductions in the *Bacteroidota* phylum are found in epilepsy models (11,12). Since this phylum is responsible for regulating CNS interleukin levels (13), reductions in its population may result in increased microglia activation and epileptogenesis (11). Furthermore, peripheral inflammation and the microbiome may also influence ASM efficacy through GI-specific metabolic interactions (14); inflammation can drastically alter hepatic CYP enzyme expression (15). Intestinal inflammation decreases ASM efficacy in the mouse pentylenetetrazole seizure model (8), implicating inflammation as an underappreciated modifier of ASM efficacy. We find carbamazepine (CBZ) to be proconvulsant under dysbiotic conditions in the TMEV model of infection-induced acute seizures (12), further emphasizing the critical role the microbiome plays in modifying seizure risk and outcomes. Overall, inflammation and the microbiome intersect to modify disease burden and treatment efficacy. Therefore, we hypothesized that alterations in the composition of the gut microbiome may also influence neurodegeneration, astrogliosis, and microgliosis in the brain following TMEV infection and ASM treatment.

To investigate this, we sought to extend our previous findings (12) to ascertain the extent to which antibiotic (ABX)-induced dysbiosis influences neuropathology in the TMEV model. We investigated how experimentally evoked gut dysbiosis could change neuropathological hallmarks in relation to seizure burden. Secondarily, we sought to characterize the neuroprotective effects of CBZ, a commonly prescribed ASM, under intact and dysbiotic conditions. We herein further establish the contributions of the gut microbiome to shaping ASyS burden and ASM neuroprotection after brain infection with TMEV in mice, with a specific finding that gut dysbiosis alters microglial reactivity and proliferation in response to CBZ administration.

Materials & Methods

Mouse brain tissue from our earlier published study was used and complete experimental methods can be found there (12). Briefly, male C56BL/6J mice (4-5 weeks old) were given oral antibiotics cocktail (ABX; ampicillin (1 g/L), metronidazole (1 g/L), neomycin sulfate (1 g/L), and vancomycin (0.5 g/L) dissolved in saline) or saline (SAL) and infected with TMEV. Then, during the acute infection period, CBZ (20 mg/kg) or vehicle (VEH; 0.5% methylcellulose) was administered prior to behavioral seizure assessment. All animal use was approved by the University of Washington Institutional Animal Care and Use Committee (protocol 4387-02) and conformed to ARRIVE guidelines (16).

Histopathology. Brain tissue was sectioned after animals were euthanized on post-infection day 7 (DPI). Fluoro-Jade C (FJC; Histochem catalog #2FJC) and Hoechst 33342 (Invitrogen catalog #62249) were utilized to detect hippocampal cell death (CA1, CA3, and dentate gyrus). We previously qualitatively assessed FJC (12), but this study aimed to extend those results quantitatively. FJC staining was performed as described (12,17). Microglia proliferation was assessed with anti-Iba1 (Fujifilm 019-19741, 1:1000) and anti-Ki67 (ThermoFisher, 14-5698-80, 1:300) primary antibodies and goat anti-rabbit IgG Alexa Fluor 555 (Abcam ab150078, 1:1000) and goat anti-rat IgG Alexa Fluor 488 (ThermoFisher, A-11006, 1:500) secondary antibodies. Astroglia and neuron proliferation was assessed with anti-Ki67 (Abcam ab16667, 1:300) primary antibody with Goat anti-Rabbit IgG Alexa Fluor 555 secondary antibody (1:500) and colabeled with Alexa Fluor 488 conjugated anti-GFAP (Sigma MAB3402X, 1:1000) or Alexa Fluor 488 conjugated anti-Neun (Sigma-Aldrich MAB377X, 1:300). Immunohistochemistry was completed as previously published (4,5). All slides were imaged on an upright fluorescent microscope (Leica DM6 B) with a 20x objective with constant acquisition settings (100 ms exposure, 1 gain; Figure S1.). FJC-, Iba-, GFAP-, and NeuN-positive cells were counted using ImageJ Fiji (1.54p). Then Iba1-, GFAP- and NeuN-positive cells co-labeled with Ki67 were identified

using the colocalization plugin (https://imagej.net/ij/plugins/colocalization.html) and counted using ImageJ.

Statistical Analysis. Two-way ANOVA with *post-hoc* Tukey t-tests was used to compare cell counts across TMEV groups. Linear regression was used to assess the impact of seizure burden and cell death on microglia, astroglia, neurons, and each of their respective proliferation levels. Statistical analysis was performed in RStudio (R 4.4.1) using the stats (4.4.1) and emmeans (1.10.7) packages, with $p < 0.05$ considered significant.

Results

When comparing across groups, we found that ASM treatment significantly impacted microgliosis in CA1 (Figure 1B, $F(1, 29) = 9.257$, $p < 0.01$), with elevated microgliosis observed in SAL-CBZ animals versus ABX-VEH ($p<0.05$) and SAL-VEH animals ($p<0.05$). However, no other significant effects of dysbiosis of CBZ on microgliosis were found in other hippocampal regions (Figure 1B). Furthermore, no other significant group differences were observed for microglia (Figure 1A), cell death (Figure S2A), astroglia (Figure S3A-B), or neuron levels (Figure 2A-B). These findings are generally consistent with prior observations of extensive hippocampal pathology in CA1 in TMEV infected mice (5,6).

While investigating the impact of seizure burden on hippocampal histopathology, we only found significant effects in the CA3. Seizure burden significantly impacted cell death in this region (i.e., FJC+ cell counts; Figure S2B; $F(4, 21) = 3.966$, $p < 0.05$, Adj. $R^2 = 0.32$). For both SAL-VEH ($\beta = 4.14$, $p < 0.05$) and ABX-CBZ groups ($\beta = 4.26$, $p < 0.01$), cell death increased with seizure burden. This result aligns with our previous research that found the highest seizure burdens in these groups (12). Seizure burden also significantly impacted NeuN+ cell counts (Figure 2C, $F(4, 16) = 7.963$, $p < 0.001$, Adj. $R^2 = 0.58$), and NeuN/ki67 colabeled cell counts (Figure 2D, $F(4, 16) = 5.508$, $p < 0.01$, Adj. $R^2 = 0.47$). The

seizure burden for SAL-VEH animals negatively correlated with NeuN+ cells counts ($\beta = -1.35$, $p < 0.05$) and NeuN/ki67+ cell counts ($\beta = -1.405$, $p < 0.05$), suggesting that untreated seizures contributes to neurodegeneration. However, ABX-VEH animals displayed a positive correlation between seizure burden and NeuN+ cells ($\beta = 10.423$, $p < 0.01$) and NeuN/ki67+ cells ($\beta = 5.478$, $p < 0.05$). In fact, ABX-VEH animals consistently displayed higher neuron counts than the SAL-CBZ ($p < 0.05$) and SAL-VEH groups ($p < 0.01$). These data suggest that gut dysbiosis preserved neuronal function despite seizure presentation, indicating that the peripheral immune system promotes neuronal degeneration in the face of acute symptomatic seizures in this model.

Finally, we sought to define whether FJC+ was associated with microglial, astroglial, or neuronal proliferation (Figure 1 and Figure 2). Only microglial proliferation in the CA1 was significantly correlated to FJC+ labeling (Figure 1B, $F(4, 21) = 13.37$, $p < 0.0001$, Adj. $R^2 = 0.66$). SAL-CBZ animals displayed a positive correlation between FJC+ cell levels and microglia proliferation ($\beta = 0.83$, $p < 0.001$), and consistently displayed more microglia proliferation than the ABX-VEH group ($p < 0.001$). These findings illustrate that with ABX-mediated gut dysbiosis, the microglial proliferative capacity was ablated in the setting of ASM administration.

Discussion

This study aimed to extend our prior work assessing the effects of experimentally-evoked dysbiosis on the phenotypic features of ASyS following a TMEV infection (12), as well as addressing how a short-course ASM administration (here CBZ) could influence seizure-related neuropathological outcomes. Our present results reveal two hippocampal region-dependent histopathological profiles following gut dysbiosis in the TMEV model: (1) CBZ-mediated effects in the CA1, and (2) dysbiosis-mediated effects in the CA3. Importantly, this current investigation provides critical insight into the

effects of the peripheral immune system priming on CNS pathology in response to an epileptogenic insult, carrying large translational potential to managing clinical ASyS (18).

In CA1, CBZ administration preserved microglia proliferation, especially relative to VEH-treated dysbiotic animals that showed marked reductions versus sham-infected saline-treated mice (Figure 1B). Moreover, dysbiosis disrupted this capacity. Previous work in our lab showed that both groups (CBZ-treated non-dysbiotic and untreated dysbiotic) demonstrated lower seizure burdens than untreated non-dysbiotic and CBZ-treated dysbiotic animals (12), indicating anticonvulsive effects that arise from different mechanisms and aligning with clinical effects of probiotic supplementation on seizure control in people with epilepsy (19). This may reflect the biphasic role of microglia in seizure onset, where initial activation is neuroprotective, while chronic activation is detrimental (20). Therefore, CBZ-induced microgliosis may promote an acute, beneficial immune response, whereas dysbiosis ablates microgliosis, potentially undermining this protective effect. While our study found a novel effect of CBZ administration on microglia, further investigation is needed to assess the long-term impact of dysbiosis on chronic inflammation, especially given its known chronic effects on microglial function (21). Overall, our results suggest that CBZ confers neuroprotection by enhancing acute microglial responses, while dysbiosis disrupts this process. Altogether this work provides a promising direction for exploring TMEV pathophysiology and the neurological consequences of dysbiosis in clinical ASyS.

In CA3, TMEV infection enhanced neurodegeneration and decreased neurogenesis, consistent with previous research (4,22), with seizure burden serving as the main predictor of histopathology. In fact, TMEV infection has been found to contribute to acute seizure occurrence through inhibitory cell loss and resulting reductions in inhibitory post-synaptic currents (IPSCs) (22). Conversely, dysbiosis reversed these effects, increasing neuronal survival and proliferation as seizure burden increased. While this effect appears to be neuroprotective, it may reflect pathological changes. Increased neurogenesis

observed acutely following status epilepticus contributes to epileptogenesis by promoting aberrant neuronal migration and integration (23). Notably, blocking neurogenesis slowed epileptogenesis (23) and normalizes epilepsy associated cognitive decline (24) reflective of a disease-modifying benefit. Dysbiosis has also been shown to alter synaptic connectivity, particularly by weakening CA3-CA1 synaptic communication and reducing spontaneous postsynaptic glutamatergic currents, effects linked with impaired microglial function (25). Our findings of dysbiosis-induced impairments in microgliosis in CA1 and elevated neurogenesis in CA3 parallel these observations, suggesting that disrupted microglial-neuronal interactions may impair regional crosstalk and inadvertently suppress seizure activity. However, while dysbiosis may transiently reduce ASyS burden through these mechanisms, its enhancement of neurogenesis may predispose the brain to future epileptogenesis (24). Further investigation is thus needed to clarify the chronic pathological consequences of dysbiosis in the TMEV model and establish whether cognitive comorbidities are worsened or alleviated by dysbiosis during an epileptogenic insult.

Conclusion

Overall, our results indicate that dysbiosis may decrease microglia function in the face of an acute neurological insult, thus impairing their ability to regulate inflammation, recruit other repair cells, clear debris, and coordinate intercellular communication. While this impairment may inadvertently reduce acute seizure burden, it likely reflects a more insidious underlying effect on disease trajectory. Thus, further research is needed to understand the long-term consequences of dysbiosis. In contrast, we herein found that CBZ enhanced microgliosis, thus offering a potential mechanism for its neuroprotective anti-seizure effects against ASyS in the TMEV model. However, this benefit was nullified by dysbiosis, highlighting the need to investigate how dysbiosis undermines CBZ anticonvulsant activty despite no changes in circulating plasma concentrations (12). Although previous

work from our lab found no dysbiosis-related changes in CBZ plasma concentration, other studies have shown that inflammation can inhibit cytochrome P450 enzymes and/or hepatic drug transporters that may influence circulating drug levels (26). Therefore, examining how dysbiosis affects drug metabolism in this translationally relevant mouse model of ASyS and acquired TLE may uncover novel intervention strategies to improve antiseizure medication activity in human patients with epilepsy.

Declarations

Individual Contributions: Sophia Shonka, Inga Erickson, Melissa Barker-Haliski: conception or design of the work. Sophia Shonka, Inga Erickson, Melissa Barker-Haliski, Hannah Park: acquisition, analysis, or interpretation of data. Sophia Shonka, Inga Erickson, Melissa Barker-Haliski: drafting or substantially revising the work.

Disclosures: None of the authors have any conflict of interest to disclose. We confirm that we have read the Journal's position on issues involved in ethical publication and affirm that this report is consistent with those guidelines

Data Availability: All clinical monitoring, behavioral, and histological data is available upon request from the corresponding author.

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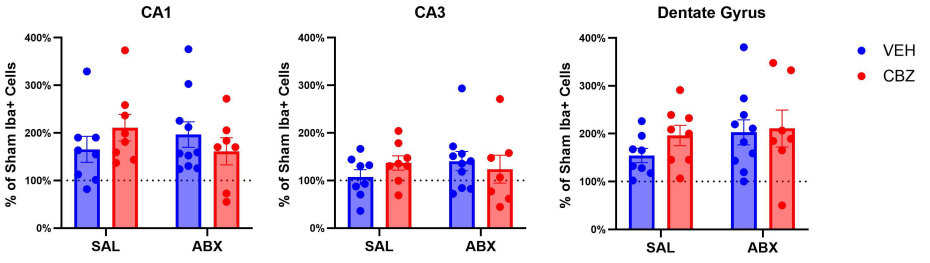
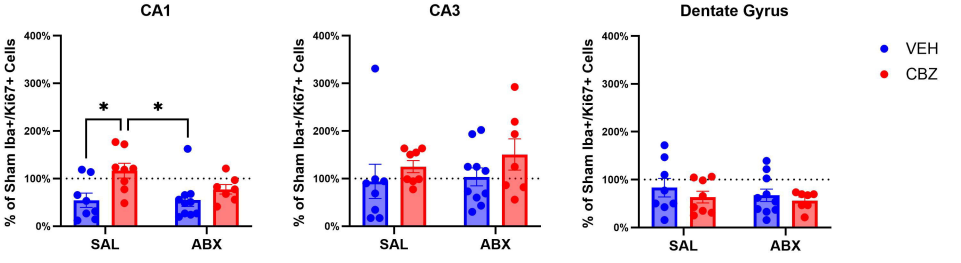
Figure 1. Microglia cell counts and microglia proliferation levels in the hippocampus. Graphs show mean ± SEM. A) There was no effect of ABX or CBZ treatment on microglia levels in the hippocampus, B) There was no effect on ABX or CBZ treatment on microgliosis in the CA3 or dentate gyrus regions of the hippocampus, C) Seizure burden did not moderate the relationship between ABX and CBZ treatment on microglia levels in the hippocampus, D) Seizure burden did not moderate the relationship between ABX and CBZ treatment on microgliosis in the hippocampus, E) Cell death count did not moderate the relationship between ABX and CBZ treatment on microglia levels in the hippocampus, F) Cell death count did not moderate the relationship between ABX and CBZ treatment on microgliosis in the CA3 or dentate gyrus regions of the hippocampus.

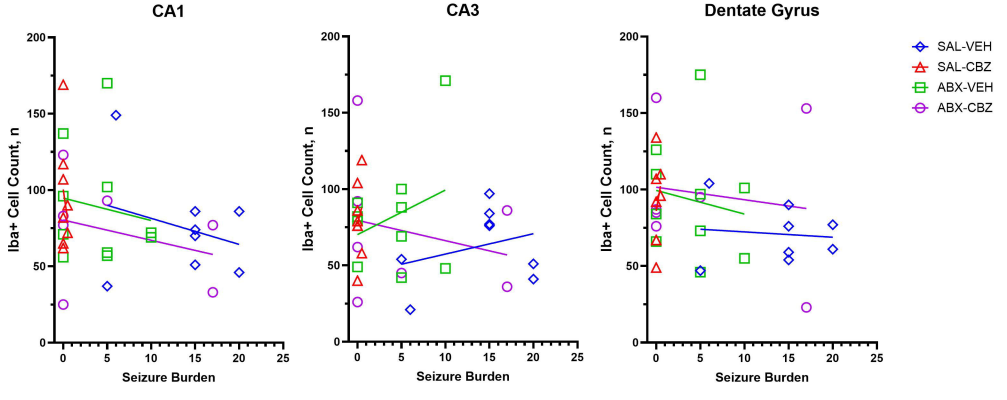
A. Microglia Levels in the Hippocampus



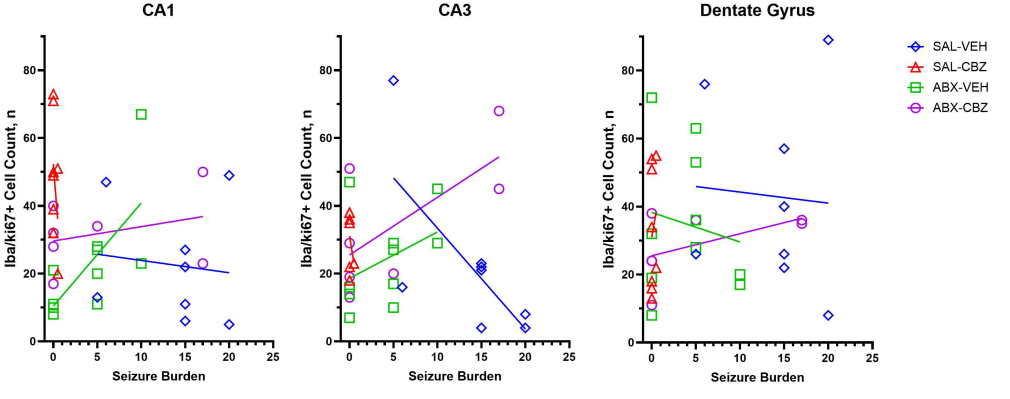
B. Microglia Proliferation in the Hippocampus



C. Impact of Seizure Burden on Microglia Levels in the Hippocampus



D. Impact of Seizure Burden on Microglia Proliferation in the Hippocampus



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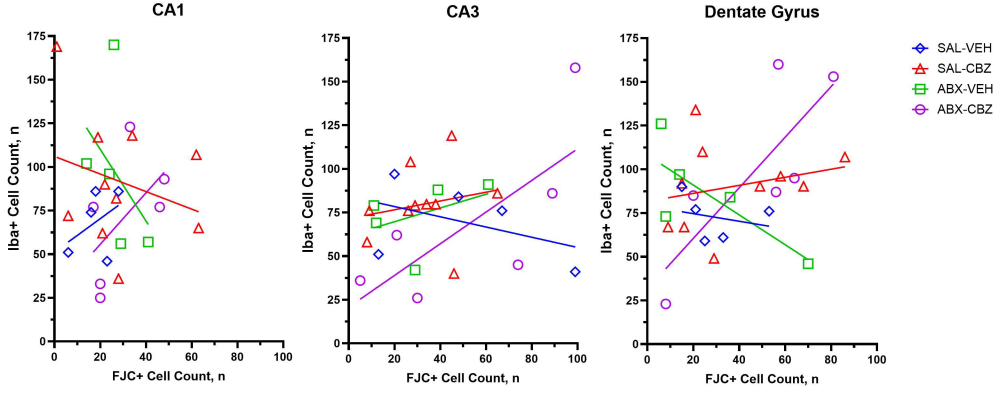

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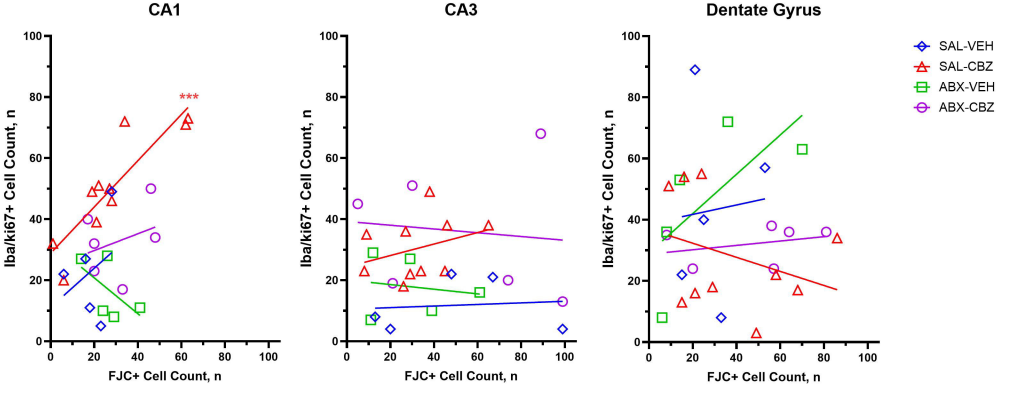
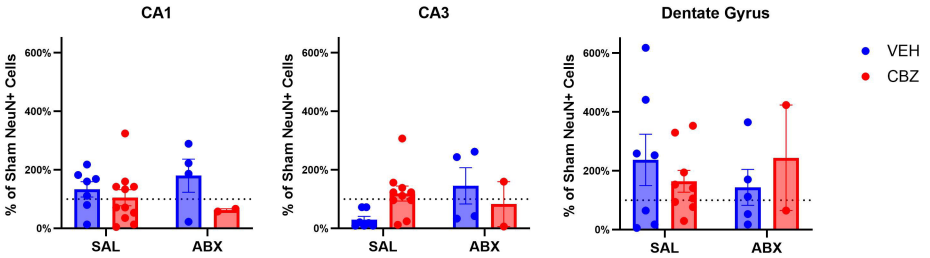
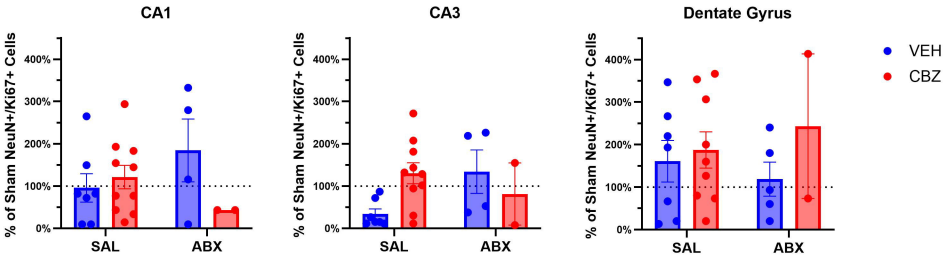


Figure 2. Neuron cell counts and neuron proliferation levels in the hippocampus. Graphs show mean ± SEM. A) There was no effect of ABX or CBZ treatment on neuron levels in the hippocampus, B) There was no effect of ABX or CBZ treatment on neuron proliferation levels in the hippocampus, C) Seizure burden did not moderate the relationship between ABX and CBZ on neuron levels in the CA1 and dentate gyrus regions of the hippocampus, D) Seizure burden did not moderate the relationship between ABX and CBZ on neurogenesis in the CA1 and dentate gyrus regions of the hippocampus, E) Cell death count did not moderate the relationship between ABX and CBZ on neuron levels in the hippocampus, F) Cell death count did not moderate the relationship between ABX and CBZ on neuron proliferation levels in the hippocampus.

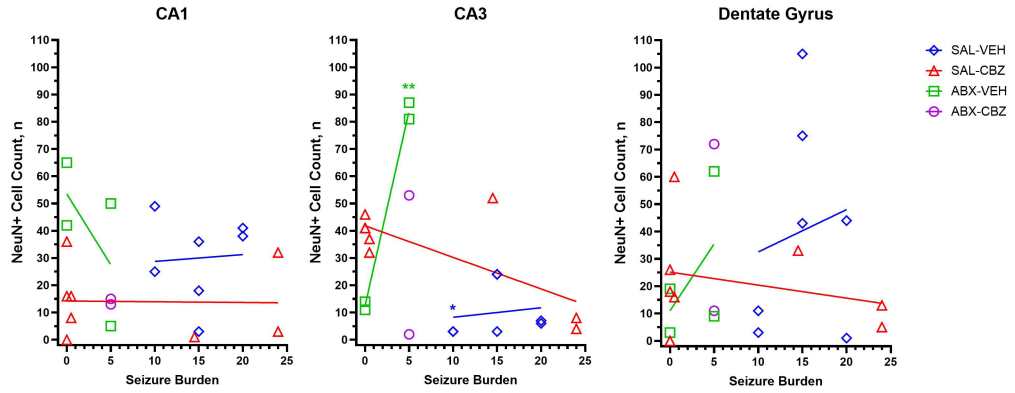
A. Neuron Levels in the Hippocampus



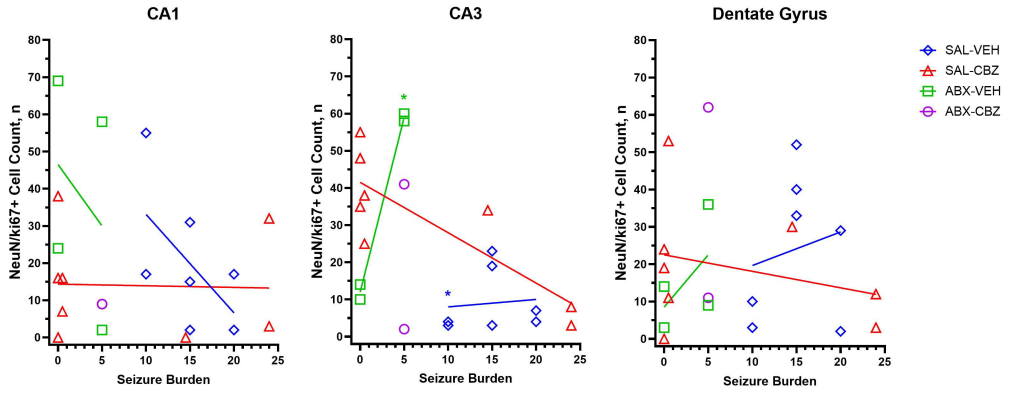
B. Neuron Proliferation in the Hippocampus



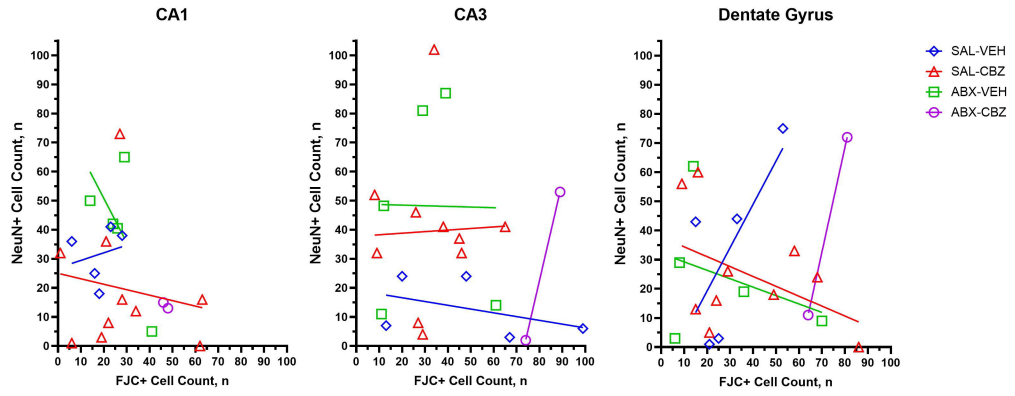
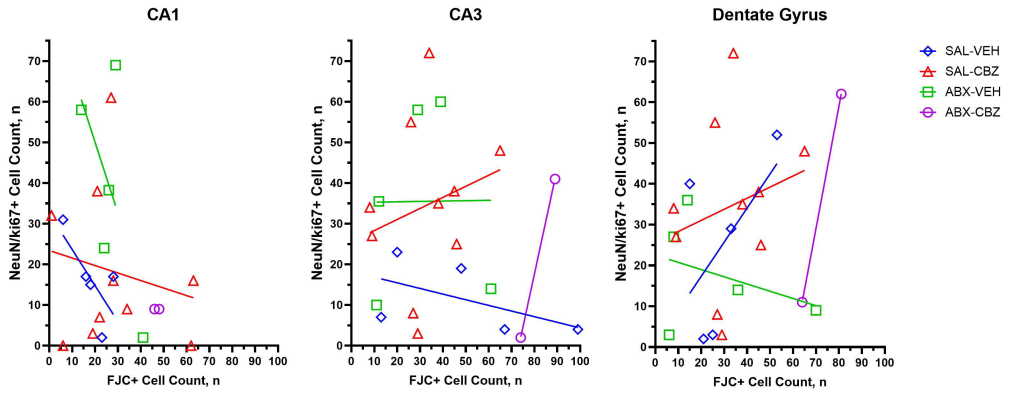
C. Impact of Seizure Burden on Neuron Levels in the Hippocampus



D. Impact of Seizure Burden on Neuron Proliferation in the Hippocampus



E. Impact of Cell Death on Neuron Levels in the Hippocampus



F. Impact of Cell Death on Neuron Proliferation in the Hippocampus