The gut-brain axis in seizure susceptibility: A role for microbial metabolite S-equol

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Dissertation submitted to the faculty of the Virginia Polytechnic Institute and State University in partial fulfillment of the requirements for the degree of:

Doctor of Philosophy

In

Translational Biology, Medicine, and Health

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4.27.2021

Roanoke, VA

Keywords: epilepsy; seizure; gut-brain axis; microbiome; rodent models

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ABSTRACT

Epilepsy is a complex, chronic neurological disorder with diverse underlying etiologies characterized by the spontaneous occurrence of seizures. Epilepsy affects all ages from neonates to elderly adults, with the most recent CDC estimates stating that ~3 million adults and over 400,000 children are currently suffering from active epilepsy in the U.S. alone. In adults, the leading cause of epilepsy worldwide in central nervous system (CNS) infection, while in neonates the most common cause of seizures is hypoxic/ischemic encephalopathy (HIE). However, in both adults and neonates, current antiepileptic drugs (AEDs) are ineffective in 30-50% of patients, despite the availability of over 20 FDA approved AEDs with diverse molecular targets. This disparity highlights a critical need for novel therapeutics in seizure-susceptibility and epilepsy.

The microbes that inhabit gut mucosal surfaces, termed the gut microbiota, have been increasingly implicated in the pathology of neurological diseases including epilepsy. This gut-brain axis is an intriguing therapeutic target in epilepsy as gut microbes can affect the CNS through multiple mechanisms including vagus nerve signaling, immune-gut interactions, and through production of microbial-metabolites including neurotransmitters, short chain fatty acids (SCFAs), lactate, vitamins, and S-equol. Furthermore, the gut microbiota is crucial for neurodevelopment, indicating that the gut-brain axis may be involved in pediatric seizure-susceptibility.

This dissertation reviews current evidence on the role of gut metabolites in seizure-susceptibility in epilepsy, highlighting the microbial-derived metabolite S-equol as a potential novel AED. We then evaluate gut microbiome alterations in the Theiler's murine encephalomyelitis virus (TMEV) adult mouse model of CNS infection-induced seizures and find

decreases in S-equol-producing bacteria in the gut microbiomes of TMEV-infected mice with seizure phenotypes. We characterize the effect of exogenous S-equol on neuronal function *in vitro*, demonstrating a reduction in neuronal excitation following S-equol exposure. We additionally characterize entorhinal cortex (ECTX) pyramidal neuronal hyperexcitability, and demonstrate the ability of exogenous S-equol to ameliorate CNS-infection-induced ECTX neuronal hyperexcitability *ex vivo*. Finally, we demonstrate that perinatal and postnatal exposure to antibiotics alters the gut microbiome and increases seizure-susceptibility following HIE exposure in p9/p10 mice, potentially via sex-specific alterations in neuronal function. Together, this dissertation evaluates the gut-brain axis in pediatric and adult mouse models of seizure-susceptibility and identifies the gut metabolite S-equol as a potential target for the treatment of seizures.

The gut-brain axis in seizure susceptibility: A role for microbial metabolite S-equol

Allison Faye Bouslog

GENERAL AUDIENCE ABSTRACT

Epilepsy, a disease defined by the occurrence of two or more spontaneous seizures, affects over 50 million people worldwide. This makes epilepsy one of the most common chronic neurological disorders across the globe. People with epilepsy suffer increased mortality, lower quality of life, and increased social stigma. There is currently a crisis in the treatment and management of epilepsy, because although over 20 different anti-epileptic drugs (AEDs) are available to patients, these drugs only work in ~70% of individuals with epilepsy, leaving 30% of patients with uncontrolled seizures. Currently available AEDs are designed to target classical central nervous system (CNS) components. However, a growing body of evidence suggests that epilepsy is related to complex systems throughout the body. Therefore, in this manuscript we explore novel therapeutic targets outside of the CNS for the management of seizures.

Over 1000 species of bacteria live in the in the human gut, and are termed the gut microbiota. Gut microbes produce a variety of chemicals that circulate through the body and can even reach the brain. Interaction of chemicals produced by the gut microbiota and brain chemistry have been shown to affect disease outcomes in Autism Spectrum Disorder, Parkinson Disease, and other brain disorders. However, very few studies have examined the possibility of a role for the gut microbiota in epilepsy. In this dissertation, we review chemicals produced by the gut microbiota that may alter epilepsy biology. We additionally examine gut microbiota alterations in a rodent model of epilepsy, and identify a novel chemical, S-equol, that is produced by the gut microbiota and impacts epilepsy biology in our rodent model. Lastly, we explore how altering the maternal gut microbiota in rodents can influence seizure-susceptibility in infants.

Acknowledgements

I want to thank my advisors Susan Campbell and Harry Sontheimer, without whom I would not be the scientist I am today. Thank you Susan for all the time and effort you have spent working with me to not only grow as a scientist but as a person over the 7+ years you have mentored me through college and graduate school. Thank you Harry for continually inspiring me through your passion for neuroscience, and for exposing me to all the great local beers in Roanoke and Blacksburg through years of lab outings. I am lucky to have an incredibly supportive committee, all of whom have really gone above and beyond in guiding me through experiments and personal growth during my PhD. Thank you Michelle Olsen, Xin Luo, and Mark Witcher for working with me and challenging me to think in new ways about science.

I am also especially lucky to have a huge group of scientists who have mentored me through both undergraduate and graduate school. Thank you Bhanu, Dipan, Ian, Robyn, Emily, Leanne, and Jess for very patiently teaching me most of the techniques in this thesis and providing me feedback and support when experiments didn't go the way I expected. Thank you also to my lab mates Joelle, Tre, Oscar, and K'Ehleyr for teaching me techniques, talking science with me in lab and over beers, and for your support and guidance.

Thank you to my mom, dad, and sister for continuously supporting me and being there for me throughout my life. Your unwavering love and endless supply of good wine have sometimes been the only things getting me through this graduate school process, and I can't express my gratitude for you enough. Thank you to my grandparents, aunts, uncles, and cousins who have been there at my birthday parties and graduations, watched Alabama football games with me, and taught me how to ski, rock climb, and eat crabs (almost) like a true marylander. I am so blessed to

have such an outpouring of love from my entire family, and I know I will feel at home almost anywhere life takes me because I'll always have family close by to look after me.

Finally, I am especially grateful for my husband Joe, whose skill in making croissants has fueled the completion of this document. Thank you for loving and supporting me through late nights in lab, stressful project deadlines, and endless presentation practicing. Most importantly, thank you for supporting me in my dreams and life goals, even when my faith in myself was wavering.

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Glossary of Abbreviations

Central nervous system (CNS)

Hypoxic/ischemic encephalopathy (HIE)

Antiepileptic drug (AED)

Short chain fatty acid (SCFA)

Theiler's murine encephalomyelitis virus (TMEV)

Entorhinal cortex (ECTX)

Autism-Spectrum Disorder (ASD)

Alzheimer's Disease (AD)

World Health Organization (WHO)

Excitation/inhibition (I/E)

γ-aminobutyric acid (GABA)

pentylenetetrazol (PTZ)

Parkinson's Disease (PD)

Topiramate (TPM)

Racemic Equol (equol)

Days post infection (dpi)

Quantitative insights into microbial ecology (QIIME)

Temporal lobe epilepsy (TLE)

estrogen receptor β (ESR2)

Linear discriminant effect size analysis (LEfSe)

Resting membrane potential (RMP)

Action potential (AP)

Phosphate buffered saline (PBS)

High-conductance calcium-activated potassium channel (BK)

Dimethyl sulfoxide (DMSO)

Neonatal intensive care unit (NICU)

Antibiotic-treated (AbTr)

Untreated (UnTr)

Long term potentiation (LTP)

After-hyperpolarization (AHP)

Inter-event interval (IEI)

Selective serotonin reuptake inhibitor (SSRI)

Spontaneous excitatory post-synaptic current (sEPSC)

G protein-coupled estrogen receptor (GPER/GPR30)

Chapter 1: Introduction

Microbial-derived metabolites in epilepsy

Allison Gallucci, Devika Shukla, Susan Campbell

This chapter is currently being prepared for submission. AG & SC were responsible for idea conception. AG & DS reviewed manuscripts and summarized data. AG was responsible for draft preparation.

Abstract

Epilepsy is a disease defined by spontaneously recurring seizures that affects over 50 million people worldwide. Over 20 antiepileptic drugs are currently FDA approved, however, ~30% of patients with epilepsy have seizures that are unresponsive to medication. There is a clear unmet need to examine novel therapeutic targets in seizure-susceptibility and epilepsy. The gut microbiome represents an important therapeutic target in neurological diseases including epilepsy, and there is mounting evidence suggesting a role for the gut-brain axis in epilepsy treatment. However, there is paucity of information on mechanistic links regarding microbial regulation of brain function in epilepsy. This review discusses current understanding of microbial-derived metabolites, including neurotransmitters, short chain fatty acids (SCFAs), lactate, vitamins, and polyphenol metabolites as causative links in the gut-brain axis in epilepsy.

Introduction

Epilepsy is one of the most common chronic neurological diseases worldwide and currently affects over 50 million people across the globe(1). The defining symptom of epilepsy is the occurrence of unprovoked spontaneous seizures, which may arise due to a variety of etiologies

including genetic disorders, developmental structural abnormalities, central nervous system (CNS) infection, tumors, or CNS trauma(2). Epilepsy is also highly comorbid with both developmental and adult-onset neurological disorders including Autism-Spectrum Disorder (ASD) and Alzheimer's Disease (AD) as well as peripheral disease states including metabolic disorders and autoimmune disease(3). Epilepsy diagnosis is linked to increased social stigma, reduced access to education and occupation, and premature mortality(1). Although the latest classification by the World Health Organization (WHO) defines epilepsy as a disease rather than a syndrome, consideration of the underlying etiology remains imperative to determine possible novel therapeutic avenues(1,4).

Seizures are generally thought to be caused by abnormalities that give rise to alterations in excitation/inhibition (I/E) balance in the CNS, where excitation is mediated through glutamatergic synaptic signaling, and inhibition is mediated through γ-aminobutyric acid (GABA)ergic synaptic signaling(5). However, astrocytic maintenance of extracellular glutamate in tripartite synaptic signaling is increasingly implicated as also having an important role in I/E balance and seizure neuropathology(6,7). Additionally, glial reactivity is a common characteristic of seizures of varied etiology, and glial reactivity alone can cause seizures in rodent models(8,9). Finally, extracellular matrix components can also play a role in I/E balance and seizure-susceptibility through influencing inhibitory neuronal capacitance(10), indicating that the neuropathology of seizures is multifaceted and involves complex neurological pathways. Outside the CNS, peripheral inflammatory immune response can also impact seizure-development(11). CNS infection is the most common cause of epilepsy worldwide, and inflammatory cytokines are associated with multiple animal models of seizure-susceptibility(1,12,13). Metabolic dysfunction is also highly associated with epilepsy across etiologies, and oxidative stress alone can cause seizures(14).

Overall, the pathophysiology of epilepsy is complex and involves multiple systems within and outside of the CNS.

Although over 20 different antiepileptic drugs (AEDs) with diverse CNS mechanisms of action are currently FDA approved, ~30% of epilepsy patients do not respond to any available AED, and are therefore considered to have refractory epilepsy(15,16). Even though multiple AEDs have been recently approved, this population of patients with refractory epilepsy has remained stagnant(17), highlighting a compelling need for novel therapeutic targets in epilepsy and seizure-susceptibility. While most available AEDs have primarily CNS targets, epilepsy is a disease that can be impacted by multiple peripheral systems. Therefore, development of novel therapeutics may benefit from exploration of targets outside of CNS synaptic signaling.

Gut microbiome

The gut microbiome consists of the genetic material of the bacteria, fungi, protozoa, and viruses that inhabit the mucosal surfaces of the intestinal tract. Over 1000 different species of bacteria with diverse genetic profiles make up the healthy human gut microbiome(18), and alterations in the diversity and composition of the gut microbiome are associated with a variety of host disease states(19). The gut microbiome exhibits dramatic shifts across development before stabilizing in adulthood(20). However, even a stable adult microbiome can be altered significantly through dietary changes, chronic drug administration, antibiotic use, and lifestyle changes(21). The intersection between diet, lifestyle, the gut microbiome, and disease state is a topic of growing interest across diverse fields of study including immunology, cardiovascular science, cancer, and neuroscience(22). Probiotic therapeutics are gaining traction in the clinic as treatment for gastrointestinal diseases, with multiple microbiome-based therapeutics, including a fecal transplant to treat *Clostridium difficile* infection, which recently concluded successful phase III

clinical trials(23). A growing body of pre-clinical evidence also supports a role for probiotic therapies in non-gastrointestinal diseases including cardiovascular disease, metabolic disease, and interestingly, neurological diseases(24–28). However, probiotic therapies are not always successful, which may be due to the complex nature of microbial niches in the gut as well as limited understanding of the mechanisms by which gut microbiome makeup can affect distal systems such as the CNS(29–31). The high number of genes and unknown functions and properties of bacteria brings a huge challenge in studying the gut microbiome in disease. More research on mechanistic links between the gut and the brain is needed, and studying metabolites produced by probiotic bacteria is a crucial step towards mechanistic understanding that could lead to the development of successful therapeutics.

Gut microbiome and epilepsy

The link between epilepsy and the gut microbiome has been extensively reviewed(32–35). Studies show that adult patients with epilepsy have altered microbiomes compared to healthy controls(36). In addition, dysbiosis of the gut microbiome is associated with patients with drug-resistant epilepsy. Patients with refractory epilepsy have altered microbiomes compared to both healthy controls and to epilepsy patients whose seizures respond to AEDs, indicating a potential link between the gut microbiome, epilepsy, and responsiveness to current therapeutics(37). Infants and children with refractory epilepsy also have altered microbiomes compared to healthy controls(38,39).

Dietary intervention, the gut microbiome, and seizures

Ketogenic diet therapy consisting of strict adherence to high fat (>70% of total calorie intake) and low carbohydrate (<10% of total calorie intake) can improve symptoms in children

with refractory epilepsy(40), and is known to alter the gut microbiome in healthy adults as well as mice(41). Correspondingly, the ketogenic diet can alter the gut microbiome in infants and children with refractory epilepsy(39,42). Importantly, alterations in the microbiome induced by ketogenic diet correspond with reduction in seizure burden(43). In rodent models of epilepsy, administration of the ketogenic diet alters the gut microbiome. For instance, treatment with microbes alone can reproduce the anti-epileptic effect of the ketogenic diet in rodents. This probiotic therapy increased hippocampal GABA/glutamate ratio and reduced gamma-glutamylated amino acids in the periphery(44). Outside of dietary alterations, recent work from our lab highlights microbial shifts in a female rat model of Rett syndrome, a rare neurological disorder that is comorbid with seizures, compared to WT rats(45). In a genetic rat model of absence seizures, the gut microbiome was also altered compared to WT rats, and fecal transplant from WT rats into rats with absence seizures significantly reduced seizure burden(46). Fecal transplant from control mice also reduced seizure burden in mice exposed to chronic stress. In turn, fecal transplant from stressed mice increased seizure-susceptibility in control mice that were not exposed to chronic stress, indicating a bi-directional effect of the gut-brain axis on seizure-susceptibility(47). Probiotic therapy can also reduce seizure burden and corresponding behavioral comorbidities in pentylenetetrazol (PTZ)-kindled rats, potentially via an increase brain GABA levels and reduce nitic oxide(48,49). Excitingly, patients with refractory epilepsy showed marked improvement in seizure-burden following probiotic therapy in a prospective trial, indicating a promising potential for gut-microbiome therapeutics in patients with epilepsy(50). However, many of these studies show correlational links between the gut microbiome and seizure-susceptibility, and there are currently very few proposed mechanisms by which the gut microbiome influences disease progression in epilepsy.

Gut microbiome: pathways to the CNS

Vagus nerve

The gut microbiota can influence the CNS through several direct and indirect pathways. The vagus nerve, or cranial nerve X, is the longest of the cranial nerves and innervates areas of the cardiovascular, respiratory, autonomic, endocrine, and immune systems. Vagus nerve signaling is important in the sensation of pain, pressure, and temperature, and in the regulation of heartbeat and blood pressure(51). Vagal afferents express multiple receptors for bacterial metabolites, making the vagus nerve a key highway for microbiome-systemic interactions(52). Multiple studies highlight the importance of vagal signaling in the gut brain-axis, as some microbiota-related effects in the CNS are abolished following vagotomy(52–54). Interestingly, vagus nerve stimulation has been shown to improve seizure-burden in patients with refractory epilepsy(55), highlighting the potential link between the gut microbiome, vagus nerve, and epilepsy. Causal studies linking vagal activation to the gut-brain axis are needed to further understand the mechanisms by which the vagus nerve is involved in the gut microbiome and seizure susceptibility.

Immune system

The gut microbiota can also influence the CNS through microbiome-immune interaction. A healthy gut microbiome is essential for the development and maintenance of a healthy immune system(56). Germ-free mice, or mice born without gut microbes that are housed in sterile environments to preserve a microbe-free state, are a common animal model used to study the gut microbiome in health and disease(57). Germ-free mice exhibit severe deficits in immune system development and have altered B and T cell populations(58). CNS infection is the most common cause of epilepsy worldwide, highlighting a clear link between the immune system and epilepsy(1).

Inflammation is also common in non-infection associated epilepsy etiologies, and reactive astrogliosis alone can cause seizures in mice(8). In turn, the ketogenic diet may be beneficial in refractory epilepsy partially due to anti-inflammatory properties(59). Taken together, these studies indicate a mechanistic insight for a gut-immune axis in seizure-susceptibility.

Gut metabolites

Lastly, the gut microbiome produces a diverse array of metabolites that are important in human health and disease. Disease-induced changes in microbiome composition can affect the production of the types and levels of gut metabolites. Many gut metabolites can cross the blood brain barrier (BBB), allowing for direct signaling between gut microbes and constituents of the CNS, including neurons. Accordingly, gut metabolites have been implicated in a variety of neurological diseases including AD, ASD, and depression(60–62). Microbial production of neurotransmitters, SCFAs, lactate, B and K vitamins, and S-equol has the potential to play a mechanistic role in the gut-brain axis in seizure-susceptibility and epilepsy. This review presents current evidence of the role of microbial-metabolites in epilepsy and related disorders, highlighting S-equol as a novel microbial-derived therapeutic target in seizure-susceptibility and epilepsy.

Gut Metabolites

Neurotransmitters

The gut microbiome produces a variety of neurotransmitters that are involved in epilepsy including glutamate, GABA, serotonin, and dopamine. However, the role of gut-derived neurotransmitters in seizure-susceptibility remains largely unexplored. Here we discuss evidence for gut-derived neurotransmitters in seizure-susceptibility and related mechanisms.

Glutamate

Glutamate acts as one of the main excitatory neurotransmitters in the CNS and in the peripheral nervous system. In the gut, glutamate is derived from free glutamate in food, as well as dietary proteins and bacteria that synthesize it. Specific gut and brain functions, including the presence of brain disorders, are influenced by changes in glutamate receptor activity and glutamate transmission along the gut brain axis(63). Gut microbiota composition is associated with cognitive function in mouse models in part through changes in peripheral glutamate metabolism(64). When examining gut glutamate levels in mouse models, mice displaying schizophrenia-relevant behaviors had decreased glutamate levels and decreased microbiome α-diversity. In contrast to gut glutamate levels, in cerebral anatomies including the hippocampus these mice were found to have elevated glutamine and GABA(65). As temporal lobe epilepsy (TLE) is one of the most common forms of acquired epilepsy(1), alterations in hippocampal neurobiology are important in determining the link between the gut microbiome and seizure-susceptibility.

While glutamate can be directly influenced by food consumed, its presence in the gut is highly dependent on the specific bacteria responsible for its metabolism. The relative abundance of *Corynebacteriaceae*, *Coriobacteriaceae* and *Burkholderiaceae* is positively associated with fecal glutamate(64). In contrast, *Streptococaceae* abundance negatively correlates with fecal glutamate(64). Individuals with ASD were found to have a decline in 2-keto-glutaramic acid and a decline in the normally abundant microbiota that are responsible for glutamate metabolism (66). Glutamate levels may also be altered through diet-microbiome-CNS interactions. The ketogenetic diet alters the gut microbiome and has been used as a neuroprotective treatment for many diseases including epilepsy(41,42). The ketogenetic diet has also been shown to protect against acute electrically induced seizures and spontaneous tonic-clonic seizures in mouse models(44). Treatment with *Akkermansia* and *Parabacteroides* in the gut confers seizure protection to mice

following 6-Hz stimulation model of seizure-induction(44). Seizure protection was associated with reductions in systemic precursors to GABA, including gamma-glutamylated amino acids, and elevated hippocampal GABA/glutamate levels(44). Taken together, these results provide evidence that microbiome alterations alter brain levels of glutamate. However, as glutamate does not cross the BBB, it is unlikely that gut-production of glutamate directly influences neural glutamatergic signaling. Further research on microbial-derived glutamate is necessary to elucidate the possible link between microbiome glutamate production and CNS disease.

GABA

GABA, is the principal inhibitory neurotransmitter in the CNS. Decreases or increases in cerebral GABA back to baseline are associated with improvements a range of disorders including schizophrenia, abdominal pain, depression, antisocial behavior, memory, alcoholism, and cirrhosis, and seizures(67). In epilepsy, elevated hippocampal GABA/glutamate levels correlate with seizure protection in genetic mouse models(44). Gut bacteria, including *Bifidobacterium dentium*, are reported to produce GABA and, in the gut, GadB is the primary bacterial enzyme responsible for generating bioactive, microbial-derived GABA(68). While GABA is synthesized by many different types of gut bacteria, GABA does not cross the BBB and is instead synthesized in the brain where glutamine is converted first to glutamate and then to GABA.

GABA is reportedly reduced in the prefrontal cortex of rats administered high fat diet (HFD) associated with reduction in the relative abundance of *Bacteroidetes* and increased relative abundance of *Firmicutes* and *Cyanobacteria*(69). Germ-free mice receiving schizophrenia microbiome fecal transplants had lower glutamate and higher glutamine and GABA in the hippocampus. These mice also displayed schizophrenia-relevant behaviors, indicating that increased GABA is key in schizophrenia patients(65). A growing body of evidence implicates

Lactobacillus sp. with alterations in brain GABA levels. Administration of *Lactobacillus rhamnosus* to mice alters GABA receptors and reduces anxiety and depression-related behaviors(6). *Lactobacillus reuteri*, a species with decreased relative abundance in the Shank3 KO mouse model of ASD, positively correlates with the expression of GABA receptor subunits in the brain(70). Inoculation with *Lactobacillus reuteri* is also associated with increased GABA production in the hippocampus and results in significantly improved memory in mice(71). Probiotic therapy via fermented milk, or kefir, treatment can also alter cerebral GABA levels. Two kefirs (Fr1 and UK4) consisting of *Lactococcus lactis, L. kefiranofaciens*, *Bifidobacterium breve*, and *Pseudomonas* sp., or unfermented milk control, were administered to mice and both kefirs increased the capacity of the gut microbiota to produce GABA, which was linked to an increased prevalence in *Lactobacillus reuteri*. The detected *Lactobacillus reuteri* strain was found to encode two enzymes associated with the production of glutamate from glutamine: glutaminase and glutamine–fructose-6-phosphate transaminase. *Lactobacillus reuteri* may converting 2-oxoglutarate to glutamate, an important precursor to GABA(72). Excitingly, *Lactobacillus* probiotic supplementation substantially reduces seizure severity in PTZ-induced kindled rats such that very few probiotic-treated animals show full kindling, possibly through altering brain GABA levels(48). These reports provide insights on possible *Lactobacillus*-mediation of CNS GABA. However, as GABA does not cross the BBB, it is unlikely that gut-derived GABA is directly responsible for these effects. Future research on gut-derived GABA and its role in CNS disease is necessary to further understand mechanistic links in the gut microbiome and seizure-susceptibility.

Tryptophan/Serotonin

The neurotransmitter serotonin is derived from dietary tryptophan, which can be found in breastmilk as well as animal products. In the CNS, serotonin is produced primarily by serotonergic

neurons in the brainstem(73). CNS serotonin is widely studied as a modulator of mood and mood-related neurological disorder(74–76). Epilepsy is highly comorbid with mood disorders(77), and a growing body of evidence implicates serotonin signaling in seizure-susceptibility and epilepsy. Increasing brain levels of serotonin generally leads to a decrease in seizure burden in rodent models of epilepsy(78–81). In turn, reducing brain serotonin levels increases rodent susceptibility to seizures(81–83). Serotonin agonists show promise in reducing seizure burden in patients with Dravet syndrome(84). However, taking selective serotonin reuptake inhibitors (SSRIs) increases risk of developing epilepsy in adults after a traumatic brain injury (TBI)(85), indicating that serotonin therapeutics have a complex relationship with seizure-burden depending on the underlying etiology.

Serotonin in the gut is essential for the development of the enteric nervous system (ENS) and for enteric neuronal maintenance of gastrointestinal motility(86). The majority of serotonin in the human body is produced in the gut by host enterochromaffin cells(87), although several gut microbes including *Streptococcus thermophilus, Escherichia coli,* and several *Lactobacillus* sp. also have the capacity to produce serotonin(88–90). Germ-free mice have altered ENS development as well as alterations in enteric neuron serotonin release and uptake, indicating microbial-derived serotonin is an important player in peripheral serotonin signaling(91). Gastrointestinal motility is related to a host of neurological disorders including depression and anxiety, and may be a contributing factor to seizure-susceptibility(92,93).

Although no studies have currently directly assessed the role of gut microbe-production of serotonin on seizure-susceptibility, a growing body of evidence suggests that the gut microbiome is involved in cerebral serotonin production. Germ-free rodents have increases in hippocampal serotonin compared to rodents with normal microbiota. These changes occur in male mice but not

female mice, indicating a sex-dependent effect of gut microbiome alteration on hippocampal serotonin levels(94). Additionally, germ free mice have increased cortical serotonin levels compared to conventionalized mice(95), denoting that bacterial metabolism of tryptophan may compete with brain production of serotonin. In contrast, probiotic studies indicate that serotonin precursor tryptophan levels are increased in rodents following ingestion of *Bifidobacteria*, show that probiotic therapeutics could be beneficial in increasing CNS serotonin levels(96). Probiotic kefir supplementation can also increase colonic serotonin levels and decrease stress behaviors in rodents(72). Although gut-derived serotonin does not cross the BBB, other mechanisms of gut-brain communication may be involved in serotonin's CNS effects. The efficacy of SSRIs can be abolished through blocking vagal signaling following oral SSRI treatment, indicating that SSRI therapy relies on vagal gut-brain signaling(97). Future studies on microbial-derived serotonin are required to understand the role of microbial neurotransmitter production on CNS disease.

Dopamine

Dopamine, a precursor to norepinephrine and epinephrine, is a neurotransmitter largely involved in movement and reward pathways in the CNS. Dopaminergic signaling is the target of therapeutics in multiple neurological diseases such as Parkinson’s Disease (PD), schizophrenia, and addiction(98–100). In epilepsy, stimulation of the dopaminergic pathway has a complex relationship with seizure-susceptibility, eliciting both pro-epileptic and antiepileptic effects depending on the stimulation of D1 or D2 dopaminergic receptors(101,102). Adult patients with epilepsy have reduced expression of D2 receptors and increased expression of D1 receptors, indicating alterations in dopaminergic signaling in patients with epilepsy(103). Children with epilepsy likewise have altered dopamine availability in their prefrontal cortex(104).

Like serotonin, dopamine is also produced in the gastrointestinal tract and is important for gastrointestinal motility(105) as well as regulating inflammation in the periphery(106). Dopamine in the gastrointestinal tract can be produced by gut epithelial cells(105), some immune cells(107), and gut microbiota species including *Bacillus* sp., *Escherichia coli*, *Hafina* alvei, and *Klebsiella pneumoniae*(108). Accordingly, peripheral dopamine is reduced in germ-free mice(109). Microbiome production of dopamine metabolites is correlated with increased mental quality of life in healthy individuals(110). Probiotic therapy can decrease dopamine metabolism disorder symptoms in patients with ASD(111). In turn, probiotic therapy with *Lactobacillus reuteri* improves behavioral symptoms in multiple rodent models of ASD, primarily through alterations in oxytocin and dopaminergic synaptic signaling. However, these effects are abolished following a vagotomy, indicating that vagus-nerve signaling, not microbial production of dopamine, is responsible for the beneficial effect of probiotics on ASD(112).

Although the gut microbiota can synthesize neurotransmitters that act locally on enteric neurons, most of these signaling molecules cannot cross the BBB and have no direct effect on the CNS. It is likely that microbiome-related effects on CNS neurotransmitter levels are due to alternate gut-brain axis pathways including vagus-nerve signaling or immune-brain crosstalk. Future work on the mechanistic role of microbial-derived neurotransmitters is necessary to elucidate the effect of these microbial metabolites on seizure-susceptibility and epilepsy.

Short chain fatty acids

Colonic bacterial fermentation of dietary fiber leads to the production of fatty acids less than 6 carbon atoms in length, termed SCFAs, the most common of which are acetate, butyrate, and propionate(113). There is a large body of evidence on the beneficial health effects of microbial-derived SCFAs(114–116), and the role of SCFAs in the gut-brain axis has been

extensively reviewed(117–121). SCFA levels are altered in patients with neurological disorders such as ASD, Rett syndrome(122,123), cerebral palsy and epilepsy(124), indicating a correlational relationship between SCFA levels and neurological diseases including epilepsy. Anti-epileptic drug valproic acid (VPA) can alter microbial production of SCFA's(125). However, VPA did not alter serum or fecal SCFA levels in patients with epilepsy, although VPA led to side-effects hypothesized to be related to SCFA content(126). In contrast, ketogenic diet therapy alters serum SCFA levels and is associated with improved clinical outcomes in patients with mild cognitive impairment(127), which is of interest as ketogenic diet therapy has been shown to be beneficial in patients with refractory epilepsy(128).

SCFAs can cross the BBB and are detected in micromolar levels in cerebrospinal fluid (117,129), making them an interesting therapeutic target in the gut-brain axis in epilepsy. In the CNS, SCFAs are important in the development and function of microglia(130), alter hypothalamic GABAergic and glutamatergic transmission(131), and alter epileptiform neuronal activity in neurons from patients with chronic seizures(132). Butyrate is a histone deacetylase (HDAC) inhibitor and affect CNS disease outcomes in both pediatric and adulthood insults through mediating neuroinflammation as well as BDNF signaling(133,134). In contrast, propionate may act in the CNS through lowering neuronal pH, which may hyperpolarize neuronal cell membranes causing neurons to be hypoexcitable(132). In rodent models, treatment with either propionate or butyrate reduces seizure burden following PTZ injection(135–137). Treatment with sodium butyrate additionally reduces seizure burden in a kindling model of seizure-genesis(138), and reduces absence seizures in a genetic model of absence epilepsy(139). Importantly, sodium butyrate therapy reduced seizure burden in a case study in a patient with intractable glycosylphosphatidylinositol (GPI)-deficiency related seizures(140). Taken together, these

findings indicate SCFAs are promising therapeutics targets in seizure-susceptibility and epilepsy. However, intracerebral propionate injection induces epileptiform spiking and behavioral seizures in rats, as well as ASD-like behaviors, indicating that dosage and route of administration can shift the effect of SCFAs from antiepileptic to pro-epileptic(141). More research on dose, mechanism of action, and route of administration is necessary to further elucidate the possible beneficial effect of SCFAs in epilepsy.

Lactate

The brain is the most metabolically demanding organ in the human body, accounting for 20% of the body's total energy expenditure. Although glucose metabolism is the primary energy source in the brain, astrocytic production and shuttling of lactate has recently been highlighted as important in the maintenance of brain energy metabolism(142,143). Additionally, astrocytic-release of lactate is essential for the formation of long-term memory(144), indicating a role for lactate in the CNS outside of energy metabolism.

Although lactate can be produced in the brain, it is also acquired through diet as well as through microbial fermentation of dietary fibers. Probiotic therapy with lactic-acid-producing bacteria has been shown to improve neurological outcomes following traumatic brain injury (TBI)(145) and neuroprotective against PTZ-induced seizures(49). Additionally, exogenous lactate reduced epileptiform activity *in vitro* in neurons from patients with epilepsy(132). However, there is no direct evidence in vivo that lactate produced by the gut microbiota can alter seizure activity. Peripheral lactate can cross the BBB (146) and can alter neuronal activity through binding to G-protein-coupled receptor 81 (GPR81)(147). Lactate is also hypothesized to alter HDAC activity in the CNS, which has been implicated in changes in gene expression associated with seizure-susceptibility(138,148). In seizures and hypoxic conditions, there is a buildup of

excess lactate in the extracellular space which is thought to be important for sustained neuronal hyperexcitability in these pathologies(142,149). Targeting lactate dehydrogenase has been posited as a novel mechanism of antiepileptic drugs(150). More studies are necessary to elucidate the potential of targeting lactic-acid producing bacteria as a novel therapeutic in epilepsy.

Vitamins

The human body cannot produce many essential vitamins and minerals and relies on dietary consumption as well as microbial production for complete nutrition. Gut microbes can produce a variety of essential vitamins including B and K vitamins(151). Although the benefit of vitamins in epilepsy treatment is up for debate(152), there is mounting evidence that vitamin supplementation can improve seizure burden in patients depending on the underlying disease etiology(153).

Folate (B9)

Folate, or vitamin B9, is a B vitamin found in some vegetables that can also be produced in the gut microbiome by a variety of *Lactobacilli* and *Bifidobacteria*(154). Vitamins like folic acid are actively transported across the BBB(155). In epilepsy, low plasma folate levels are associated with psychosis in patients with epilepsy compared to patients with epilepsy that do not experience psychosis(156,157). Additionally, patients with epilepsy that experience depression symptoms have lower plasma folate than patients with epilepsy that do not experience depressive symptoms(158). In rodents, serum folate concentration is reduced in rats exposed to the pilocarpine model of seizure-induction which exhibits spontaneous recurrent seizures compared to controls(159). Folic acid in conjunction with the AED topiramate (TPM) can reduce PTZ-induced seizures in rats as well as reduce hippocampal cell death in comparison to TPM treatment alone(160). Furthermore, maternal supplementation that includes folate leads to a reduction in

absence epilepsy in rodent offspring of folate-treated dams(161). However, in humans, a clinical trial revealed that folate therapy did not reduce seizure burden or related cognitive dysfunction in patients with epilepsy, casting doubt on the benefit of folate-related therapy(162).

Pyridoxine (B6)

Pyridoxine, or vitamin B6, is found in foods such as fish and sweet potato, and can also be produced in the gut microbiome by organisms such as *Bacteroides fragilis* and *Bifidobacterium longum*(163). Vitamin B6 is reduced in the serum of patients with nodding syndrome(164), and vitamin B6 metabolism is altered in pediatric patients with epilepsy(165). In rodents, cortical vitamin B12 concentrations were lower in seizure-prone rats compared to seizure-free rats(166), and pyridoxine deficiency can lead to alteration in cortical GABA levels and behavioral seizures. Importantly, these deficits can be reversed through pyridoxine supplementation(167). In humans, a high dose of vitamin B6 shows preliminary efficacy in treating infantile spasms(168). Additionally, combination therapy of valproic acid and vitamin B6 reduced seizure-burden, but standard of care adrenocorticotropic hormone therapy is more effective(169). More studies must be done to elucidate the promise of vitamin B6 therapeutics.

Biotin (B7)

Biotin, or vitamin B7, is produced by *Bacteroides* bacteria and is metabolized in the body from dietary sources including dairy, nuts and seeds(163,170). In rodents, biotin deficiency leads to increased seizure severity and reduced seizure latency in a kindling model of seizure-susceptibility. Furthermore, biotin deprivation *in vitro* alters neuronal cell death following inflammatory challenge via avidin(171). Seizures also occur in pediatric patients with biotin deficiency(172), and biotin therapy has been shown to reduce seizure burden in two small cohorts

of children with biotinidase deficiency-associated seizures(173,174). Taken together, these findings suggest that B7 supplementation may improve seizure-burden in pediatric epilepsy patients depending on the underlying disease etiology.

Cobalamin (B12)

Bacteria including *Lactobacillus plantarum* and *Bifidobacterium animalis* express gene pathways to produce Cobalamin, or vitamin B12(163). Vitamin B12 can also be acquired through dietary sources such as animal products including eggs and milk. In humans, cobalamin deficiency is associated with infantile spasms(175), and patients with cobalamin deficiency present with seizures(176). In rodents, intracranial injection of cobalamin can decrease epileptiform activity in rats with penicillin-induced seizures(177). Additionally, treatment with a mixture of thiamine, pyridoxine, and cobalamin reduced seizure severity following kainate injection(178). In some studies, AED use in epilepsy patients is associated with a reduction in serum cobalamin, and supplementation with cobalamin can prevent AED-induced arterial damage (hyperhomocysteinemia)(179–181). These studies suggest that supplementation of vitamin B12 alongside AED therapy may reduce side-effects in patients with epilepsy.

Riboflavin (B2)

Riboflavin, or vitamin B2, is found in animal products and green vegetables but is also produced in the gut microbiome by species such as *Lactobacillus fermentum*(163). Children with epilepsy have decreased nutrient intake of microbiome-associated vitamins including riboflavin, possibly due to differences in eating habits(182). Adults with epilepsy have alterations in serum levels of riboflavin, which may be due to AED interactions with B vitamins(183). Like cobalamin, riboflavin has been shown to improve AED-induced side effects including

hyperhomocysteinemia(184). However, more research must be done to further examine the role of riboflavin, and specifically microbial-derived riboflavin, in seizure susceptibility and epilepsy.

Vitamin K

Vitamin K is found in animal products and is also produced by bacteria including *Escherichia coli* and *Streptomyces*(185). Serum levels of microbial-produced vitamin K is associated with cognitive function in patients with Alzheimer's disease(186). Vitamin K analogues show promise as novel antiepileptic therapeutics in rodent and zebrafish models of epilepsy(187). In contrast, however, vitamin K levels are higher in children with febrile seizures compared to age matched controls(188), indicating a complex relationship between vitamin K levels and neurological disease.

Despite growing interest in the gut microbiome as a therapeutic target in neurological disease, and the role of B and K vitamins in epilepsy and seizure-susceptibility, there is a paucity of experimental research on gut-microbe derived vitamins in seizure models. Although the link between gut microbe production of vitamins and outcomes in epilepsy remains to be elucidated, there is promising evidence that probiotic therapies may result in increased vitamin production and improved nutrition in patients.

S-equol

Polyphenols are micronutrients found abundantly in plants that can be obtained through some plant-based foods. Polyphenols consist of over 8000 structurally diverse plant metabolites with widespread biological significance. Like vitamins, dietary polyphenols are associated with positive health effects, however, new evidence suggests that many of these beneficial health outcomes may be associated with downstream metabolites produced by gut microbes(189). The

gut microbiome processes a multitude of polyphenols into metabolites that act systemically in the host. Phytoestrogens are chemicals produced by plants that may bind to estrogen receptors. Microbial conversion of phytoestrogens from soy isoflavones is one of the most well-defined processes by which the gut microbiome produces bioactive metabolites including S-equol(190).

S-equol is a gut microbial metabolite of dietary isoflavone daidzein, which can be found in soy and other plant products, that is highly permeable in the gut and the BBB(191). Unlike previously discussed metabolites including neurotransmitters, lactate, and vitamins, S-equol is solely produced by the gut microbiome, and thus germ-free mice have non-detectable levels of S-equol in the periphery(192). A growing list of bacteria that produce equol includes members of family *Coriobacteriaceae*, *Bifidobacterium* sp., and *Clostridium* sp.(193). Although bacteria exclusively produce the S-enantiomer of equol, chemically synthesized R-equol and racemic equol mixtures both have biological effects(194). Equol supplements have been shown in the clinic to reduce hot flashes in menopausal women, likely due to the high binding affinity of equol to estrogen receptor β(195). In menopausal women, S-equol supplements reduced hot flashes which was likely due to the high binding affinity of S-equol to estrogen receptor β(196), and S-equol supplements may prevent cardiovascular disease in obese patients(197). Equol-production status is also associated with improved benefit following isoflavone treatment in AD, and the role of S-equol in AD is the subject of ongoing clinical trials(198).

S-equol is increasingly implicated as beneficial in neurological disease. Rats treated with equol exhibit less anxiety and depressive behaviors compared to untreated rats(199). S-equol has also been found to have cytoprotective and neuroprotective effects by decreasing cytotoxicity in human neuroblastoma cells(200), to have antioxidant effects(201), to reduce nitric oxide that contributes to inflammation(202), enhance anti-inflammatory response, and cell oxidative damage

protection(203). In cultured astrocytes, equol induces cell proliferation and migration as well as increases F-actin rearrangements, which indicates that equol may enhance cerebral development(204). A S-equol intake in mice causes a reduction in phosphorylation of the NR2B AMPA receptor subunit responsible for plasticity in the Shaffer collateral CA1 synapse. This results in a decrease in long term potentiation (LTP) in the ventral hippocampus and decreased contextual fear memory and plasticity in mice(205). HIV rat models have also been able to provide further insight on equol's restorative properties. S-equol improved stimulus-response learning, pre-attentive processes, and perceptual sharpening back up to control levels in HIV-1 Tg rats(206). Increased dose intake of S-equol improves learning and sustained attention in HIV-1 Tg rats(207). *In vitro*, S-equol can reduce glutamate excitotoxicity in cultured hippocampal neurons(208), and S-equol is neuroprotective in neurons exposed to hypoxic conditions(209), indicating a possible neuroprotective effect in epilepsy neuropathology. Overall, S-equol is a promising novel target in the gut-brain axis in epilepsy, and more research is necessary to elucidate the possibility of S-equol as a therapeutic target in seizure-susceptibility.

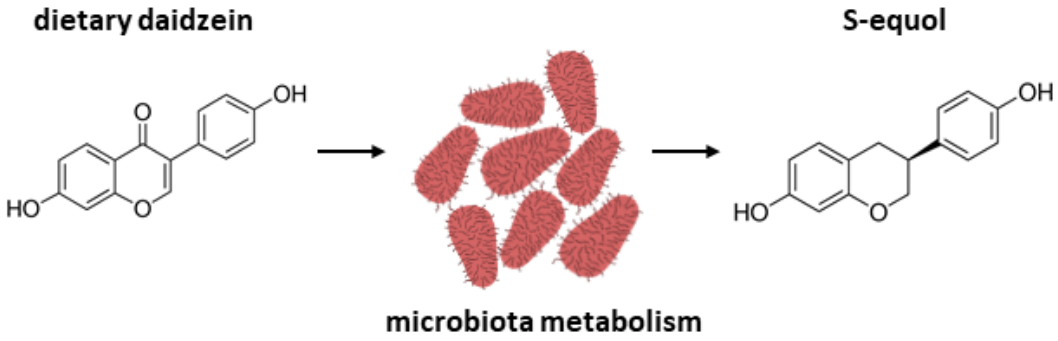
Conclusions

Epilepsy is a complex neurological disorder with multiple underlying etiologies that is also highly comorbid with a variety of neurological, immune, and metabolic diseases. Although there is a large body of evidence correlating microbiome alteration and outcomes in epilepsy, there are very few proposed mechanisms by which the gut microbiome influences CNS disease. Gut metabolites such as neurotransmitters, SCFAs, lactate, vitamins, and equol may act as key mechanistic players in the gut-brain axis. There is an unmet need for novel AEDs with mechanisms of action outside of synaptic signaling, and this review presents current knowledge of gut

metabolites and their involvement in epilepsy and highlights S-equol as a novel therapeutic target in seizure-susceptibility and epilepsy.

Figures

Figure 1


dietary daidzein
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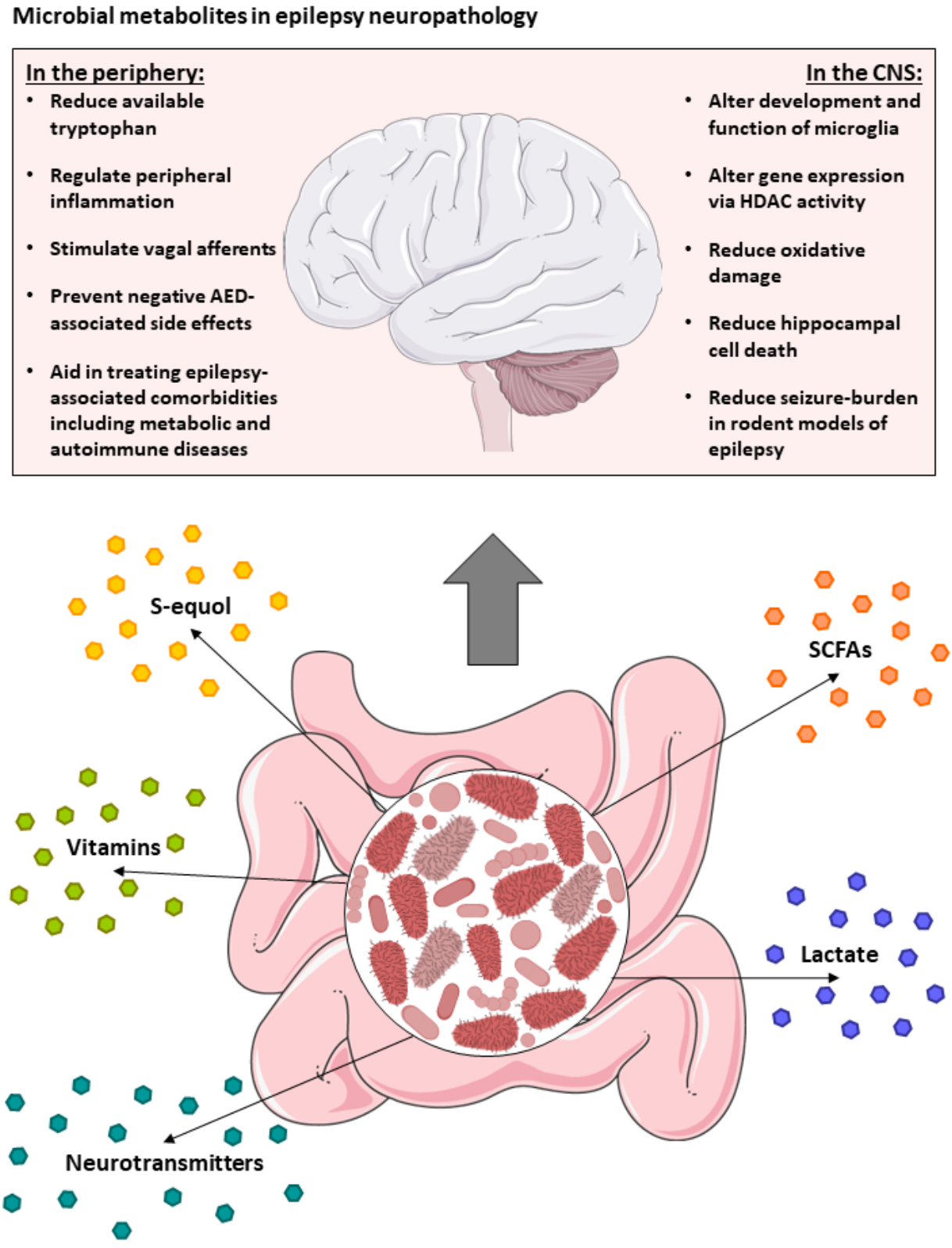
Graphical abstract



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Chapter 2:

Gut metabolite S-equol ameliorates hyperexcitability in entorhinal cortex neurons following TMEV-induced acute seizures

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This chapter is currently under review at Epilepsia. AG, DP, SC designed research question and project. AG, KT, RG were responsible for bioinformatics pipeline development and execution. AG, JT, DS were responsible for data analysis. AG collected experimental data. AG, KT, DS, SC drafted text and figures. All authors contributed to text and figure revisions.

Summary and Keywords

Objective

A growing body of evidence indicates a potential role for the gut-brain axis as a novel therapeutic target in treating seizures. Intracranial injection of Theiler's murine encephalomyelitis virus (TMEV) leads to the development of acute seizures that are difficult to treat with several current antiepileptic drugs (AEDs). The present study sought to characterize the gut microbiome in TMEV-induced seizures, and to evaluate the effect of microbial metabolite S-equol on neuronal physiology as well as TMEV-induced neuronal hyperexcitability *ex vivo*.

Methods

We infected C57BL/6J mice with TMEV and monitored the development of acute behavioral seizures 0-7 days post infection (dpi). Fecal samples were collected at 5-7 dpi and processed for 16S sequencing and bioinformatics were performed with QIIME2. Finally we conducted whole-

cell patch-clamp recordings in cortical neurons to investigate the effect of exogenous S-equol on cell intrinsic properties and neuronal hyperexcitability.

Results

We demonstrated that gut microbiota diversity is significantly altered in TMEV-infected mice at 5-7 dpi, exhibiting separation in beta diversity in TMEV-infected mice dependent on seizure phenotype and lower abundance of genus *Allobaculum* in TMEV-infected mice regardless of seizure phenotype. In contrast, we identified specific loss of S-equol-producing genus *Adlercreutzia* as a microbial hallmark of seizure phenotype following TMEV infection. Electrophysiological recordings indicated that exogenous S-equol alters cortical neuronal physiology. We found that entorhinal cortex (ECTX) neurons are hyperexcitable in TMEV-infected mice and exogenous application of microbial-derived S-equol ameliorated this TMEV-induced hyperexcitability.

Significance

Our study presents the first evidence of microbial-derived metabolite S-equol as a potential mechanism for alteration of TMEV-induced neuronal excitability. These findings provide new insight for the novel role of S-equol and the gut-brain axis in epilepsy treatment.

Keywords: Gut-brain axis, seizures, TMEV, S-equol, electrophysiology

Key Points

- TMEV infection in C57BL/6J mice leads to altered gut microbiota at 5-7 dpi.

- Loss of S-equol-producing bacteria is a microbial hallmark of seizure phenotype following TMEV infection.
- Exogenous S-equol alters cortical neuronal physiology in a concentration and time-dependent manner.
- ECTX neurons from TMEV-infected mice are hyperexcitable compared to PBS-injected controls, and exogenous application of S-equol ameliorates TMEV infection-induced ECTX hyperexcitability.

Introduction

Epilepsy is one of the most common chronic neurological disorders worldwide, with a global incidence of ~5 million people each year(1). People with epilepsy suffer from social stigma, debilitating comorbidities, and premature death(1). The most common cause of epilepsy worldwide is central nervous system (CNS) infection, however there are few anti-epileptic drugs (AEDs) that specifically target infection-induced seizures. This gap in available treatments is likely because viral infection-induced epilepsy is difficult to model in rodents due to high mortality rates among infected rodents(2,3). Work in the last decade has highlighted Theiler's murine encephalomyelitis virus (TMEV) as a low-mortality model of viral-induced epilepsy(4,5). Intracranial TMEV injection leads to temporal lobe epilepsy (TLE)-like neuropathology, widespread astrogliosis and microgliosis in cortical and limbic regions, and ictogenesis peaking at 5 days post infection (dpi) in ~50% of adult C57BL/6 mice(4). Viral infections like TMEV can lead to seizure-genesis by directly killing neurons or via increased cerebral inflammation(6), however, it is still unclear what molecular mechanisms are involved in TMEV-induced seizures.

The bacteria that inhabit the mucosal surfaces of the body, termed the microbiota, are known to modulate a wide variety of peripheral diseases and have recently been implicated in a

multiple neural disorders(7–9). The gut microbiota can influence the CNS directly through vagus nerve signaling as well as indirectly through circulating metabolites and cytokines produced via microbiota-immune system interaction(10–12). In epilepsy, adults and children with drug-resistant seizures have altered microbiomes compared to adults with seizures that respond to drug therapy and age-matched healthy controls, respectively(13,14). Additionally, probiotic supplementation has been shown to improve seizure burden in adults with seizures that are refractory to drug treatment(7). In rodents, gut microbiome alteration alone can reproduce the anti-seizure effects of the ketogenic diet(15), and microbiome alteration decreases seizure susceptibility in rodents with chronic stress(16). Most of the current research findings are highly correlative showing that changes in the gut microbiota can affect seizure outcomes. Therefore, there is a gap in our knowledge of specific mechanisms by which gut microbes modulate seizures, which in turn impedes efforts to develop potential gut-brain axis therapeutics in epilepsy.

S-equol is a metabolite produced exclusively by the metabolism of soy isoflavones by the gut microbiota(17). It is highly available in serum in its free form(18) and can cross the blood brain barrier(19). S-equol is known to bind with high affinity to estrogen receptor β (ESR2) which are widely expressed in the brain (20)(21). In Alzheimer's, S-equol is currently being studied to improve mitochondrial function by activating estrogen receptors(22). Oral supplementation of S-equol improves cognitive performance in a rat model of HIV infection(23), which is of note as the prevalence of epilepsy in patients with HIV is significantly higher than that of the general population(24). Additionally, S-equol is a potent antioxidant(25,26) and antioxidants can attenuate seizures in some models of epilepsy(27). S-equol was reported to be protective against oxidative stress by downregulating neuronal apoptosis and increasing neurite growth(28). Importantly, S-equol is also neuroprotective against glutamate excitotoxicity in cultured hippocampal

neurons(29), indicating a potential role in protecting against hippocampal excitotoxicity and cell death commonly found in epilepsy patients and rodent models(30–32). Together these studies demonstrate that the microbial-derived metabolite can affect the CNS, and may therefore be relevant to neurological function and disorders. Although multiple studies point to neuroprotective effects, no studies have yet examined the role of microbial-derived S-equol in neuronal physiology.

In the present study, we show significant alterations in the gut microbiome of C57BL/6J mice following TMEV infection. Additionally, we identify key S-equol-producing microbial taxa associated with TMEV-induced seizure phenotype relative to TMEV-infected mice that do not display seizures. We demonstrate the ability of exogenous S-equol to alter neuronal physiology in a concentration and time-dependent manner. Lastly, we also show marked increase in excitability of ECTX neurons in TMEV-infected mice, and demonstrate the ability of microbial-derived S-equol to ameliorate TMEV-induced hyperexcitability. Collectively, our results indicate a central role for a microbial-derived metabolite in regulating neuronal excitability and propose a mechanistic link between the gut-brain axis and TMEV-induced seizures.

Methods

2.1 Animals

Animals were housed and handled according to the guidelines of the National Institutes of Health Committee on Laboratory Animal Resources. Prior approval of the Virginia Polytechnic Institute and State University Institutional Animal Care and Use Committee was obtained for all experimental protocols. All efforts were made to minimize animal pain. C57BL/6J mice (stock #000664) aged 5-6 weeks were purchased from the Jackson Laboratory (Bar Harbor, ME, USA). Mice were allowed to acclimatize for at least 7 days prior to experiments. Mice were provided chow (Envigo 2918) and water *ad libitum* and kept in a facility providing 12 hours light/dark cycle.

TMEV-infected mice were housed separately from PBS-injected mice due to the infectious nature of the virus, however, all TMEV-infected mice were housed together regardless of seizure phenotype. All the experiments included almost equal numbers of male and female mice.

2.2 TMEV Infection and Seizure Monitoring

6-8 week old C57BL/6J mice were anesthetized with isoflurane and injected intracortically with 2 x 10^5 PFU (plaque-forming units) of the Daniels strain of TMEV (DA-TMEV) or phosphate-buffered saline (PBS) as previously described(33). Acute behavioral seizures were induced twice daily 2-7 days post infection (dpi) by briefly shaking the cage to agitate the mice. Behavioral seizure intensity was graded using the following modified Racine seizure scale: Stage 1 - mouth and facial movements; Stage 2 - head nodding; Stage 3 - forelimb clonus; Stage 4 - forelimb clonus and rearing on hind limbs; Stage 5 - forelimb clonus, rearing, and falling; and Stage 6 - intense running, jumping, repeated falling, and severe clonus(34). Seizure phenotype was defined as the presence of at least 1 handling-induced behavioral seizure of any intensity during twice daily monitoring between 2-7 dpi.

2.3 Fecal Collection and DNA isolation

Fecal pellets were dissected from the distal colon of TMEV-infected and PBS-treated control mice 5-7dpi using clean tweezers immediately following cervical dislocation. Pellets were stored in DNA-free Eppendorf tubes (Cat. No. 022600028) at -80C.

Mouse fecal DNA was isolated using the DNA Fecal/Soil Microbe Miniprep Kit (Zymo Research; Irving, CA) according to manufacturer's instructions. One fecal pellet was used for each isolation. DNA was quantified and samples were stored at -80C until sequenced.

2.4 16S Sequencing and Analysis

The universal primers 515F and 926R were chosen to amplify the V4–V5 region of the 16S rRNA gene following the Earth Microbiome Project protocol (https://www.earthmicrobiome.org/). The polymerase chain reaction (PCR) reaction mixture included 13.0 µL of PCR-grade water, 1.0 µL of template DNA, 10 µM of each primer, and 10.0 µL of 5PRIME HotMasterMix (2x) (Quantabio, Beverly, MA). Samples were amplified in duplicate under the following thermocycler conditions: 94°C for 3 min for initial denaturing, then 35 cycles of 94°C for 45 s, 50°C for 60 s, and 72°C for 90 s. A final elongation step occurred at 72°C for 10 min followed by a hold at 4°C. After pooling duplicates, all amplicons were visualized on a 2% agarose gel and quantitated on a Qubit fluorometer (FisherScientific, Hampton, NH). Normalization was performed based on Qubit results, and amplicons were purified on a Pippin Prep (Sage Science, Beverly, MA) targeting a 520bp range. This pool was then quantitated using quantitative PCR. Amplicons were loaded at 9.5 pM and sequenced using the MiSeq v3 600-cycle kit on the Miseq platform (Illumina, Inc., San Diego, CA). Sequencing yielded 19.6 million paired-end reads. Quality scores were within Illumina specifications.

Sequencing data was analyzed with QIIME2 v2019.10(35). Forward and reverse reads were quality filtered, trimmed, and joined with DADA2, DADA2 was also used to denoise joined reads to amplicon sequence variants (ASVs) (via q2-dada2)(35–37). ASVs were aligned to a phylogenetic tree using an insertion method to the greengenes reference database (via q2-fragment-insertion-sepp)(35,37–41). Alpha and beta diversity metrics were calculated using samples rarified to 12909 sequences per sample (via q2-diversity)(35–52). Differential abundance was measured using ANCOM, and the features were filtered if they did not at least occur 2 times in 2 samples(35–37,49,50). Operational Taxonomic Unit (OTU) tables from QIIME were utilized to predict abundances of KEGG orthologs (KOs) and collapse KOs into KEGG pathways for

functional analysis. KEGG pathways were analyzed and graphed using STAMP(53). Linear Discriminant Effect Size Analysis (LEfSe) was performed with default parameters(54). OTU tables generated in QIIME were assigned LDA scores and graphed utilizing the Galaxy web application.

2.5 Slice Preparation

Coronal slices for naïve experiments were prepared as reported previously(55). Briefly, mice were decapitated and their brains were quickly removed and immersed in ice-cold cutting solution containing (in mmol/L): 135 N-methyl-d-glucamine (NMDG), 1.5 KCl, 1.5 KH2PO4, 23 mmol/L choline bicarbonate, 25 d-glucose, 0.5 mmol/L CaCl2, and 3.5 MgSO4 (Sigma-Aldrich). 300 μm coronal brain slices were made and recovered for 40-60 minutes in oxygenated recording solution in mmol/L: 125 NaCl, 3 KCl, 1.25 NaH2PO4, 25 NaHCO3, 2 CaCl2, 1.3 MgSO4, and 25 d-glucose at 32°C and maintained at room temperature before recordings. For horizontal slices for ECTX recordings, TMEV-infected or PBS-injected mice were anesthetized with ketamine/xylazine and transcardially perfused with ice-cold cutting solution containing (in mmol/L): 92 NMDG, 2.5 KCl, 1.2 NaH_2PO_4, 30 $NaHCO_3$, 20 HEPES, 25 D-(+)-glucose, 5 sodium-L-ascorbate, 3 sodium pyruvate, 0.5 $CaCl_2$, 10 $MgCl_2$ for 1.5 minutes before quickly extracting the brain. Horizontal brain slices (300 uM) were prepared by following the NMDG protective recovery method(56). Briefly, the brain slices were allowed to recover from slicing insult in the cutting solution at 32 °C for total 25 minutes. During this recovery period, sodium concentration of the incubation solution was gradually increased from about 40 mM present in the cutting solution to 128 mM by adding NaCl solution in the incubation chamber every 5 minutes. Subsequently, the slices were transferred to the holding aCSF containing (in mmol/L): 92 NaCl, 2.5 KCl, 1.2 NaH_2PO_4, 30 $NaHCO_3$, 20 HEPES, 25 D-(+)-glucose, 5 sodium-L-ascorbate, 3

sodium pyruvate, 2 $CaCl_2$, 2 $MgCl_2$ at room temperature. The slices were incubated in the holding aCSF for at least 1 hr before using them for electrophysiological recordings. The NMDG protective recovery method significantly improved the slice health and neuronal morphology (CITE Ting et al., 2018). All electrophysiological recordings were performed in the recording aCSF containing (in mmol/L) 125 NaCl, 3 KCl, 1.25 NaH_2PO_4, 25 $NaHCO_3$, 5 HEPES, 12.5 D-(+)-glucose, 2 $CaCl_2$, 2 $MgCl_2$ with either 1 uM S-equol (Cayman Chemicals) or equal DMSO concentration (<0.05%).

2.6 Electrophysiology

Individual brain slices were transferred to a recording chamber and continuously perfused (4 mL/min) with oxygenated recording solution. Whole-cell recordings were conducted using borosilicate glass capillaries (KG-33 glass, Garner Glass) and filled with internal solution containing (in mmol/L): 134 K-gluconate, 1 KCl, 10 HEPES, 2 mg-ATP; 0.2 Na-GTP and 0.5 ethylene glycol tetraacetic acid (EGTA). The pH was set to 7.3 with KOH, and the osmolality was measured (~290 mOsm/kg). All recordings were performed at 32 ± 1°C. Individual cells were visualized using a Zeiss Axioscope (Carl Zeiss) microscope equipped with Nomarski optics with a 40× water-immersion objective lens. Tight seals were made using electrodes with a 3–5-MΩ open-tip resistance. Signals were acquired from pyramidal entorhinal cortex cells with an Axopatch 1B amplifier (Molecular Devices), controlled by Clampex 11 software via a Digidata 1440 interface (Molecular Devices). Action potential threshold current and input-output curves were calculated as described previously(57). For the experiments involving S-equol, slices were pre-incubated in 1uM S-equol (Cayman Chemicals) for at least 2 hours before recordings.

2.7 Statistics

Alpha diversity values were calculated with observed features and evenness metrics. Beta diversity analyses were calculated with weighted and unweighted unifrac distance. LDA score for LEfSe analysis was performed on differential abundance tables generated with QIIME. Microbiome differences between experimental groups were assessed using ANCOM analysis and Kruskal-Wallis H tests with Dunn's multiple comparisons correction. All p-values for KEGG pathways are FDR adjusted. Statistical details of experiments are mentioned in the figure legends. Data are sufficiently normal distributed and variance within groups is sufficiently similar to be used for parametric tests when specified. Experimental designs with two treatment groups were analyzed by two-tailed unpaired *t* tests. Welch's correction was applied where variances of both the groups were statistically different. Experimental designs with more than two groups were analyzed using one-way ANOVA or two-way ANOVA followed by Tukey's or Sidak's post-hoc multiple comparison tests where specified. Data analysis was performed using GraphPad Prism 8.0, Microsoft Excel, and Origin 2016 (OriginLab).

Results

3.1 TMEV infection and seizure phenotype are associated with altered gut microbiota diversity

We first characterized the gut microbiota of C57BL/6J mice infected with TMEV. Typically, TMEV infection induces acute seizures 3-10 dpi in about 50-65% of infected mice(5). Analysis of fecal microbiota of TMEV-injected and PBS-injected aged matched mice at 5-7 dpi (Figure 1a), showed no difference in alpha diversity richness metrics (observed features) across any group (PBS mean = 143 ± 14.4, TMEV-No Seizure 140.5 ± 9.7, TMEV-Seizure 127.9 ± 10;

n.s., Kruskal-Wallis test; Figure 1b). Although, alpha diversity measures of evenness (Pieluo index) revealed a trend towards lower diversity in TMEV-infected mice with seizure phenotypes, compared to both PBS-injected controls as well as TMEV-infected mice which did not develop seizures, the result was not significant (PBS mean = 0.54 ± 0.03, TMEV No-Seizure 0.53 ± 0.03, TMEV-Seizure 0.44 ± 0.4; p=0.08., Kruskal-Wallis test; Figure 1c). Interestingly, beta diversity measured by weighted unifrac distance showed significant separation of all 3 experimental groups, indicating distinct communities of microbes across all three experimental conditions (p=0.001, PERMANOVA; Figure 1d). Taken together, these findings indicate that TMEV infection as well as seizure phenotype following TMEV infection are associated with altered gut microbiome diversity.

3.2 Selective alteration in microbial taxa in TMEV-infected mice

As diversity analyses revealed separation of all three experimental groups, we examined which taxonomies were altered in TMEV-infected mice. Regardless of experimental condition, the microbiomes of all mice in this study are dominated by Firmicutes and Bacteroidetes phyla (Figure 2 a). Although Firmicutes/Bacteroidetes ratio has been shown to be altered in some models of neurological disease, we found no difference across any experimental group (PBS mean = 2.33 ± 0.5, TMEV No Seizure 1.14 ± 0.3, TMEV Seizure 3.19 ± 0.8; n.s., Kruskal-Wallis test; Figure 2b). We next performed ANCOM analysis via QIIME2 to examine alterations in the composition of microbes across our experimental groups(50). Regardless of seizure phenotype, mice infected with TMEV display lower abundances of *Allobaculum*(PBS mean = 0.250 ± 0.071, TMEV No-Seizure 0.037 ± 0.018, TMEV-Seizure 0.019 ± 0.013; Reject null hypothesis, ANCOM; Figure 2c).

To further identify key alterations in microbial taxa following TMEV infection, the well-established feature analysis, LEfSe, an algorithm which employs linear discriminant analysis (LDA) to identify differential features among experimental groups was used(54). Figure 2d shows the clades that best describe PBS injected controls compared to TMEV infected mice. Again, loss of genus *Allobaculum* was identified as a key biomarker of TMEV infection regardless of seizure phenotype. Notably, LEfSe analysis further indicates loss of genus *Prevotella* in TMEV-infected rodents, mirroring published findings at acute timepoints in TMEV-infected SLJ mice(58). Additionally, TMEV-infection reduced the abundance of genus *Bifidobacterium*. Together these findings indicate that TMEV infection leads to specific alterations in the composition of the gut microbiota regardless of seizure phenotype.

3.3 Seizure phenotype following TMEV infection is associated with loss of key microbes

As we are interested specifically in the link between seizure phenotype and gut microbe alterations, we aimed to identify microbial biomarkers of seizure phenotype. LEfSe analysis of TMEV-infected mice stratified by seizure phenotype identified genus *Lactobacillus* in class *Bacilli* as well as *Streptococcaceae* bacterium *RF32* as biomarkers of seizure phenotype following TMEV infection (Figure 3a,b). In contrast, phyla Tenericutes, classes *Bacteroidia* and *Mollicutes,* and genera *Roseburia*, *Anaeroplasma*, *Ruminococcus*, and *Adlercreutzia* are identified as biomarkers of TMEV-infected mice that do not develop acute seizures. Currently there are few mechanistic links between gut microbiome populations and seizure susceptibility. Given that gut microbes produce a variety of metabolites known to have widespread CNS targets(59–61), we performed a thorough literature search to identify metabolites produced by the hallmark taxonomies identified in our LEfSe analyses. Interestingly, the majority of the taxonomies underrepresented in TMEV-

infected mice with seizure phenotypes contained genera associated with the production of the bacterial metabolite S-equol(62–68) (Table 1). Specifically, genus *Adlercreutzia* (in family *Coriobacteriaceae*) currently only contains one species: *A. equolifaciens*, a relatively understudied gut microbe known to produce S-equol from dietary daidzein(66). Considering that S-equol crosses the blood brain barrier(19) and has been shown to reduce glutamate excitotoxicity in cultured neurons(69), we next evaluated the effect of exogenous S-equol on neuronal physiology.

3.4 S-equol alters cortical neuronal physiology in naïve rodents

Few studies have previously examined the effects of exogenous S-equol *in vitro* at timescales appropriate for acute slice experiments(70). As 300 μM exogenous S-equol has been shown to acutely alter calcium signaling in GLUTag cells(71), we first examined the effects of 300 μM S-equol on cortical principal neuron firing properties and action potential threshold (Figure 4a, b). Whole cell patch-clamp recordings were obtained from visually identified layer 2/3 pyramidal neurons in naïve adult mice. Short current injections with 2 pA incremental increase in amplitude were used to determine changes in action potential current threshold. 30-minute exogenous application of high S-equol (300 μM) concentration increased the action potential firing threshold (ACSF = 193.111 ± 24.26 pA; 300 μM S-equol = 260.4 ± 29.08 pA; $p = 0.0102$, Student's paired *t* test; Figure 4a). Next, action potentials were elicited with 1s long current injections of increasing amplitude, and the effect of 300 μM S-equol was tested. We found that 300 μM S-equol decreased the number of action potentials following increasing current injection steps (ACSF vs. 300 μM S-equol, $p = 0.0001$, repeated measures two-way ANOVA with Bonferroni correction for comparisons; Fig. 4b) without altering the resting membrane potential (RMP) of cortical neurons (ACSF = -64.33 ± 5.2 mV, 300 μM S-equol = -60.01 ± 11.55 mV; n.s., Student's paired *t* test; Figure 4c).

Although *in vitro* literature on S-equol ranges in concentrations up to 300uM(71–73), *in vivo* S-equol treatment in mice has only been shown to lead to serum S-equol concentrations approaching 10 μM(74), we next examined the effect of 10 μM S-equol on neuronal physiology. A lower concentration of S-equol (10 μM), also increased the action potential firing threshold (ACSF = 232.5 ± 101.7 pA; 10 μM S-equol = 289.4 ± 101.3 pA; p = 0.0026, Student's paired *t* test; Figure 4d), as well as decreased the number of action potentials following increasing current injection, although to a lesser extent than high dose of S-equol (ACSF vs. 10 μM S-equol , p = 0.0003, repeated measures two way ANOVA with Bonferroni correction for comparisons; Figure 4e), but did not alter the RMP (ACSF = -68.11 ± 3.0 mV; 10 μM S-equol -66.8 ± 9.758 mV; n.s., Student's paired *t* test; Figure 4f). These results demonstrate concentration-dependent alterations in neuronal firing properties following 30 min exposure to exogenous S-equol .

As exogenous S-equol has previously been shown to exert a time-dependent effect *in vitro*(72), we lastly pre-incubated acute slices in 1 μM S-equol >2h to examine the effects of prolonged S-equol exposure at low levels. Indeed, pre-incubation of 1 μM S-equol increased the action potential firing threshold (ACSF = 130.6 ± 34.36 pA; 1 μM S-equol = 195.8 ± 71.42 pA; p = 0.0168, Student's *t* test; Figure 4g). Pre-incubation with 1 μM S-equol lead to a trend towards a decrease in the number of action potentials, but did not reach significance (ACSF vs. 1 μM S-equol, n.s., repeated measures two-way ANOVA with Bonferroni correction for comparisons; Figure 4h) and the RMP is also not changed (ACSF = -65.06 ± 2.2 mV; 1 μM S-equol = -68.39 ± 2.24 mV; n.s., Student's *t* test; Figure 4i). Together, these data demonstrate that S-equol alters cortical neuronal physiology in a concentration and time-dependent manner.

3.5 Microbial-derived metabolite S-equol ameliorates TMEV-induced ECTX hyperexcitability

As our microbiome data showed the loss of S-equol producing bacterial in TMEV-seizure mice and S-equol decreased basal neuronal activity, we set out to examine the effect of S-equol on neuronal excitability in TMEV-infected mice with seizures. Seizures are generally thought to be caused by a disruption of the excitation/inhibition balance in the brain, where neurons become hyperexcitable due to either increased excitatory signaling or decreased inhibitory signaling (or a combination of both). A large body of research correlates hyperexcitability of cortical neurons to seizure susceptibility(75–77). Although cortical astrogliosis has been documented in TMEV-infected mice(4), and limbic brain regions exhibit pronounced neuropathological changes and serve as a region of seizure initiation following TMEV infection(78,79), no study has examined electrophysiological properties of entorhinal cortex (ECTX) neurons following TMEV-induced seizures. Therefore, we first set out to establish the activity of ECTX neurons from TMEV-infected mice with seizures and PBS-injected controls.

Indeed, ECTX neurons from TMEV-infected mice with confirmed seizure phenotypes have decreased thresholds to fire a single action potential following current injection (Figure 5a). After finding that the pyramidal neurons of ECTX from TMEV-infected mice are intrinsically hyperexcitable during acute seizure period, we next tested the effects of S-equol by pre-incubating acute slices from TMEV-infected mice with confirmed seizure phenotypes and PBS controls in 1 μM S-equol. S-equol (1 μM) application in *ex vivo* brain slices significantly increased the action potential firing threshold of ECTX neurons from TMEV-infected mice (PBS = 174.35 ± 12.7 pA; TMEV-Seizure 113.25 ± 8.6 pA; TMEV-Seizure + 1 μM S-equol = 166.72 ± 13.6 pA; PBS + 1 μM S-equol = 226.7 ± 82.67 pA; p = 0.0001, one-way ANOVA with Tukey's multiple comparisons; Figure 5a). This effect is not due to changes in the RMP, as it was not different between neurons from TMEV-infected mice compared to controls (PBS mean = -61.5 ± 1.3 mV;

TMEV-Seizure = -62.9 ± 1.8 mV; TMEV Seizure + 1 μM S-equol = -61.2 ± 2.3 mV; PBS + 1 μM S-equol = -66.66 ± 4.76; n.s., one-way ANOVA; Figure 5b). Furthermore, TMEV-infection altered the input-output curve of ECTX neurons following current injection (Figure 5c). Neurons from PBS controls displayed typical input-output curves with increasing numbers of action potentials with increasing current injection. In contrast, neurons from TMEV-infected mice showed higher excitability by firing more action potentials at lower current injection compared to PBS control. In response to higher current injections (>150 pA), neurons from TMEV-infected mice displayed a depolarization block (Fig. 5c), a silent state of a neuron in response to excessive excitation(80). In contrast, 1 μM S-equol application prevented the depolarization block observed in ECTX neurons from TMEV-infected mice, and decreases the number of APs fired in neurons from PBS-injected mice at 120 pA current injection (PBS vs. TMEV vs. TMEV + S-equol vs. PBS + S-equol, P = 0001, repeated measures two-way ANOVA with Bonferroni correction for comparisons, Figure 5c). Taken together, these findings indicate that S-equol can ameliorate TMEV-induced hyperexcitability in ECTX neurons, which suggests that the effects of S-equol on TMEV-induced seizures and epilepsy should be further evaluated.

Discussion

In the present study, we show significant alterations in the gut microbiome of C57BL/6J mice following TMEV infection. Additionally, we identify key microbial taxa associated with TMEV-induced seizure phenotype. We demonstrate the ability of exogenous S-equol to alter neuronal physiology in a dose and time-dependent manner. Lastly, we also show marked alterations in neuronal physiology in the entorhinal cortex (ECTX) of TMEV-infected mice, and demonstrate the ability of microbial-derived S-equol to ameliorate TMEV-induced hyperexcitability. Collectively, our results indicate a central role for a microbial-derived

metabolite in regulating neuronal excitability and propose a mechanistic link between the gut-brain axis and virus-induced seizures.

In our study although the microbiomes of all experimental groups were comprised primarily of Firmicutes and Bacteroidetes phyla, ANCOM analysis revealed significant reduction of genera *Allobaculum* in the microbiomes of TMEV-infected mice. Interestingly, *Allobaculum* has been associated with 5-HT levels in a mouse model of depression(81), and was similarly reduced in a mouse model of schizophrenia(82), indicating a potential role for loss of *Allobaculum* in neurological disease. We additionally utilized LEfSe to further elucidate microbiome alterations in TMEV-infected mice compared to controls. Although no previous published work has examined the gut microbiome in infection-induced seizures, alterations in the gut microbiome of SJL mice infected with TMEV have been previously reported(58). TMEV brain infection in SJL mice leads to multiple sclerosis (MS)-like symptoms rather than seizures(83). In the TMEV-induced mouse model of MS, viral infection leads to sustained alteration of the gut microbiome, consisting of reduction in *Alloprevotella* in the acute disease phase, *Akkermansia* and *Anaerotruncus* during disease progression, and *Streptococcus* in the chronic disease phase(58). Our results mirror these findings, as we show a similar reduction in *Prevotella* in TMEV-infected mice regardless of seizure phenotype. Notably, TMEV-infected mice with seizures show increased *Clostridia* genera as well as decreased *Ruminococcus* compared to controls, replicating findings in human patients with encephalitis-induced seizures(84). These results indicate a potential specific, translational link between encephalitis-induced seizures and gut-microbiota alteration. Additionally, our study highlights alterations in *Bifidobacterium* following TMEV-infection. *Bifidobacterium* probiotics are beneficial in models of rodent anxiety(85), and previous studies in TMEV-infected mice with seizure phenotypes show increased anxiety is associated with seizure phenotype(86). Importantly,

Bifidobacterium probiotics have been shown to reduce seizure burden in human patients as well as rodent models of epilepsy(87,88). Our findings underscore a potential role for *Bifidobacterium*-based probiotics as potential therapeutics in CNS infection-induced seizures.

LEfSe analysis additionally revealed loss of genus *Adlercreutzia* in TMEV-infected mice with seizure phenotypes. Genus *Adlercreutzia* currently only contains one species: *A. equolifaciens*, a bacterial species known to produce S-equol(66). S-equol is a gut microbial metabolite of dietary isoflavone daidzein, which can be found in soy and other plant products, that is highly permeable in the gut and the blood brain barrier(19). The neuroprotective effects of equol are well documented. *In vitro*, S-equol decreases cytotoxicity in human neuroblastoma cells(89), increases cell viability, enhances anti-inflammatory response, and protects against oxidative damage in cultured microglia(90,91). S-equol was also shown to reduce glutamate excitotoxicity in cultured neurons(69). In vivo, rats treated with S-equol exhibit less anxiety and depressive behaviors(92) and show increased dendrite arborization of Purkinje cells and neurite growth(93). Additionally, rodents treated with S-equol have altered plasticity in the Shaffer collateral CA1 synapse, have reduced LTP in the ventral hippocampus, and in turn display decreased contextual fear learning(94). Furthermore, in HIV rat models, S-equol treatment improves neurocognitive deficits(95), further lending support to the hypothesis that S-equol is neuroprotective in virus-associated and seizure-associated neural pathways.

Loss of CA1 hippocampal neurons and altered neuronal physiology in CA3 and DG neurons is a hallmark of TMEV-associated seizures(32). Additionally, there is widespread cortical astrogliosis following TMEV-injection, indicating the presence of cortical pathogenesis alongside classical hippocampal pathogenesis. The entorhinal cortex has long been a structure of interest in the pathogenesis of TLE(96–98). ECTX neurons are highly connected to the hippocampus(99–

102) are hyperexcitable in kainic-acid(103) and pilocarpine models of epilepsy(75,104), and serve as a model for testing anti-epileptic compounds *in vitro*(103). However, no study has examined electrophysiological properties of ECTX neurons following TMEV-infection. In the current study, we demonstrated that ECTX neurons from TMEV-infected mice with seizure phenotypes have a reduced threshold to fire an AP following current injection, and display prominent depolarization block at high levels of sustained current injection. Excitingly, we show that exogenous application of microbial metabolite S-equol (1uM) ameliorates TMEV-induced ECTX neuronal hyperexcitability.

We posit that S-equol may affect neuronal physiology through a variety of mechanisms. S-equol binds with high affinity to estrogen receptor β (ESR2)(20), ESR2 is expressed widely in the brain(21) and cortical ESR2 expression is similar between male and female rodents(105,106). Estradiol, which binds to both estrogen receptor α and ESR2, has been shown to decrease seizure susceptibility in a kainic acid model of epilepsy(107), and estrogen supplementation is neuroprotective in hippocampal cells in rodents after status epilepticus(108). Alternatively, S-equol alters the activity of high-conductance calcium-activated potassium channels (BKs) in smooth muscle cells *in vitro* by binding to the beta1 subunit(109). As both loss and gain of function mutations of BK channels can lead to seizures and epilepsy in humans(110), it is also possible that S-equol acts via regulation of BK channel activity. Future identification of the mechanism by which S-equol alters neuronal physiology will enhance the translational potential of S-equol as an anti-seizure therapeutic.

TLE patients often experience a disease progression consisting of an initial insult(111), followed by a highly variable quiescent period during which massive cellular alterations occur(112) and finally the onset of chronic seizures(113). Many rodent models of epilepsy follow

a similar progression of acute injury, during which seizures may present, followed by a period of epileptogenesis which leads to the development of spontaneous recurrent seizures(5,114–117). Probiotics have been shown to be beneficial in rodent models during the initial insult phase of TLE, reducing the occurrence of acute seizures following Pentylenetetrazole (PTZ)-induced kindling(88) or 6-Hz stimulation(15). Microbiota transplant is additionally protective in rodents against PTZ-induced seizures following chronic stress(16). Using a genetic rat model of absence seizure, a recent study showed altered beta diversity of the gut microbiome prior to seizure phenotype(118). Regarding chronic seizures, adults with drug resistant chronic seizures have altered microbiomes, and probiotic supplementation has been shown to improve seizure burden in adults with refractory chronic seizures(14,87). Probiotic therapy has also been shown to reduce chronic seizure burden in a *Kcna1*-/- rodent model of epilepsy(15). The current study provides evidence for microbiome alteration and potential therapeutic benefit of exogenous S-equol during the acute phase of the TMEV model of virus-induced epilepsy. Given that there is evidence for microbiota involvement in chronic seizures in both rodents and humans, it is possible that the beneficial effects of S-equol will also be present during epileptogenesis and chronic epilepsy phases. More studies are necessary to elucidate the involvement of the gut microbiota and S-equol beyond acute seizure periods.

Studies in rats indicate that serum equol is highly associated with phytoestrogen content of rodent chow(18). We performed a preliminary study to assess equol levels in our facility in TMEV-infected mice compared to PBS-injected controls. As we used male and female mice in our microbiome data, we also assessed differences in male and female serum equol. Interestingly, our preliminary data show trends ($p=0.068$) in reduction in serum equol levels in TMEV-infected mice that experience seizure phenotypes compared to PBS-injected controls at 3 and 5 dpi, followed by

a significant increase in serum equol levels in TMEV-infected mice compared to controls at 7 dpi (Figure S1a). Unexpectedly, serum levels of equol are reduced in female mice compared to male mice regardless of experimental condition (Figure S1b). Future studies are warranted to further examine the sex-specificity of serum equol levels in larger, more appropriately powered datasets.

As we present trends in reduction of serum S-equol in relationship to the onset of acute seizures following TMEV-infection, as well as data suggesting S-equol has antiepileptic properties *in vitro*, we next performed a pilot study to assess the potential of S-equol as an antiepileptic drug *in vivo*. Published in vivo pharmacology experiments utilizing equol vary widely in terms of route of administration, equol dose, dosing paradigm, and specificity of S-equol or racemic equol (Table S1)(19–26). In the present study, we injected S-equol dissolved in DMSO suspended in 4% carboxymethyl cellulose intraperitoneally (i.p.) once daily at a dose of 10mg/kg for 3 days prior to TMEV injection as well as throughout seizure monitoring (0-7 dpi) (Figure S2a). S-equol treatment was well tolerated, and S-equol treated mice experienced similar weight fluctuations to those of experimental and control mice respectively (Figure S2b). However, S-equol treatment did not alter seizure-burden in mice injected with TMEV compared to PBS-injected controls, and this effect did not vary by sex (Figure S2c-e). The present study has several limitations, including that only a single dose of S-equol was tested, a novel vehicle/injection method combination was used compared to previously published studies, and that S-equol was only administered once daily for a relatively short time prior to TMEV-infection. Taken together, these limitations suggest that a multi-dose, longer duration pharmacology study be performed to address the potential anti-epileptic effects of S-equol *in vivo*.

Taken together, our results demonstrate a clear role of the gut-brain axis in TMEV-induced seizures. TMEV infection and seizure phenotype are associated with alterations in microbial

diversity, and loss of S-equol-producing microbes is a hallmark of TMEV-induced seizures. In turn, exogenous application of S-equol restores neuronal firing properties in acute slices from TMEV-infected rodents with seizure phenotypes. These data highlight the role of S-equol and the gut-brain axis in virus-induced seizures and identify a new target in the study of the gut-brain axis therapeutics in epilepsy.

Acknowledgements

Sequencing was performed at the Genomics Sequencing Center which is part of the Fralin Life Science Institute at Virginia Tech (FLSI-illuminaseq@vt.edu 540-231-1229).

Figures

Figure 1

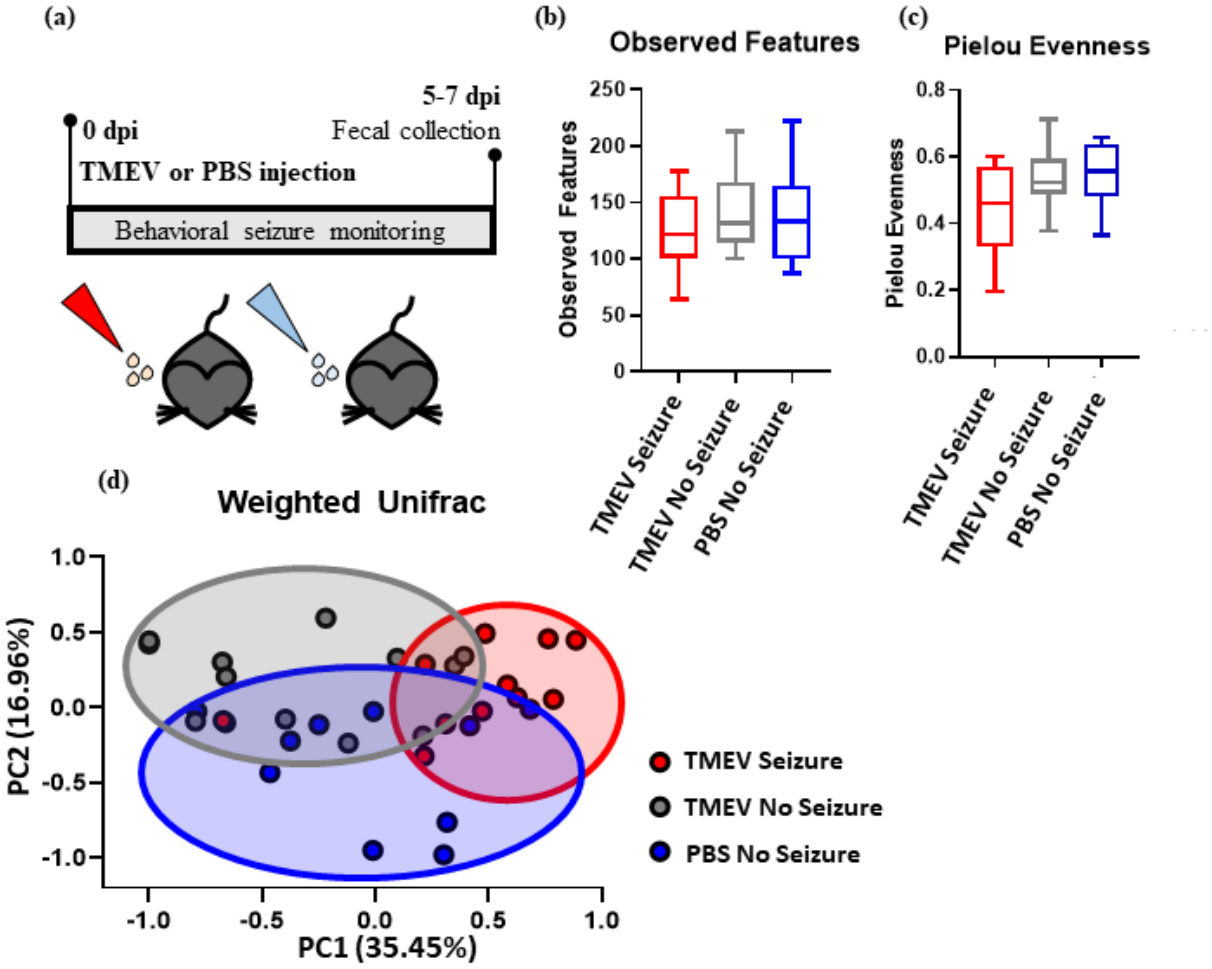

Figure 1: (a) Timeline of TMEV infection, behavioral seizure monitoring, and fecal collection for 16S sequencing. TMEV Seizure n=11 mice; TMEV No Seizure n=12 mice, PBS No Seizure n=11 mice. (b) Alpha diversity measured via Observed Features (n.s. using Kruskal Wallis H test). (c) Alpha diversity measured via Evenness index(p=0.08 using Kruskal Wallis H test). (d) Beta diversity measured via weighted unifrac distance (PERMANOVA p=0.001).

Figure 2

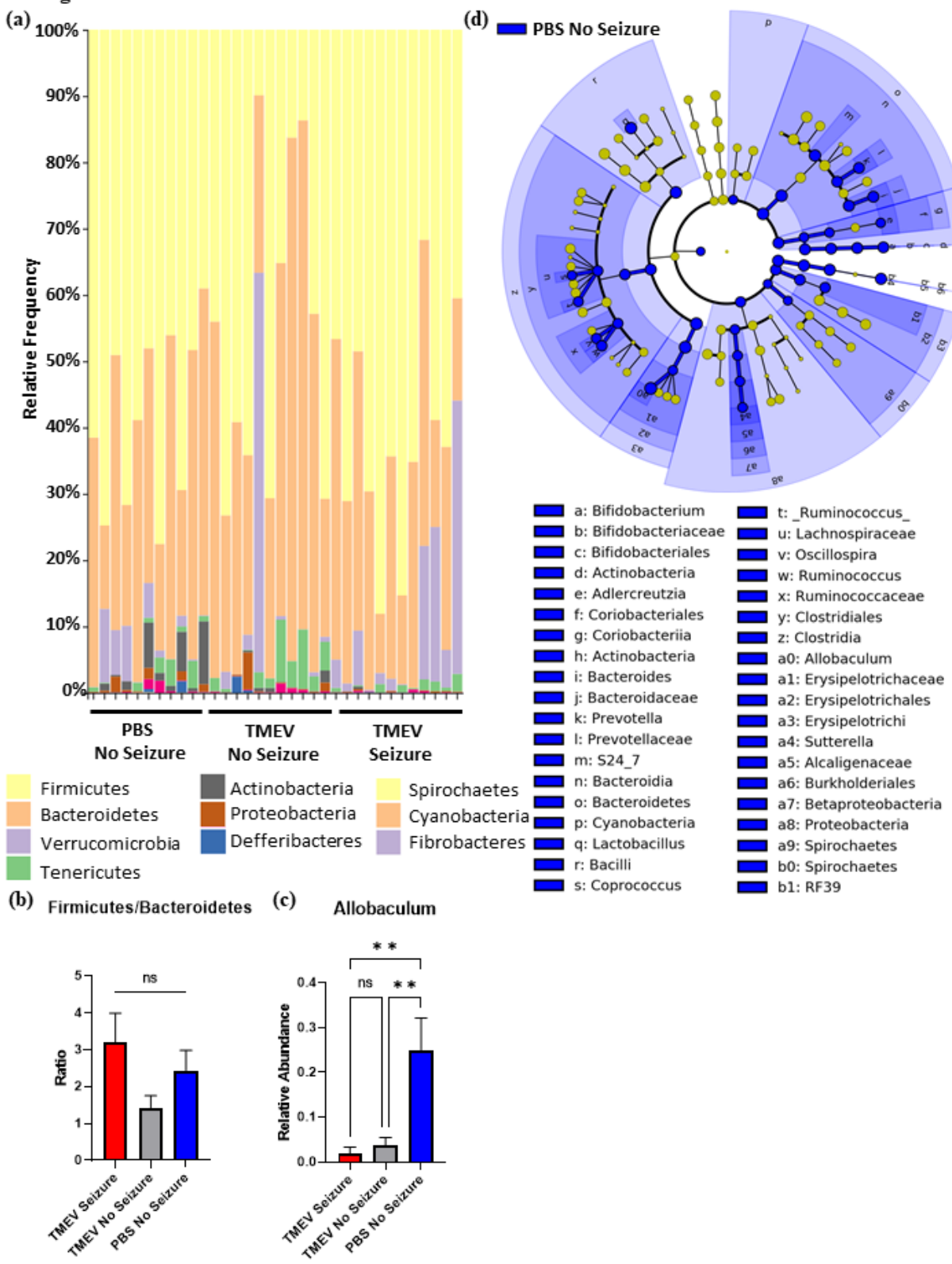
(a)
100%
90%
80%
70%
60%
50%
40%
30%
20%
10%
0%
Relative Frequency
PBS No Seizure
TMEV No Seizure
TMEV Seizure
Firmicutes
Bacteroidetes
Verrucomicrobia
Tenericutes
Actinobacteria
Proteobacteria
Defferibacteres
Spirochaetes
Cyanobacteria
Fibrobacteres
(d)
PBS No Seizure
a: Bifidobacterium
b: Bifidobacteriaceae
c: Bifidobacteriales
d: Actinobacteria
e: Adlercreutzia
f: Coriobacteriales
g: Coriobacteriia
h: Actinobacteria
i: Bacteroides
j: Bacteroidaceae
k: Prevotella
l: Prevotellaceae
m: S24_7
n: Bacteroidia
o: Bacteroidetes
p: Cyanobacteria
q: Lactobacillus
r: Bacilli
s: Coprococcus
t: _Ruminococcus_
u: Lachnospiraceae
v: Oscillospira
w: Ruminococcus
x: Ruminococcaceae
y: Clostridiales
z: Clostridia
a0: Allobaculum
a1: Erysipelotrichaceae
a2: Erysipelotrichales
a3: Erysipelotrichi
a4: Sutterella
a5: Alcaligenaceae
a6: Burkholderiales
a7: Betaproteobacteria
a8: Proteobacteria
a9: Spirochaetes
b0: Spirochaetes
b1: RF39
(b)
Firmicutes/Bacteroidetes
Ratio
ns
TMEV Seizure
TMEV No Seizure
PBS No Seizure
(c)
Allobaculum
Relative Abundance
ns
**
**
TMEV Seizure
TMEV No Seizure
PBS No Seizure

Figure 2: Relative abundance (a) taxa barplot showing bacteria phyla represented in each sample. (b) Firmicutes/Bacteroidetes ratio (n.s.) (c) Relative abundance of Allobaculum genera. ANCOM result: Reject Null Hypothesis. ** $p<0.01$ via Kruskal Wallis H-test with Dunn's multiple comparison analysis. (d) Functional characterization of OTUs represented in the gut microbiota of PBS-injected mice compared TMEV-infected mice. Significant OTUS have been identified by linear discriminant analysis in addition to effect size (LEfSe). Significance is represented by LDA >2.

Figure 3

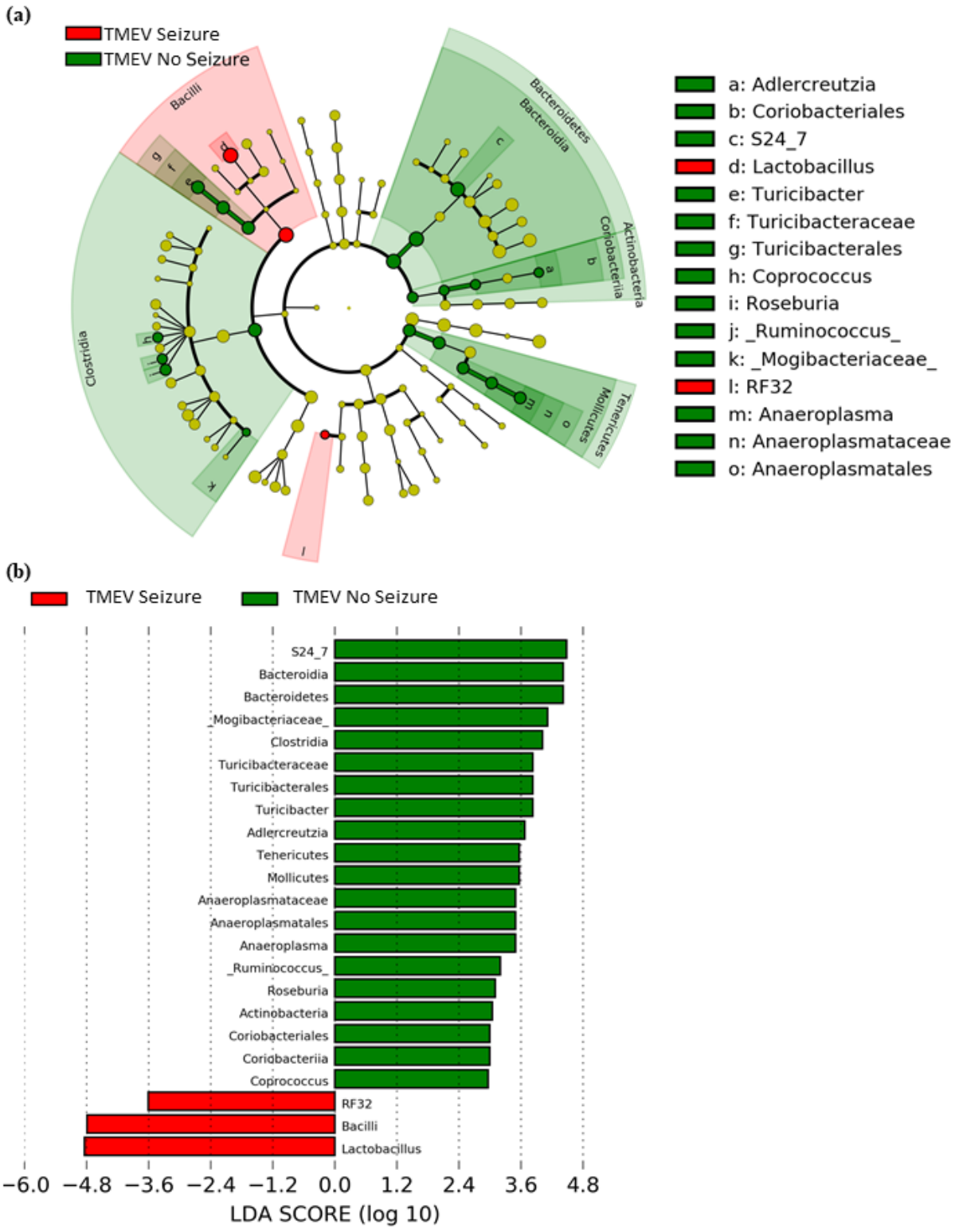

Figure 3: Functional characterization of OTUs represented in the gut microbiota of TMEV-

infected mice stratified by seizure phenotype. Significant OTUS have been identified by linear discriminant analysis in addition to effect size (LEfSe). Significance is represented by LDA >2. (a) Cladogram of biomarkers identified in LEfSe analysis. (b) Histogram of LDA scores of taxonomies identified in LEfSe analysis. Green histograms represent biomarkers of TMEV-infected mice with no seizures; Red histograms represent biomarkers of TMEV-infected mice with seizure phenotypes.

Figure 4

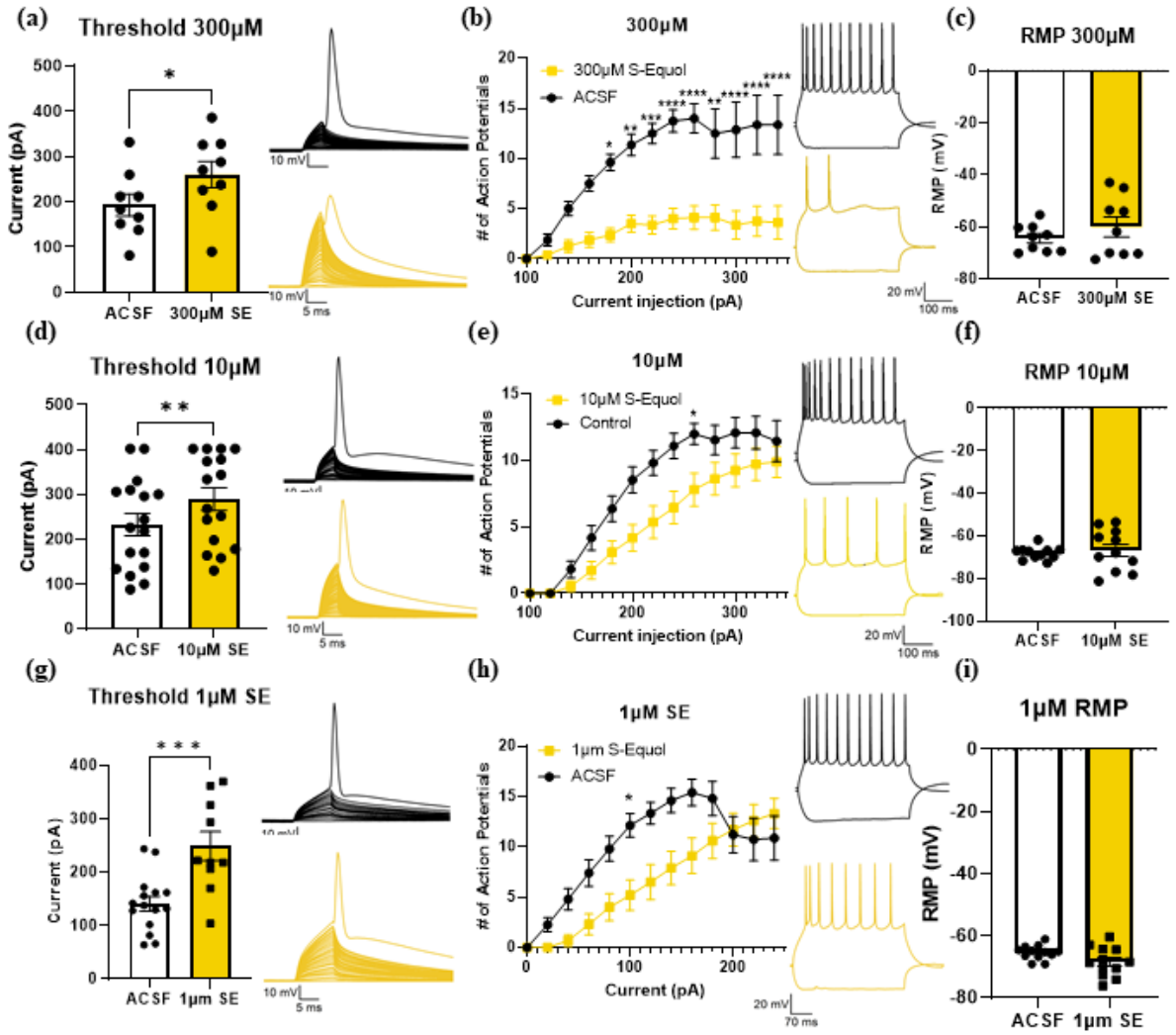

Figure 4: (a) Threshold to fire first action potential following current injection at 2pA steps before and after 30min 300uM S-equol wash. * represents $p < 0.05$ using student's t test. (b) Input/output curve of number of action potentials following current injection at 20 pA steps before (n=14 cells; 4 mice) and after 30 min 300uM S-equol wash (n=8 cells; 3 mice). * represents $p<0.05$, ** $p<0.01$, *** $p<0.001$, **** $p<0.00001$ using 2 way ANOVA. Representative traces at 100 pA current injection (c) Resting membrane potential before and after 30min 300uM S-equol wash (n.s. using student's t test.). (d) Threshold to fire first action potential following current injection at 2pA steps before and after 30min 10uM S-equol wash. ** represents $p < 0.01$ using student's t test. (e)

Input/output curve of number of action potentials following current injection at 20 pA steps before (n=20 cells; 8 mice) and after 30 min 10uM S-equol wash (n=20 cells; 8 mice). * represents $p<0.05$ using 2 way ANOVA. Representative traces at 100 pA current injection (f) Resting membrane potential before and after 30min 10uM S-equol wash (n.s. using student's t test.). (g) Threshold to fire first action potential following current injection at 2pA steps following pre-incubation in 1uM S-equol (n=10 cells; 3 mice) or ACSF+DMSO (n=10 cells; 3 mice) * represents $p <0.05$ using student's t test. (h) Input/output curve of number of action potentials following current injection at 20 pA following pre-incubation in 1uM S-equol n.s. using 2 way ANOVA. Representative traces at 100 pA current injection (i) Resting membrane potential following pre-incubation in 1uM S-equol (n.s. using student's t test.).

Figure 5

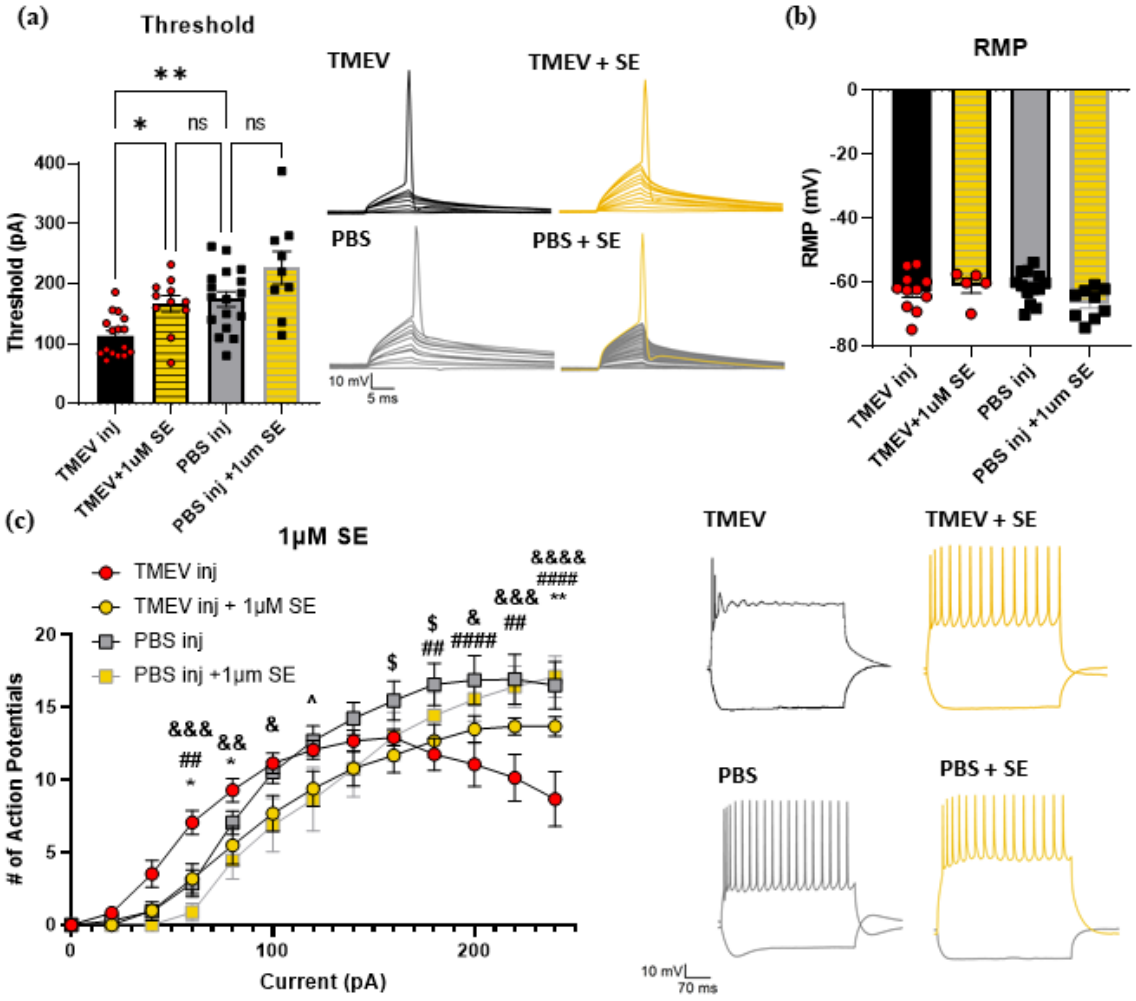

Figure 5: (a) Experimental timeline TMEV inj (n=16 cells; 6 mice); TMEV inj + SE (n=11 cells;

4 mice); PBS inj (n=15 cells; 5 mice); PBS inj + SE (n=9 cells; 3 mice). (b) Threshold to fire first action potential following current injection at 2pA steps. * represents p <0.05, ** p<0.01 (c) Resting membrane potential (n.s. using Kruskal Wallis H test). (d) Input/output curve of number of action potentials following current injection at 20 pA steps. *represents difference between TMEV and TMEV+1uM SE *p<0.05, **p<0.01; #represents difference between TMEV and PBS #p<0.05 ##p<0.01, ###p<0.001, ####p<0.0001; &represents difference between TMEV and PBS+1uM SE &p<0.05, &&p<0.01, &&&p<0.001, &&&&p<0.0001; ^represents difference between PBS and PBS+1uM SE ^p<0.05 using 2 way ANOVA. Representative traces at 240pA current injection

Figure S1

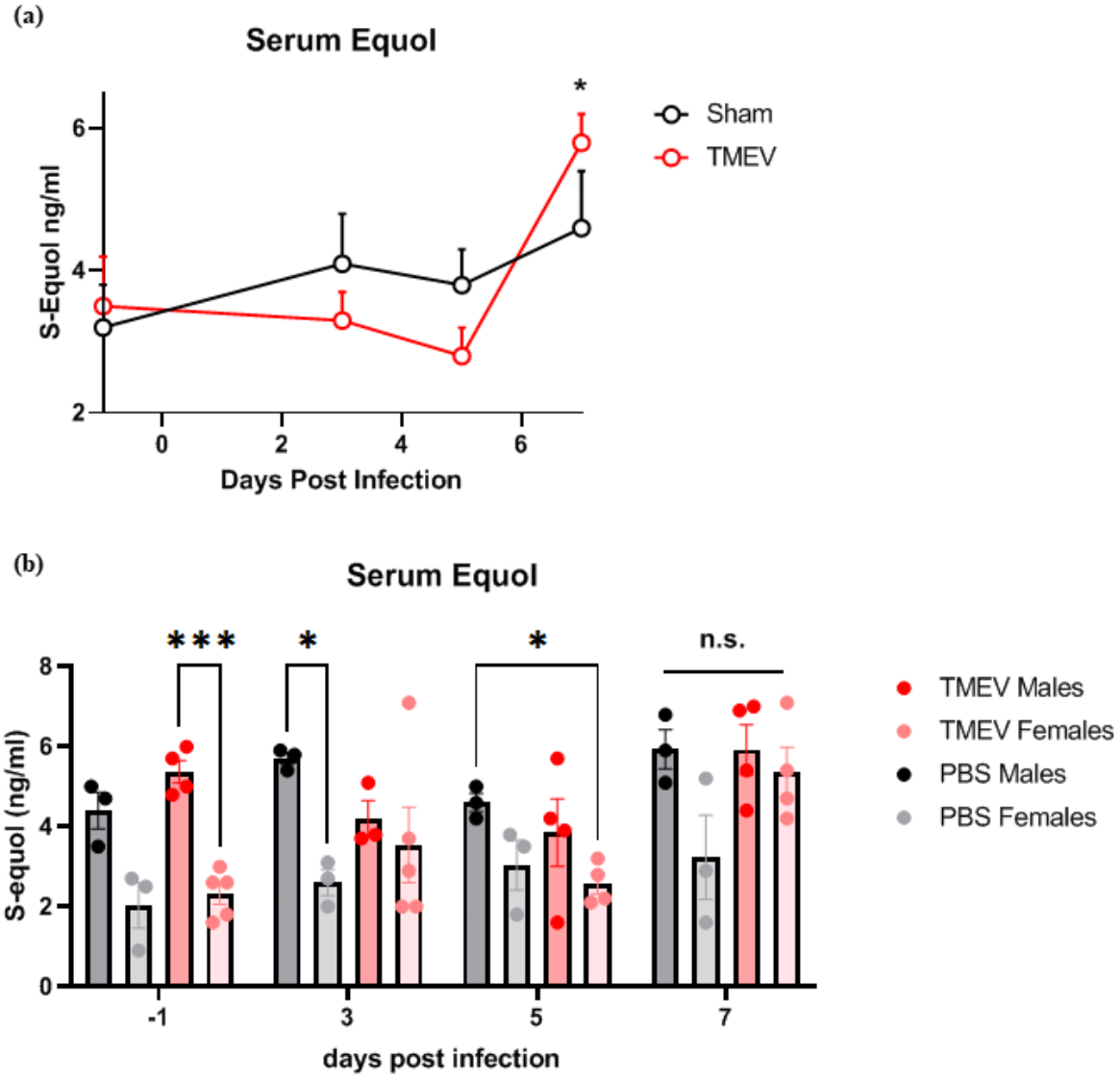

Figure S1 (a) serum concentrations of equol in PBS-injected and TMEV-injected mice with confirmed seizure phenotypes. * represents $p<0.05$ using two-way ANOVA FDR adjustment (b) serum concentrations of equol in male vs. female PBS-injected and TMEV-injected mice. * represents $p<0.05$ using two-way ANOVA FDR adjustment.

Figure S2

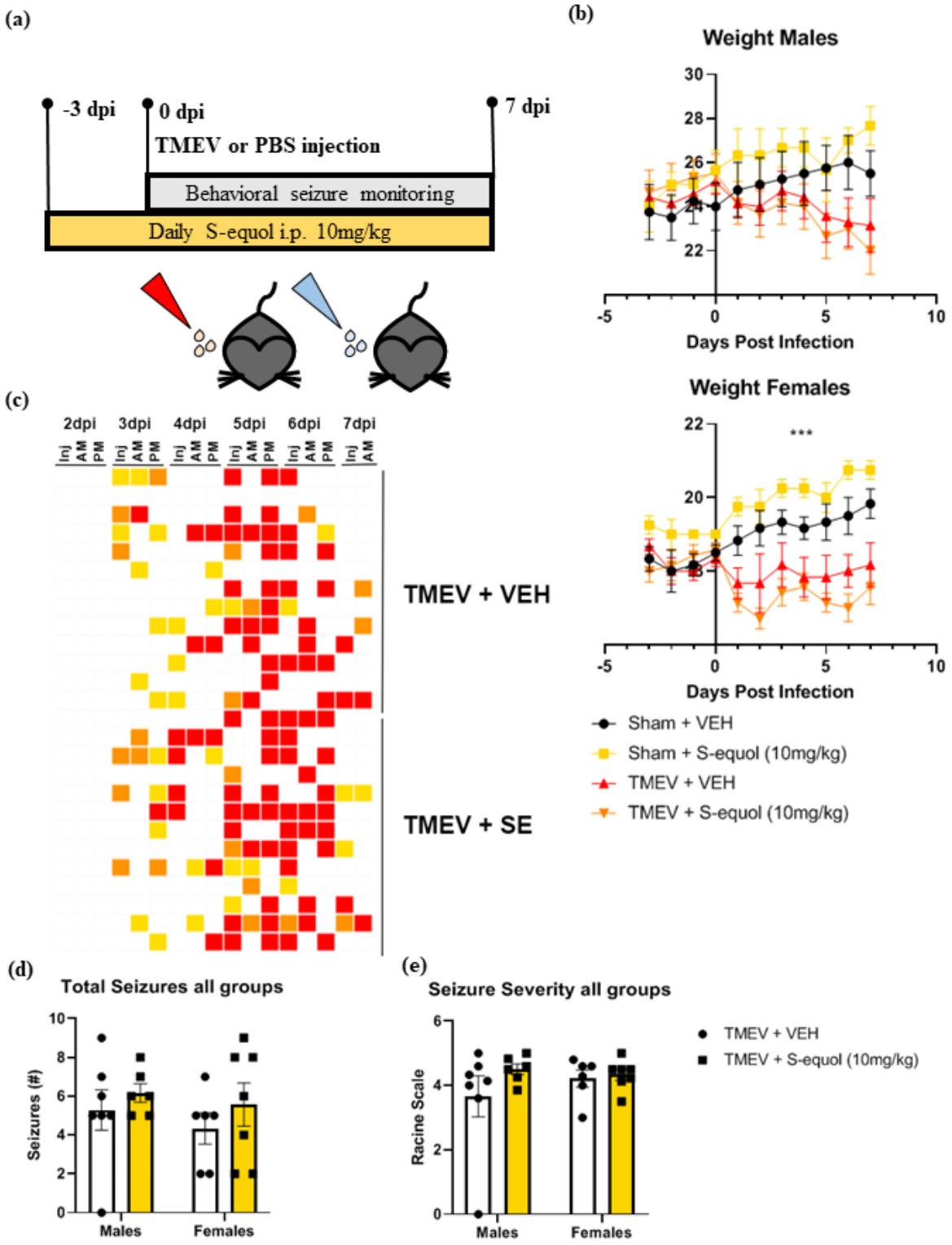

Figure S2 (a) Experimental paradigm. (b) Weight of PBS-injected vs TMEV-injected mice. *** represents $p<0.001$ using 2-way ANOVA with FDR adjustment. (c) Heatmap depicting seizure burden and severity in TMEV-infected mice. (d) Quantification of total seizures in males and

females infected with TMEV. n.s. using 2-way ANOVA with FDR adjustment. (e) Quantification of total seizure severity in males and females infected with TMEV. n.s. using 2-way ANOVA with FDR adjustment.

Figure S3

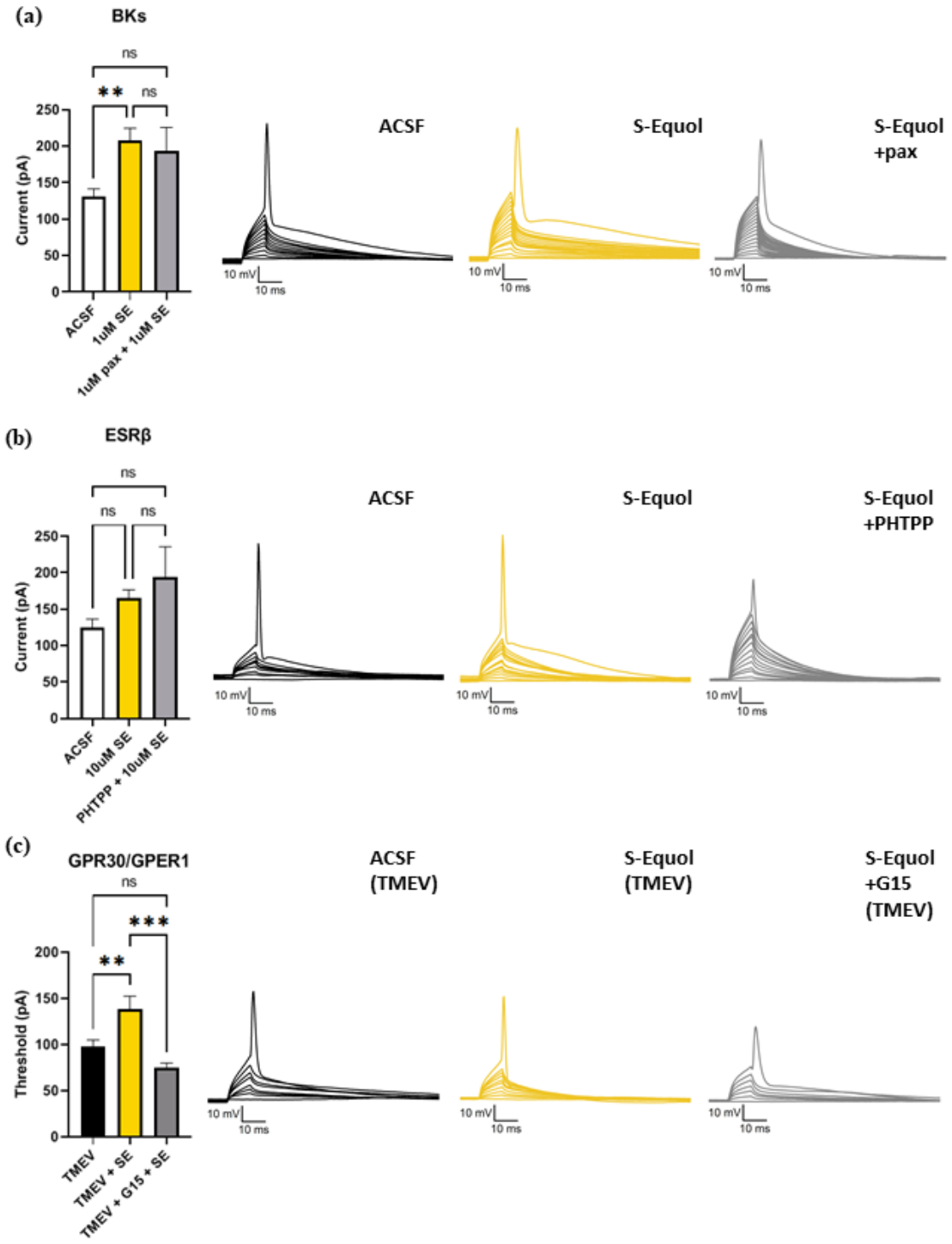

Figure S3 Threshold to fire a single action potential following current injection in (a) neurons from naïve mice exposed to ACSF, 1uM S-equol, or 1uM S-equol + 1uM paxilline. ** represents $p<0.01$ using 1-way ANOVA with FDR adjustment. (b) neurons from naïve mice exposed to ACSF, 10uM S-equol, or 10uM S-equol + 1uM PHTPP. & represents $p=0.07$. (c) neurons from TMEV-infected mice with confirmed seizure phenotypes exposed to ACSF, 1uM S-equol, or 500nM G15. ** represents $p<0.01$, *** represents $p<0.001$ using 1-way ANOVA with FDR adjustment.

Table S1

Citation	***Model***	***Soy free diet***	***Sex***	***Ovariectomized***	***Dosing Method***	***Dose(s)***
Selvaraj et al., 2004	Mouse	Yes	F	Yes	daily injections OR diet pellets	daily injections (0, 4, 8, 12, or 20 mg [kg body weight]−1 day−1) or in the diet (0, 500, or 1000 ppm) for 12 days.
Bax et al., 2019	Mouse	Yes	M&F	N	Oral administration	daily oral administration of 10 mg S-equol/kg body weight in 2.5% dimethyl sulfoxide/0.5% carboxymethyl cellulose
Loutchanwoot et al., 2015	Rat	Yes	M	-	Oral Gavage	two doses of 100 and 250 mg/kg body weight (BW)/day in olive oil for 5 days

Onoda et al., 2011	Mouse	Yes	F	Y	Diet	ad libitum access to diet
Moran et al., 2019	Rat	Yes	F	Y	S-equol pellets	treated daily with placebo or SE pellets (0.05, 0.1, or 0.2 mg)
Blake et al., 2011	Rat	Yes	M&F	N	Subcutaneous (s.c.) injection	5.0 mg/kg body weight of equol
Choi 2009	Mouse	No	F	N	Oral administration via drinking water	5 or 25 mg/kg body weight/day for 1, 3, or 7 wk
Yu et al., 2016	Rat	No	M	-	Intraperitoneal (i.p.) injection	0.625, 1.25 and 2.5 mg·kg immediately before experiment

Table S1 depicts published *in vivo* dosing methods of equol in rodent models.

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Chapter 3:

Maternal antibiotic treatment alters offspring gut microbiome and leads to increased offspring seizure susceptibility

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This chapter is currently in preparation. AG, LC, SC designed research question and project. AG was responsible for bioinformatics pipeline development and execution. AG, JM, and EM were responsible for data analysis. LC and AG collected experimental data. AG, EM, and SC drafted text and figures. All authors contributed to text and figure revisions.

Abstract

Seizures in neonates are associated with poor outcomes later in life. The most common cause of neonatal seizures is hypoxic/ischemic encephalopathy (HIE). HIE can present in neonates in neonatal intensive care units (NICU)s due to a variety of underlying conditions. Antibiotics are some of the most commonly prescribed drugs in NICUs, and up to 40% of pregnant women are prescribed antibiotics during pregnancy. Perinatal and postnatal antibiotic use can drastically alter the composition of the gut microbiome. The present study sought to examine the effect of maternal antibiotic treatment on offspring microbiome composition and seizure susceptibility following hypoxia exposure.

Introduction

Seizures occur in ~1.5% of full term neonates(1) and are associated with poor developmental outcomes including increased risk of cerebral palsy, microcephaly, and autism(2–4). Hypoxic/ischemic encephalopathy(HIE) is the most common cause of neonatal seizures,

accounting for 30-53% of clinical cases(5,6). Neonates that experience severe HIE are at increased risk of developing epilepsy along with worsened cognitive outcomes later in life(7–9). Despite a high prevalence of cases, there are few treatment options available for neonates presenting with HIE-induced seizures(10). Available antiepileptic drugs (AEDs) are not effective in over half of neonates(11) and are harmful to the developing brain(12). Further understanding of the risk factors for neonatal seizures is imperative for developing novel therapeutics. The infant brain is exposed to hypoxia during various conditions including perinatal asphyxia, premature birth, congenital heart disease, and pulmonary disease(13). Infants in neonatal intensive care unit (NICU) environments for these conditions are often prescribed antibiotics(14,15). Additionally, close to 40% of pregnant women are prescribed antibiotics during pregnancy or lactation(16–19). Although use of antibiotics during gestation is a risk factor for neonatal seizures(20,21), no study has yet examined the molecular underpinnings of seizure susceptibility following perinatal and postnatal exposure to antibiotics.

Maternal antibiotic use during gestation, as well as antibiotic treatment in neonates, leads to pronounced changes in the infant microbiome(22–25). Infants with epilepsy have altered microbiomes compared to healthy age-matched controls, and microbiome alteration correlates with reduction in seizure burden in epileptic infants treated with ketogenic diet(26). Children with drug resistant epilepsy who respond to ketogenic diet treatment also have altered gut microbiomes compared to children who do not experience a reduction in seizure burden(27). In rodents, gut microbiome alteration alone (without diet intervention) can have a similar anti-epileptic effect to the ketogenic diet(28), indicating that the anti-seizure effects of the ketogenic diet in infants may also be mediated via microbiome alteration. The maternal microbiome has additionally been shown to modulate offspring neurodevelopment in rodents, affecting axogenesis, white matter

architecture, and long term potentiation (LTP)(24,29,30). Although previous studies have highlighted the potential link between the pediatric gut microbiome, diet, and epilepsy, no study has examined the role of the gut microbiome in pediatric models of epilepsy outside of dietary intervention.

Because the offspring microbiome composition is determined by the maternal microbiome, we hypothesized that antibiotic-induced changes in maternal microbiome would affect offspring and lower seizure threshold. In the current study, we parallel previously published findings that maternal antibiotic treatment in rodents leads to marked shifts in offspring microbiota. Additionally, we find that maternal antibiotic treatment leads to increased offspring seizure susceptibility following hypoxia exposure, corresponding with altered offspring neuronal physiology. Furthermore, we show these alterations in offspring neuronal physiology are sex-specific. Taken together, these findings indicate that maternal antibiotic treatment leads to offspring microbiome alteration and an increase in offspring seizure-susceptibility and altered neuronal function.

Results

Maternal antibiotic treatment leads to offspring microbiota alteration

Antibiotics are some of the most prescribed medications in NICUs, with Ampicillin, Gentamicin, and Vancomycin among the most common(31,32). Although the infant microbiome can be lastingly altered after even a single course of antibiotics(33), brain development and outcomes following hypoxia exposure are heavily influenced by both perinatal and postnatal events(34). We therefore treated mice with a cocktail of broad-spectrum antibiotics consisting of ampicillin, colistin, metronidazole, vancomycin, gentamicin, and kanamycin in drinking water to maximally reduce gut microbes in breeding pairs (Fig. 1a). This maternal antibiotic treatment led

to a decrease in the number of culturable bacterial colonies present 24 hours after plating fresh fecal samples (Fig. 1 b,c). As many bacterial species in the gut microbiota are not culturable, we additionally performed 16S sequencing on fecal samples from antibiotic-treated and control mating pairs. Samples from antibiotic-treated mice separated from controls in beta diversity (Fig. 1 d), indicating significant alterations in gut microbiome composition. These findings indicate that perinatal and postnatal antibiotic exposure significantly altered offspring gut microbiota.

Maternal antibiotic treatment leads to increased offspring seizure susceptibility

It is well established that loss of gut microbiome diversity through perinatal and postnatal antibiotic treatment or germ-free rearing alters neurodevelopment(24,29,35–37). Additionally, there is evidence in adult models of epilepsy that antibiotic treatment or germ-free rearing leads to increased seizure susceptibility(28). However, it is unclear how antibiotic-induced perinatal and postnatal microbiome alteration affects pediatric seizure susceptibility. To explore this question, we exposed postnatal day 9-10 offspring of antibiotic-treated and untreated dams to increasing levels of hypoxia known to elicit behavioral and electrographic seizures in rodent pups(38). Pups from antibiotic-treated dams experienced significantly more seizures at 9% O2 compared to pups from control dams, while increasing O2 deprivation led to similar seizure-occurrence in both experimental groups (Fig. 2 b). Additionally, offspring of antibiotic-treated dams displayed increased seizure duration at both 9% O2 and 6% O2 compared to offspring of control dams (Fig. 2 c). Overall, pups from antibiotic-treated dams experienced increased number of seizures throughout the duration of the experiment (Fig. 2 d), and seized for a significantly longer duration across increasing hypoxia conditions (Fig. 2e). Furthermore, pups from antibiotic-treated dams experienced a reduced latency to first seizure across hypoxic conditions (Fig. 2f). These findings

suggest that perinatal and postnatal antibiotic treatment leads to increased seizure susceptibility following hypoxia exposure.

Maternal antibiotic treatment alters offspring neuronal firing properties in a sex-specific manner in vitro

Seizure-susceptibility is often associated with neuronal hyperexcitability *in vitro*. We next examined the function of layer 2/3 pyramidal cells from male and female offspring of antibiotic-treated dams. Neurons from male and female antibiotic-treated offspring had similar resting membrane potentials, and required the same amount of current injection to elicit a single action potential compared to neurons from controls (Fig. 4 a,b). Furthermore, neurons from male antibiotic-treated offspring fired the same number of action potentials in response to current injection as neurons from untreated male controls. In contrast, neurons from female antibiotic-treated offspring fired more action potentials in response to the same current injection (Fig. 4c), indicating increased neuronal excitability.

AP properties are associated with alterations in ion channel activation(42–44). To further elucidate sex-specific alterations in neuronal physiology, we examined AP parameters in neurons from antibiotic-treated and un-treated offspring. Neurons from untreated male offspring had higher AP peak amplitudes than any other experimental group, indicating both a sex and antibiotic treatment-dependent effect on AP amplitude (Fig. 4d). Additionally, neurons from antibiotic-treated male offspring had higher after-hyperpolarization (AHP) amplitudes than those of neurons from untreated males (Fig. 4e). In contrast, antibiotic treatment did not alter the hyperpolarizing activated SAG amplitude in neurons from male offspring, but neurons from female antibiotic-treated offspring exhibited markedly decreased SAG amplitudes (Fig. 4f). There was no difference

among experimental groups in AP half width (Fig. 4g). These results highlight sex-specific alterations in offspring neuronal properties following perinatal and postnatal antibiotic exposure.

Maternal antibiotic treatment alters spontaneous excitatory postsynaptic currents (sEPSCs) in a sex-specific manner in vitro

Altered synaptic transmission is associated with seizure-susceptibility in several rodent models of adult epilepsy(45–47). The total frequency and amplitude of EPSCs was unchanged in both male and female mice regardless of antibiotic treatment (Fig. S1). Additionally, there was not a significant shift in the cumulative distribution of EPSC amplitude in any experimental group (Fig. 5b,c). Interestingly in females there was a significant leftward shift in the cumulative distribution of inter-event intervals (IEI) (Fig. 5d). Specifically, neurons from antibiotic-treated female offspring exhibited increased IEIs lasting 0-500 ms, and decreased IEIs lasting greater than 2000 ms. In contrast, there was a significant rightward shift in the cumulative distribution of IEI in male offspring, driven by an increase in IEIs lasting 1500-2000 ms in antibiotic-treated males (Fig. 5e). These data suggest that maternal antibiotic treatment alters passive as well as resting neuronal properties in offspring in a sex-specific manner.

Discussion

In the present study, we show a significant impact of perinatal and postnatal exposure to antibiotics on gut microbiome composition. In turn, perinatal and postnatal antibiotic exposure increases offspring seizure-susceptibility in hypoxic conditions. Furthermore, we present evidence that the increase in seizure-susceptibility may be due to sex-specific alterations in neuronal properties following antibiotic exposure. Overall, our results indicate that maternal antibiotic-treatment leads to notable alteration in offspring gut microbiome composition, as well as shifts in neuronal function that may ultimately lead to increased offspring seizure-susceptibility.

Possible mechanisms in the gut-brain axis in pediatric seizure-susceptibility

Development of the infant gut microbiome depends heavily on maternal factors such as diet, behavior, and antibiotic use(48). Our data replicate previous findings that perinatal and postnatal exposure to antibiotics commonly prescribed in the NICU, including ampicillin, gentamycin and vancomycin, reduces microbiome diversity and alters the composition of the developing gut microbiome(31–33,49–51). We also present novel findings that this perinatal and postnatal antibiotic exposure increases seizure-susceptibility in hypoxic conditions. Perturbations in the developing gut microbiome impact neonatal brain development and may impact seizure-susceptibility via multiple pathways including alterations in neurotransmitters, neuroinflammation, and gut metabolite production(37). For instance, dietary tryptophan is largely processed in the gut into serotonin by enterochromaffin cells and several gut microbes including *Lactobacillus* sp. (52–55). As breastfeeding and vaginal delivery increase the relative abundance of *Lactobacillus* sp. in the infant gut microbiome(56,57), it is possible that improved cognitive health outcomes associated with vaginal delivery and breastfeeding may be due in part to increased peripheral serotonin during neonatal brain development. Furthermore, germ-free mice have altered peripheral tryptophan metabolism and increased hippocampal serotonin levels in a sex-dependent manner compared to mice with intact microbiomes, indicating developmental perturbations in gut microbes influence neural serotonergic function in adults(58). Importantly, use of selective serotonin reuptake inhibitors (SSRIs) during pregnancy is associated with increased risk of neonatal seizures in offspring(59), indicating a possible link between maternal serotonin, microbiome perturbations, and offspring seizure susceptibility.

The developing gut microbiome is also closely linked to the developing immune system(60). Germ free mice display alterations in B and T cell function, increased susceptibility

to infection, and increased autoimmunity(61–63). Dysregulated immune response or inflammation during pregnancy or early neonatal life is associated with increased risk of ASD, depression, and epilepsy(64). In turn, immune-targeted therapies can reduce seizure burden in multiple adult rodent models of seizure-susceptibility, indicating that immune-brain crosstalk is important in the pathogenesis of epilepsy(65). In addition to contributing to the development of peripheral immune cell populations, the gut microbiota is also necessary for the development of functional microglia populations in the brain(66). As dysfunction in microglia or astrocytes alone can cause seizures in genetic rodent models, it is reasonable to posit that microbial influence over microglial populations may lead to seizure-susceptibility in pediatric models as well(67,68). Further studies on microbiome-immune-brain crosstalk are necessary to further elucidate the mechanism by which antibiotic treatment may lead to increased seizure-susceptibility.

One mechanism by which the gut microbiome alters the development of microglia is through microbiome production of metabolites including short chain fatty acids (SCFAs)(66). SCFAs are produced in the gut by multiple bacterial populations including *Bifidobacterium* sp. through breaking down indigestible dietary fiber(69). Peripheral SCFAs are reduced in adult patients with epilepsy, as well as in patients with commonly comorbid developmental disorders including ASD and Rett syndrome, indicating that gut-derived metabolites may play a role in development as well as seizure-susceptibility(70–73). Treatment with SCFAs can reduce seizure burden in adult models of seizure-susceptibility, however, no study as yet examined SCFAs in a pediatric model of seizure-susceptibility(74,75). More studies on neurotransmitters, immune activation, and microbial metabolites in the developing gut-brain axis are necessary to elucidate specific mechanisms by which gut microbe alterations affect neonatal seizure-susceptibility.

Sex specificity in neuronal physiology and HIE

Our data indicate that perinatal and postnatal antibiotic exposure alters neuronal function in a sex-dependent manner. Neurons from antibiotic-treated female mice fire more action potentials following increasing current injection steps and have altered SAG compared to neurons from untreated female mice. In contrast neurons from antibiotic-treated male mice fire similar numbers of action potentials following increasing current injection, but display increased action potential amplitude and increased after-hyperpolarization peaks compared to neurons from male untreated mice. Additionally, neurons from antibiotic-treated female mice exhibit reduced sEPSC inter-event intervals compared to neurons from untreated female mice, and neurons from male antibiotic-treated mice exhibit increases sEPSC inter-event intervals compared to neurons from male untreated mice. Sex-dependent alterations in neuronal development and function have been previously reported in multiple disease states including seizure-susceptibility, and exposure to sex-specific hormones during development is critical for the formation of typical brain circuitry in males and females(76). Interestingly, the gut microbiome produces phytoestrogen metabolites such as S-equol that can cross the BBB and act on estrogen receptors in the brain(77,78). Alterations in microbial production of phytoestrogens are one mechanism by which perinatal and postnatal antibiotic treatment may exert a sex-specific effect on neuronal physiology.

Although the current study does not examine the impact of sex on seizure-susceptibility following antibiotic exposure, a large body of literature demonstrates sex-specific seizure behaviors and neurological changes following HIE(79). Males have increased cerebral glutamate levels compared to females during early development, which corresponds to increased neuronal susceptibility to excitotoxic damage in males(80,81). Additionally, males are more vulnerable to seizures, exhibit increased oxidative stress, and increased neuroinflammation following HIE(79). In contrast, our data demonstrated increased neuronal excitation in neuron from antibiotic-treated

females that was not present in neurons from antibiotic-treated males. This may suggest that antibiotic treatment impairs the generally neuroprotective effect of estrogen in the developing female brain, potentially indicating that microbial-derived phytoestrogens may play a larger than expected role in estrogen signaling in early life. Our data indicate that future studies should examine sex-specificity in the negative effects of antibiotic treatment in the pediatric gut-brain axis.

In conclusion, our data indicate that alterations in gut microbiome composition via perinatal and postnatal antibiotic exposure may increase seizure-susceptibility by altering neuronal function in a sex-specific manner. These data provide novel insights on the role of the gut microbiome in neonatal seizure-susceptibility, and highlight the importance of consideration of the gut microbiome when prescribing antibiotics to pregnant mothers and infants. Future studies on the mechanistic link between gut microbes and seizure-susceptibility, as well as on long term outcomes of HIE in antibiotic-treated pups, are necessary to elucidate the potential of targeted microbiome therapeutics in neonatal epilepsy.

Methods

Animals

Animals were housed and handled according to the guidelines of the National Institutes of Health Committee on Laboratory Animal Resources. Prior approval of the Virginia Polytechnic Institute and State University Institutional Animal Care and Use Committee was obtained for all experimental protocols. Mice were provided chow (Envigo 2918) and water *ad libitum* and kept in a facility providing 12 hours light/dark cycle. All efforts were made to minimize animal pain. C57BL/6 (Jax) mice were chronically treated with antibiotics via drinking water for at least one

generation before being included in experiments. Untreated mice were given autoclaved sterile drinking water.

Fecal collection and microbiome analysis

Fecal pellets were collected from clean sterile cage floor from momentarily individually housed antibiotic-treated and untreated mice. In bacterial culture experiments, fresh fecal samples were homogenized in sterile water at 10mg/mL and plated on MRS agar plates. Plates were placed in anaerobic chambers and allowed to grow at 37C overnight. Bacterial colonies were then counted manually. Additionally, mouse fecal DNA was isolated using the DNA Fecal/Soil Microbe Miniprep Kit (Zymo Research) according to manufacturer's instructions. One fecal pellet was used for each isolation. DNA was quantified and samples were stored at -80C until sequenced. The universal primers 515F and 926R were chosen to amplify the V4–V5 region of the 16S rRNA gene following the Earth Microbiome Project protocol (https://www.earthmicrobiome.org/). The polymerase chain reaction (PCR) reaction mixture included 13.0 µL of PCR-grade water, 1.0 µL of template DNA, 10 µM of each primer, and 10.0 µL of 5PRIME HotMasterMix (2x) (Quantabio, Beverly, MA). Samples were amplified in duplicate under the following thermocycler conditions: 94°C for 3 min for initial denaturing, then 35 cycles of 94°C for 45 s, 50°C for 60 s, and 72°C for 90 s. A final elongation step occurred at 72°C for 10 min followed by a hold at 4°C. After pooling duplicates, all amplicons were visualized on a 2% agarose gel and quantitated on a Qubit fluorometer (FisherScientific, Hampton, NH). Normalization was performed based on Qubit results, and amplicons were purified on a Pippin Prep (Sage Science, Beverly, MA) targeting a 520bp range. This pool was then quantitated using quantitative PCR. Amplicons were loaded at 9.5 pM and sequenced using the MiSeq v3 600-cycle kit on the Miseq platform (Illumina, Inc.,

San Diego, CA). Sequencing yielded 19.6 million paired-end reads. Quality scores were within Illumina specifications. Sequences were analyzed using QIIME as previously described(82).

HIE protocol and seizure monitoring

Hypoxia protocol to induce seizures was as previously described(38). Briefly, p9-p10 pups form both antibiotic-treated dams as well as from untreated dams were placed in airtight)2 regulated chambers on heating pads to maintain pup body temperature. Nitrogen gas was administered at increasing amounts to reach the experimental O2 concentrations described. Mice were removed from the hypoxic chamber for 5 minutes of rest in atmospheric air between conditions. Behavioral convulsive seizures were assessed using a the following modified Racine scale(83): Stage 4: hindlimb and forelimb clonus, Stage 5: rearing and falling or loss of tone resulting in falling.

Acute slice preparation

Acute slices were prepared from antibiotic-treated and untreated mice following weaning as previously described(84). Mice were decapitated and their brains were quickly removed and immersed in ice-cold cutting solution containing (in mmol/L): 135 N-methyl-d-glucamine (NMDG), 1.5 KCl, 1.5 KH2PO4, 23 mmol/L choline bicarbonate, 25 d-glucose, 0.5 mmol/L CaCl2, and 3.5 MgSO4 (Sigma-Aldrich). Coronal brain slices (300 μm) were made and recovered for 40-60 minutes in oxygenated recording solution in mmol/L: 125 NaCl, 3 KCl, 1.25 NaH2PO4, 25 NaHCO3, 2 CaCl2, 1.3 MgSO4, and 25 d-glucose at 32°C and maintained at room temperature before recordings.

Whole cell recordings

Individual brain slices were transferred to a recording chamber and continuously perfused (4 mL/min) with oxygenated recording solution. Whole-cell recordings were conducted using

borosilicate glass capillaries (KG-33 glass, Garner Glass) and filled with internal solution containing (in mmol/L): 134 K-gluconate, 1 KCl, 10 HEPES, 2 mg-ATP; 0.2 Na-GTP and 0.5 ethylene glycol tetraacetic acid (EGTA). The pH was set to 7.24 with KOH, and the osmolality was measured (~290 mOsm/kg). All recordings were performed at 32 ± 1°C. Individual cells were visualized using a Zeiss Axioscope (Carl Zeiss) microscope equipped with Nomarski optics with a 40× water-immersion objective lens. Tight seals were made using electrodes with a 3–5-MΩ open-tip resistance. Signals were acquired from layer II/III pyramidal cells with an Axopatch 1B amplifier (Molecular Devices), controlled by Clampex 10 software via a Digidata 1440 interface (Molecular Devices). Excitatory postsynaptic currents (EPSCs) were obtained in the presence of bic (10 μmol/L; Sigma-Aldrich) to block GABAA receptors.

Statistics

Beta diversity analyses were calculated with bray curtis distance. Electrophysiology experiments were analyzed using student's t tests, ANOVA with Bonferroni adjustment, and KS test when appropriate. Changes in evoked E/IPSCs were analyzed using Clampfit Software 10.0 (Molecular Devices). Statistics were generated and graphed using Origin 7.5 Pro software (Origin), and Graphpad Prism 9.0, with significance set at $P < 0.05$.

Figures

Figure 1

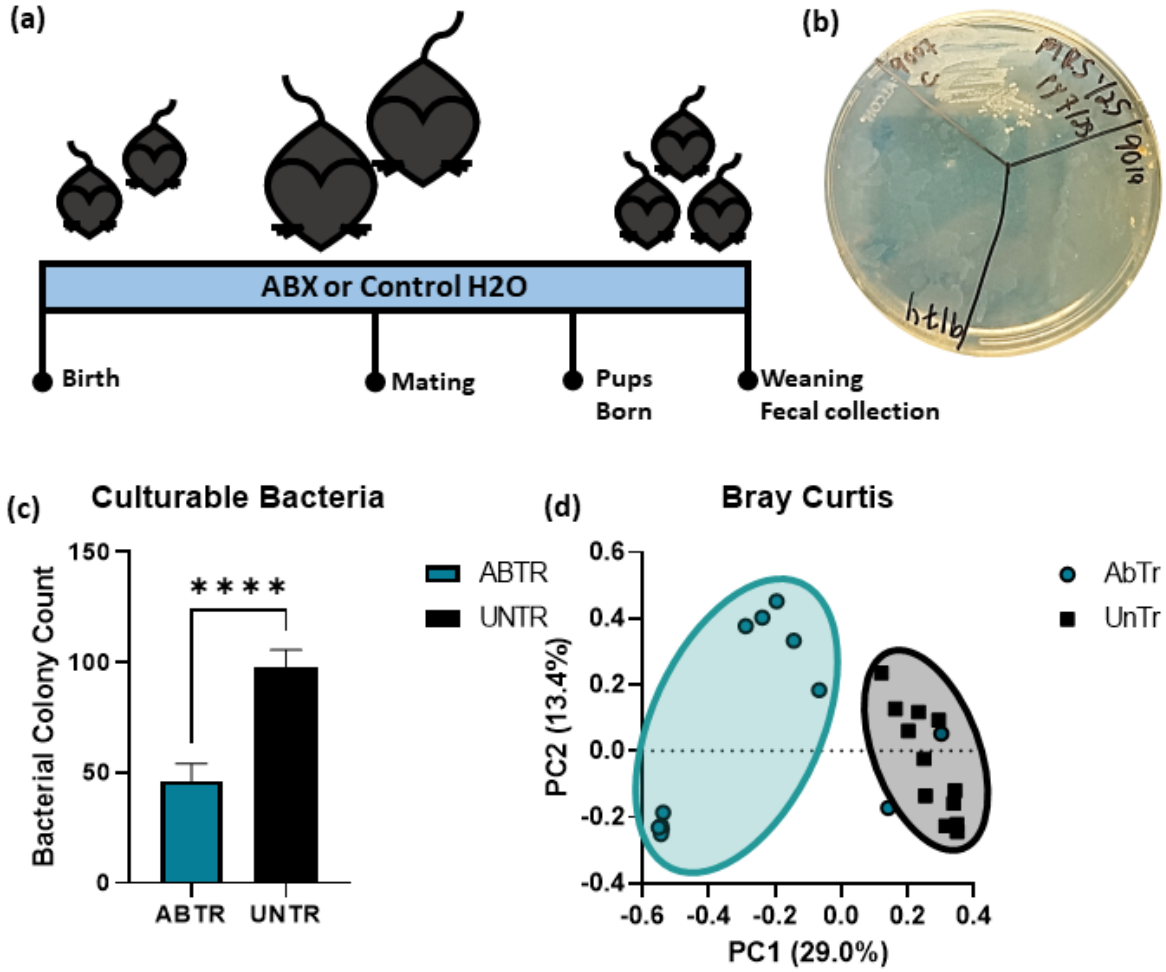

Figure 1. (a) experimental timeline. Mice were given ABX in drinking water ad libitum at least one month prior to mating. Pups were exposed to ABX during prenatal and perinatal development. Hypoxia experiment was performed on p9/p10 pups. **(b)** example culture plate 24h following fecal plating demonstrating lack of culturable bacteria in AbTr mice. **(c)** quantification of fecal culture plates following 24h incubation. **(d)** Bray Curtis Index measurement of beta diversity in AbTr and UnTr fecal samples.

Figure 2

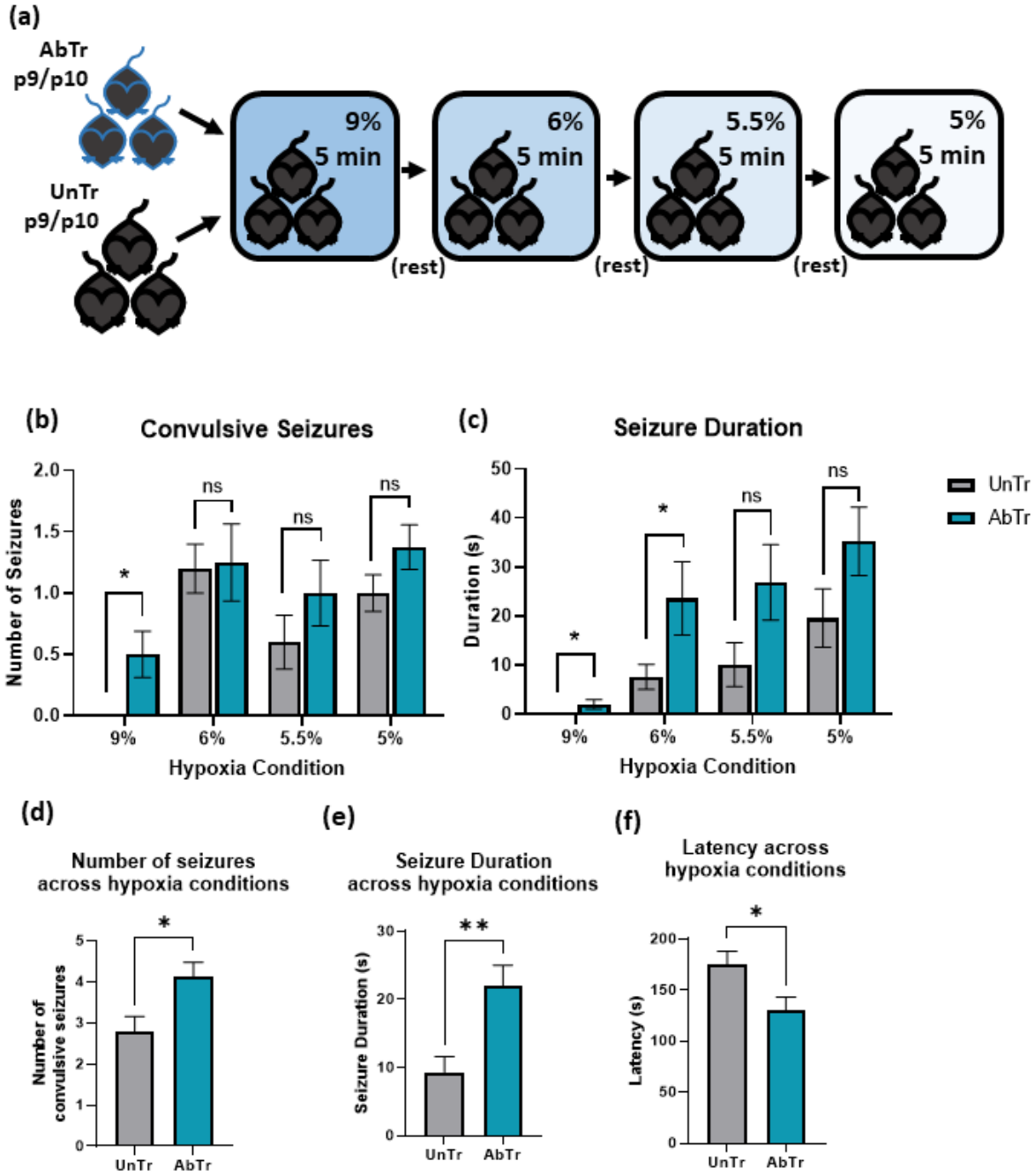

Figure 2. **(a)** Hypoxia exposure protocol. **(b)** number of convulsive seizures at increasing levels of hypoxia. * indicates p <0.05 using two way ANOVA with FDR adjustment **(c)** Seizure duration at increasing levels of hypoxia * indicates p <0.05 using two way ANOVA with FDR adjustment **(d)** Total seizures across hypoxia exposure conditions. * indicates p<0.05 using student's t-test. **(e)** Total seizure duration across hypoxia exposure conditions. * indicates p<0.05 using student's t-test. **(f)** Latency to first seizure across hypoxia exposure conditions. * indicates p<0.05 using student's t-test.

Figure 3

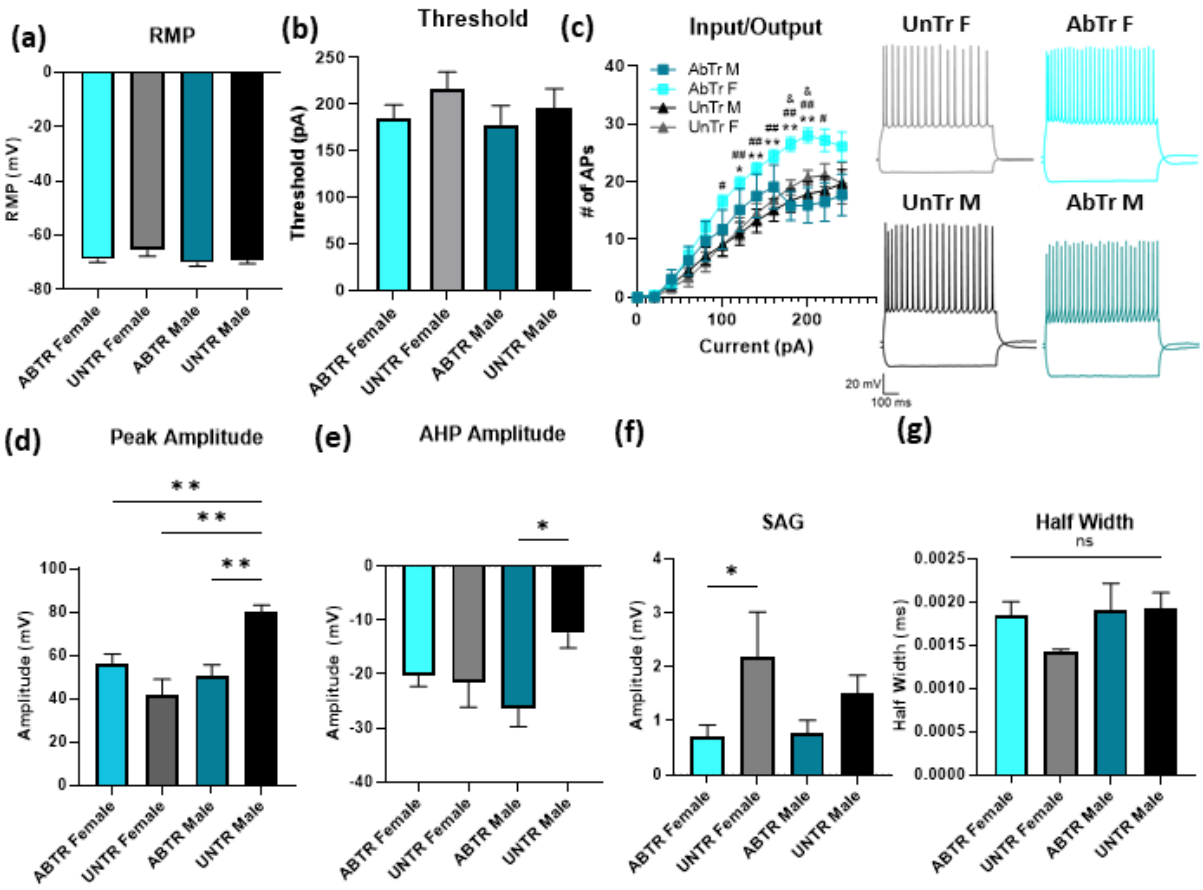

Figure 3. (a) Resting membrane potential. n.s. using 2-way ANOVA. **(b)** Threshold to fire first AP following current injection at 2pA steps. n.s. using 2-way ANOVA **(c)** Input/Output curve of number of APs following current injection at 20pA steps. *represents difference between AbTr F and UnTr F *p<0.05; #represents difference between AbTr F and UnTr M #p<0.05; &represents difference between AbTr F and AbTr M &p<0.05 using 2-way ANOVA. Traces deptic 180pA current input. **(d)** Average AP peak amplitude. **p<0.01 using 2-way ANOVA. **(e)** Average after-hyperpolarization amplitude. *p<0.05 using 2-way ANOVA. **(f)** Average SAG amplitude. *p<0.05 using 2-way ANOVA. **(g)** Average AP half width. n.s. using 2-way ANOVA.

Figure 4

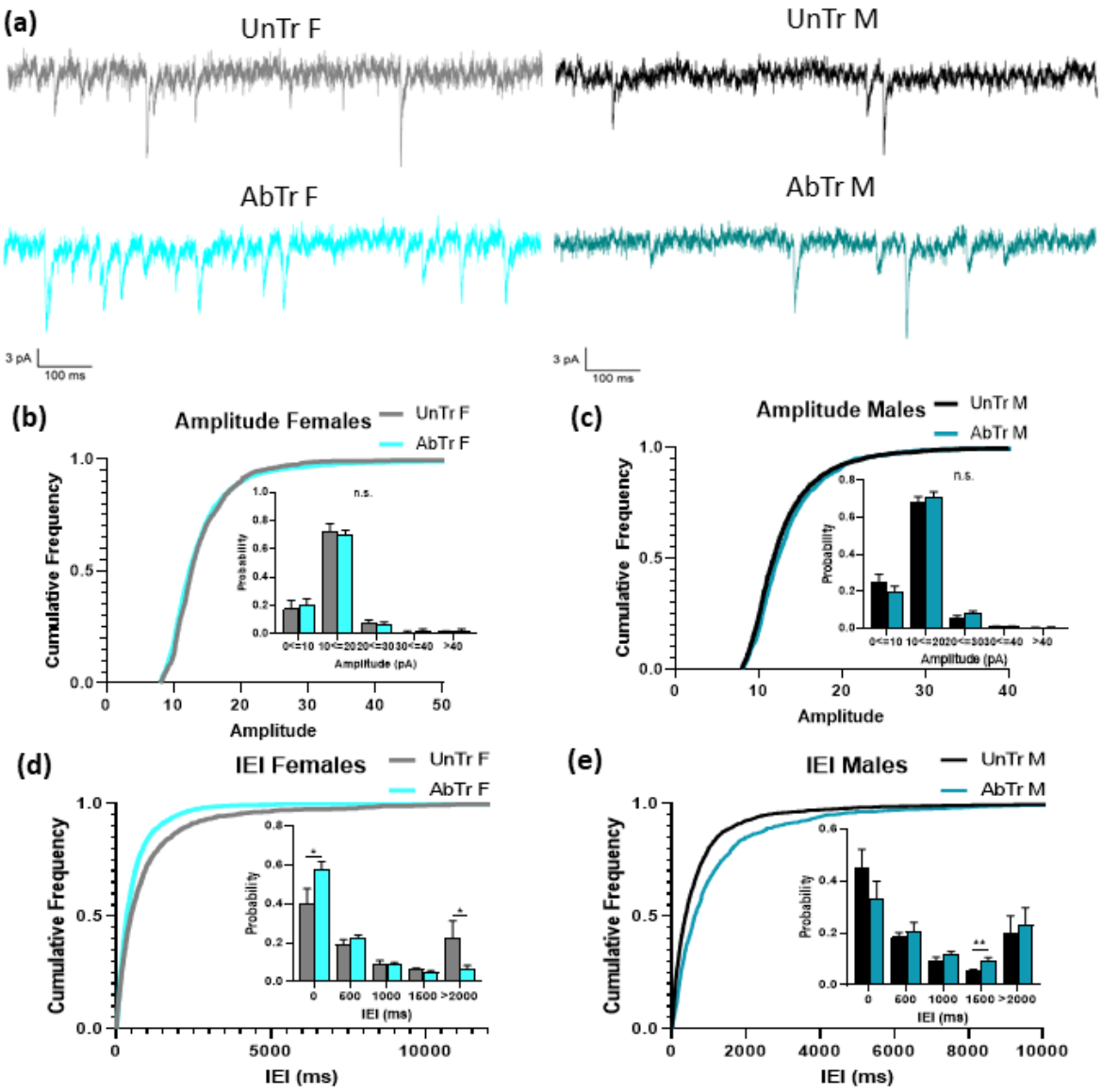

Figure 4. (a) Recordings of sEPSCS from layer2/3 cortical neurons from AbTr and UnTr male and female mice. **(b)** Cumulative distributions of sEPSC amplitudes in neurons from female AbTr and UnTr mice (n.s. using KS test). **(c)** Cumulative distributions of sEPSC amplitudes in neurons from male AbTr and UnTr mice (n.s. using KS test). **(d)** Cumulative distributions of IEIs from female ABTr and UnTr mice showing a leftward shift of the AbTr distribution (KS test, $p<0.001$). **(e)** Cumulative distributions of IEIs from Male ABTr and UnTr mice showing a rightward shift of the AbTr distribution (KS test, $p<0.001$).

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Chapter 4. Summary and Discussion

This thesis presents data describing a role for the gut-brain axis in the pathogenesis of seizure-susceptibility. Previous work has highlighted a correlational relationship between gut microbiome alterations and seizure-burden both in rodent models of epilepsy and human epilepsy patients(1–12). However, there is a paucity of data on potential mechanistic targets in the gut-brain axis in epilepsy, and no study had previously addressed the gut-brain axis in CNS-infection-induced seizures nor examined the gut-brain axis in pediatric rodent models of seizure-susceptibility. In chapter 1, we outline current evidence on microbial-metabolites and their various potential roles in epilepsy and seizure-susceptibility, and introduce S-equol as a unique candidate for further exploration. In chapter 2, we further explore the effect of S-equol on TMEV-induced alterations in neuronal physiology, and present a novel description of alterations in the gut microbiome in a model of CNS-infection-induced seizures. Finally, in chapter 3, we describe alterations in the gut microbiome following perinatal and postnatal exposure to antibiotics, and demonstrate that antibiotic treatment alters seizure-susceptibility and neuronal physiology, highlighting the need for future studies to assess the role of the gut microbiome in this antibiotic induced seizure-susceptibility. Together, these findings provide new insights on gut microbe perturbations in adult-onset CNS-infection-induced and pediatric seizure-susceptibility, and identify microbial-derived S-equol as a mechanistic link between the gut microbiome and CNS-infection induced seizures.

Infection-induced seizures and the gut microbiome

CNS infection is the most common cause of epilepsy worldwide and disproportionately affects low income populations across the globe(13). CNS infections can cause seizures by directly killing neurons, often in the temporal lobe, or through general neuroinflammation, as

neuroinflammation alone can cause seizures in genetic rodent models(14,15). Infection-induced seizures occur in ~30% of CNS infections but do not always result in the subsequent development of epilepsy(16,17). CNS infection-induced epilepsy has historically been difficult to model in rodents due to high mortality rates following inoculation with viruses that cause seizures in humans(16). Lack of translational rodent models has led to a severe unmet need for targeted therapeutics in infection-associated seizures and epilepsy. Recent publications have touted Theiler's murine encephalomyelitis virus (TMEV) as a low-mortality model of viral-induced epilepsy(18,19). Like CNS infection-induced epilepsy in humans, seizures caused by TMEV-infection are generally refractory to available AEDs, making the TMEV model of epilepsy ideal for studying alternative therapeutic strategies for treating uncontrolled seizures(20).

The gut microbiome is increasingly implicated as a possible therapeutic target in a variety of neurological disease including epilepsy(21–23). Although infection-induced seizures are highly prevalent worldwide, and a growing body of evidence highlights a role for the gut microbiome in seizure-susceptibility, alterations in the gut microbiome in rodent models of CNS infection-induced seizures have not been previously reported. In chapter 2, we identify substantial alterations in microbial communities in TMEV-infected mice compared to PBS-injected mice at 5-7 dpi, including marked reduction in genus *Allobaculum*. In neurological disease, *Allobaculum* reduction has been described in mouse models of both depression and schizophrenia(24,25). Interestingly, both schizophrenia and depression are correlated with increased neuroinflammation, and early-life infection may play a role in later neurological disease development(26,27). In turn, probiotic supplements that improve outcomes following influenza infection also increase gut abundance of *Allobaculum*, indicating that *Allobaculum* may be a key player in the immune-gut-brain axis(28). Within TMEV-infected rodents, we also identify several bacterial taxonomies that are increased

in TMEV-infected mice that do not exhibit seizure-phenotypes compared to TMEV-infected mice that develop acute seizures 5-7 dpi. We further explore possible roles for these altered taxonomies in host biology, and focus on microbial production of S-equol.

Limitations of our study include that we only examined the gut microbiome in TMEV-infected mice at a single timepoint, 5-7dpi. These data do not address the timeline of microbiome alteration in relation to TMEV-induced disease progression, nor do they provide causal evidence of microbiome differences leading to seizure-susceptibility or of seizures leading to microbiome alterations. Future studies should examine the gut microbiome at multiple relevant disease timepoints, including before TMEV-infection (-1dpi), during TMEV-infection but before seizure onset (1-2 dpi), the peak of seizure onset (5-7 dpi, current study), during epileptogenesis (1-5 weeks post infection) and during spontaneous recurrent seizures (>2months post infection) to fully explore the relationship of gut microbiome alterations and seizure-susceptibility following TMEV-infection. Additionally, data in chapter 2 suggest that loss of *Allobaculum* is related to TMEV-infection regardless of seizure phenotype. As probiotic supplementation increases *Allobaculum* abundance rodents infected with influenza(28), and probiotics reduce seizure burden in PTZ models of seizure-susceptibility(1,11), probiotic supplementation should be tested in both infection and seizure outcomes following TMEV infection.

Role of S-equol in the gut-brain axis

Our work provides evidence for the role of S-equol as a mechanistic link in the gut-brain axis in seizure-susceptibility, particularly in TMEV-induced seizures. We also explore two possibly synergistic hypotheses: 1. Gut perturbations alter the susceptibility of mice to TMEV-induced seizures via alterations in serum S-equol, and 2. S-equol alters neuronal physiology via

GPR30. Experimental evidence and future considerations for each of these hypotheses is outlined below.

In chapter 2, we describe alterations in the gut microbiome related both to TMEV-infection status as well as seizure-phenotype following TMEV-infection. Specifically, we note loss of bacterial taxonomies related to the production of S-equol in TMEV-infected rodents with seizure-phenotypes, indicating a possible link between microbial production of S-equol and seizure susceptibility following TMEV-infection. S-equol is produced solely by gut microbiome metabolism of dietary daidzein(29). The levels by which mice are able to convert dietary daidzein to S-equol vary significantly by mouse strain(30). Seizure-susceptibility and microbiome content are also reported to vary by strain as well as company of origin(31,32). Interestingly, mouse strains reported to be poor converters of daidzein into S-equol, including C57 and C3H strains, are also separately reported to exhibit spike-wave discharge activity under control conditions, indicating absence seizure-like activity, while swiss inbred (SW) mice, which are reported to be high converters of daidzein to equol, do not exhibit seizure-like epileptiform activity under control conditions(33). Together, these studies indicate a possible connection between mouse strain/origin, seizure susceptibility, and microbial production of S-equol.

Although we report in chapter 2 a reduction in S-equol producing microbial populations at 5-7 dpi in a combination of male and female mice with seizure phenotypes following TMEV-infection, our preliminary findings did not reach statistical significance in reduction of serum equol at 5 dpi, and unexpectedly we report a significant increase of serum equol in TMEV-infected mice at 7 dpi. As TMEV-infected mice lose weight, presumably due to decreased food intake, we posit that serum equol levels may be predominantly controlled during acute seizures by a reduction of dietary daidzein intake in TMEV-infected mice with seizure phenotypes. By 7 dpi, mice may begin

to normalize food intake as seizure burden subsides, which would account for the significant increase in serum equol at this timepoint. A controlled study monitoring food intake, seizure burden, and serum equol levels must be performed in order to elucidate serum equol dynamics following seizure susceptibility.

As serum equol trends lower in TMEV-infected rodents with seizure phenotypes, and as we demonstrate the ability of S-equol *in vitro* to reduce TMEV-induced neuronal hyperexcitability, in chapter 2 we additionally performed a pilot pharmacology study to assess the potential of S-equol as an AED in TMEV-induced seizures. However, once daily dosing of 10mg/mL S-equol in 4% CMC did not alter TMEV-induced seizure burden in male of female mice. Some limitations of this experiment were that S-equol dosing began only 3 days before TMEV-infection, and S-equol serum concentration obtained during dosing is unknown. Additionally, our data only cover equol dynamics during the acute seizure phase of TMEV infection, while equol-producing bacteria and serum equol dynamics may be altered during epileptogenesis and/or the spontaneous recurrent seizure phase following TMEV-infection. Studies examining acute, latent, and spontaneous recurrent seizure phases of TMEV pathogenesis are necessary to fully examine the role of the gut microbiome and S-equol in CNS infection-induced epilepsy.

S-equol alters neuronal physiology

In chapter 2, we present data on the effect of supraphysiological (300μM) exogenous S-equol on brain slices from naïve rodents. 300μM exogenous S-equol lowers the threshold for pyramidal neurons in cortical layer 2/3 to fire and single action potential following current injection, and decreases the number of action potentials fired following increasing 20pA current steps. Although previous studies have also utilized supraphysiological concentrations of S-equol to assess the role of S-equol in physiology(34), we additionally examined S-equol at lower, more

physiological concentrations. As discussed above, serum equol is highly dependent on mouse strain as well as dietary phytoestrogen content(30). Oral gavage of racemic equol in Sprague Dawley rats leads to serum equol concentrations as high as 27μM (35). Conversely, subcutaneous equol injection leads to serum equol concentrations up to 7.5μM in mice. Addition of equol to mouse diet leads to serum equol concentrations up to 8.1μM (36). In context of these findings, we additionally examined the effect of 10μM S-equol on acute brain slices, and found that 10μM exogenous S-equol also lowers the threshold for pyramidal neurons in cortical layer 2/3 to fire and single action potential following current injection, and decreases the number of action potentials fired following increasing 20pA current steps. Finally, based on data from rodents fed chow with similar phytoestrogen content to our own chow, we estimated that physiological levels of S-equol produced by the gut microbiome might be close to 1μM(37,38). Additionally, preliminary pharmacology studies indicate that human serum concentrations approach 1μM following a single bolus of S-equol, indicating that 1uM is a translational, pharmacologically-relevant concentration(39). We demonstrated a time-dependent effect of exogenous 1μM S-equol on neurons from naïve rodents as well as a reduction in neuronal hyperexcitability in neurons from rodents infected with TMEV. However, subsequent analysis of the serum content of our mice revealed baseline serum equol concentrations ranging from 4-8 ng/ml in our mouse population (Figure S1). As nM concentrations of exogenous S-equol can alter astrocytic and neuronal biology in cell culture following 48h incubation(40), it is possible that concentrations that reflect microbial-derived S-equol may alter neuronal physiology at longer timescales than addressed in this text. Further studies in cultured slices or neurons are necessary to elucidate the role of microbial-derived S-equol on neuronal physiology. However, our results demonstrate the ability of exogenous S-equol to reduce neuronal hyperexcitability following TMEV-infection and alter

neuronal physiology in a concentration and time-dependent manner. Furthermore, as our results examine S-equol concentrations achievable through S-equol dosing in vivo, the current text presents evidence on the potential of S-equol as a novel antiepileptic therapeutics.

S-equol potential mechanism of action

BK channels

There are several possible mechanisms by which S-equol may alter neuronal physiology and decrease neuronal hyperexcitability. The expression of large-conductance calcium-activated potassium (BK) channels is altered in various models of seizure-susceptibility, and both loss and gain of function mutations in BK channels can lead to epilepsy in humans(41). Equol has been shown to increase cerebral blood flow in a BK channel-activation dependent fashion, and equol increases K^+ currents in smooth muscle cells and HEK cells expressing BK channels(42). This in vitro effect was accordingly found to be dependent on equol acting on the beta1 subunit of BK channels(42). Equol also decreases serotonin-induced vascular contractions in rat carotid arteries in a BK-dependent manner(43), and while equol increases BK currents, it decreases hKv1.5, hKv4.3, IK, and IhERG currents in HEK cells expressing various cardiac specific K^+ channels(44). We therefore assessed the effect of co-application of paxilline, a selective inhibitor of BK channels, and S-equol on neuronal threshold to fire an action potential following current injection. Preliminary analyses suggest that paxilline does not interfere with the ability of S-equol to reduce the threshold to fire an action potential following current injection in neurons from naïve mice, indicating that S-equol is acting via a mechanism distinct from BK channel activation (Figure S3a). These discrepant findings may be explained by differential expression of beta subunits in smooth muscle BK channels and neuronal BK channels. BK channels in smooth muscle cells express the β1 BK subunit, while BK channels in neurons predominantly express the β4 subunit(45), and

previous studies in smooth muscle cells indicate that equol acts by directly binding to the β1 subunit of BK channels(42).

Estrogen receptor beta

S-equol is also known to bind to estrogen receptor beta (ESRβ)(29). ESRβ is widely expressed throughout the CNS(46), and estradiol has been shown to be protective against kainic-acid induced seizures via ESRβ activity(47). Additionally, estrogen supplementation is neuroprotective in hippocampal cells in rodents after status epilepticus(48). However, estrogen and estradiol therapies make poor therapeutic targets for epilepsies due to their widespread effects on multiple organ systems, especially in females. While S-equol binds to ESR2, it has not been shown to have feminizing peripheral effects, making it an interesting candidate for ESR2-mediated therapies(49). S-equol exhibits similar anti-inflammatory activity in microglia exposed to LPS to that of estradiol(50), is cytoprotective in human endothelial cells via ESRβ activation(51), and is neuroprotective in vivo against cocaine-induced loss of synaptic spines via ESRβ(52). Furthermore, equol may increase intracellular calcium and alter vitamin D expression via ESR2 transcriptional activation in intestinal HT29 cells. Therefore, we assessed the effect of S-equol on neuronal function in the presence of PHTPP, a selective ESRβ inhibitor. However, our preliminary analyses show no effect of PHTPP on the ability of S-equol in increasing neuronal threshold to fire an action potential following current injection (Figure S3b). Although equol has a low binding affinity for estrogen receptor alpha (ESRα), in neuroblastoma cells, equol may be neuroprotective against amyloid beta toxicity via ESRα activation(53). While S-equol is produced by gut microbes in an enantiomer-specific fashion, racemic equol and R-equol can be chemically synthesized(29). R-equol has a higher affinity for ESRα than S-equol, possibly confounding published data on the effect of racemic equol supplementation on health outcomes(54). Additionally, racemic equol has

been shown to alter estrogen receptor gamma (ESRγ) activity in cell culture. Further examination of ESRα and ESRγ binding of S-equol in neurons is necessary for a comprehensive examination of the mechanisms of the effect of S-equol on neuronal physiology(55).

GPER1/GPR30

Estrogen receptor-mediated changes in neuronal properties also occur in the brain through non-transcriptional pathways, including via activation of G protein-coupled estrogen receptor (GPER1) also known as G protein-coupled receptor 30 (GPR30)(56). GPR30 expression is reduced in microglia from female (but not male) patients with refractory epilepsy due to cortical malformations(57), and GPR30 knockdown rats experience shorter latency to status epilepticus following pilocarpine injection compared to WT controls. GPR30 knockdown rats also exhibit increased hippocampal neuron loss and neuroinflammation compared to WT(58). Furthermore, GPR30 agonist G1 decreases spontaneous excitatory postsynaptic current (sEPSC) frequency in cultured neurons, and in turn, GPR30 antagonist G15 increases sEPSC frequency, highlighting the role of GPR30 in excitatory synaptic transmission(57). S-equol alters dendritic arborization in cultured neurons and F-actin rearrangement in cultured astrocytes via GPR30 activation(40). Furthermore, S-equol inhibits nitric oxide production by cultured astrocytes following LPS exposure via GPR30 activation indicating that neuroprotective and glioprotective effects of S-equol may be mediated through non-genomic estrogen signaling rather than classical genomic estrogen signaling(59). Interestingly, our preliminary analyses suggest that co-administering S-equol and GPR30 antagonist G15 ameliorates the effect of S-equol on the threshold to fire an action potential following current injection in neurons from TMEV-infected mice (Figure S3c). These data indicate that S-equol may alter neuronal physiology via activating GPR30.

GPR30 is widely expressed in cortical and hippocampal pyramidal neurons, and GPR30 activation may alter neuronal physiology through several pathways(60,61). GPR30 activation reduces cultured hippocampal cell death following glutamate excitotoxicity and H2O2 exposure(62–64). *In vivo*, GPR30 activation reduces hippocampal cell death following traumatic brain injury in rats(65), and reduces anxiety behaviors via blocking stress-induced downregulation of GABAARs in the amygdala(66). Furthermore, GPR30 activation is neuroprotective following neuronal injury by reducing phosphorylation of the NR2B NDMAR subunit, suggesting that GPR30 plays a role in excitation-inhibition balance(67). Alternatively, GPR30 activation is involved in hippocampal acetylcholine production(68,69), which may be related to severity of status epilepticus in rodent models of epilepsy(70). Furthermore, GPR30 is also expressed in both astrocytes and microglia, and GPR30 expression in microglia negatively correlates with seizure frequency in women with refractory epilepsy(57), suggesting that S-equol activation of GPR30 may be neuroprotective via glial mechanisms. In cell culture, GPR30 activation increases astrocyte GLT-1 expression and accordingly increases astrocytic glutamate uptake, indicating that S-equol activation of GPR30 may be neuroprotective in glutamate excitotoxicity in epilepsy through altering astrocytic glutamate processing(71).

Overall, our findings provide evidence to support a neuroprotective effect of S-equol in TMEV-induced hyperexcitability, possibly through S-equol activation of GPR30. As GPR30 activation in neurons and glial cells plays a role in epilepsy pathology, as well as pathology of other neurological diseases, these findings suggest benefit of future studies on the interaction of microbial-derived S-equol, glia, and neurological disease.

Development, seizures, and the gut microbiome

Seizures are one of the most common causes of hospitalization in neonates and are predominantly caused by HIE, cerebral malformations, or fever(72). The developing brain is acutely susceptible to seizures due to generally enhanced neuronal excitability and increased plasticity leading to aberrant re-wiring following acute injury(73). Maternal lifestyle, diet, and drug use all play a role in neurodevelopment(74–76). Importantly, the developing infant microbiome is sourced largely from maternal microbes, and the maternal microbiome consequently plays a large role in healthy brain development(77,78). Infants with epilepsy have altered gut microbiomes compared to healthy age-matched controls, and ketogenic diet alterations in the infant microbiome correlate with diet-induced reductions in seizure burden, indicating that the gut microbiome may play a role in infantile seizure-susceptibility(4). However, no study has previously examined the gut microbiome in a pediatric model of seizure-susceptibility or epilepsy.

The gut-brain axis in pediatric seizure-susceptibility

We provide data in chapter 3 suggesting a link between perinatal and postnatal microbiome alteration and pediatric seizure susceptibility, potentially via alterations in neuronal physiology. We replicate previous reports that perinatal and postnatal administration of antibiotics reduces gut microbiome diversity and alters gut microbiome composition, and present new evidence that these changes correlate with increased seizure-susceptibility at p9/p10 following increasing exposure to hypoxic conditions. Additionally, we provide data on sex-specific alterations in neuronal physiology following perinatal and postnatal antibiotic exposure, suggesting that antibiotic-induced gut microbiome perturbations may affect steroid-driven neural developmental pathways. Together, these data provide evidence on a novel role for the gut-brain axis in pediatric seizure-susceptibility.

The current findings address acute seizures following perinatal and postnatal antibiotic exposure, but do not provide evidence on later-in-life outcomes following pediatric acute seizures. In human patients, HIE is associated with poor cognitive outcomes and increased incidence of epilepsy later in life(79). Follow-up experiments on the effect of perinatal and postnatal antibiotic exposure on subsequent development of spontaneous recurrent seizures are necessary to further examine the role of the gut microbiome and pediatric epilepsy.

Limitations of these data include the correlative nature of our findings on the role of the gut microbiome in pediatric seizure-susceptibility and neuronal physiology. A reasonable alternative hypothesis based on the data presented in chapter 3 is that direct effects of antibiotics on the brain result in increased seizure-susceptibility and alterations in neuronal physiology independent of the effect of antibiotic treatment on the gut microbiome. To tease apart these disparate findings, future studies including microbiota rescue or identification of the mechanism(s) of action by which the gut microbiome may mediate pediatric seizure-susceptibility are necessary.

S-equol in pediatric S-equol susceptibility

In chapter 3 we discuss multiple distinct mechanisms by which the gut microbiome may exert neuroprotective effects in the pediatric brain, including modulation of neurotransmitters, neuroinflammation, and microbial metabolite production. In chapters 1 and 2, we highlight S-equol as a microbial metabolite of interest in adult seizure-susceptibility and demonstrate the ability of exogenous S-equol to alter neuronal physiology in acute slices from naïve adult mice, potentially via activation of cerebral estrogen receptors. Cerebral estrogen receptors, including GPR30, play important roles in the shaping of neurodevelopment by estrogen and estradiol(80–82), and S-equol is posited to play a positive role in cerebral development by altering astrocyte migration dynamics via GPR30(40). As antibiotic treatment reduces plasma equol concentrations(83), and we

demonstrate sex-specific alterations in neuronal physiology following antibiotic treatment, it is possible that loss of S-equol signaling via GPR30 or genomic estrogen receptors is a mechanism by which antibiotic treatment leads to increased seizure-susceptibility in pediatric mice. Future studies are warranted on the effect of exogenous S-equol on neurons from pediatric mice, as well as on seizure behavior in pediatric models of epilepsy.

Conclusions

Collectively, we have provided evidence for gut-brain interactions in rodent models of seizure-susceptibility. The vast majority of the current AEDs targets ion-channels and are ineffective in 1/3 of people with epilepsy and the remaining 2/3 of epilepsy patients are not cured from the disease. The current body of work addresses this need by targeting the gut microbiome in models of epilepsy and has promise for the development of novel microbial-derived therapeutics. We described gut microbiome alterations in TMEV-infected mice, and further outlined gut microbiome alterations specific to TMEV-induced seizure phenotype. This work demonstrates a functional role for gut-metabolite S-equol in reducing TMEV-induced neuronal hyperexcitability *ex vivo*. We have additionally characterized neruomodulative properties of S-equol *in vitro* in acute brain slices from naïve mice. Finally, we provided evidence that perinatal and postnatal antibiotic exposure reduces gut microbiome diversity and increases seizure susceptibility in a pediatric model of HIE-induced seizures, and demonstrated that this antibiotic exposure alters neuronal physiology in a sex-dependent fashion. Together, these findings implicate gut microbe alterations in seizure-susceptibility in adult and pediatric models, and pose a novel role for microbial-derived S-equol in the gut-brain axis.

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