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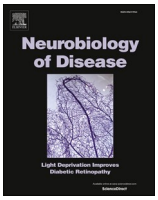

Gut microbiome effects on neuronal excitability & activity: Implications for epilepsy

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ABSTRACT

It is now well established that the bacterial population of the gastrointestinal system, known as the gut microbiome, is capable of influencing the brain and its dependent functions. Links have been demonstrated between the microbiome and a variety of normal and pathological neural functions, including epilepsy. Many of these microbiome-brain links involve the direct or indirect modulation of the excitability and activity of individual neurons by the gut microbiome. Such links may be particularly significant when it comes to microbiome modulation of epilepsy, often considered a disorder of neuronal excitability. In this review we consider the current evidence of a relationship between the gut microbiome and the excitability or activity of neurons in the context of epilepsy. The review focuses particularly on evidence of direct, causal microbiome effects on neuronal excitability or activity, but also considers demonstrations of microbiome to host interactions that are likely to have an indirect influence. While we identify a few common themes, it is apparent that deriving general mechanistic principles of microbiome influence on these parameters in epilepsy will require considerable further study to tease out the many interacting factors, systems, and conditions.

1. Introduction

The bacterial population of the human and other mammalian gastrointestinal system both depends upon and influences its host. This bi-directional relationship is mediated through a variety of biological pathways and is modulated by environmental factors including diet, stress, sleep, exercise, and social interactions. Microbe-to-host interactions are long established and indeed biologically expected (Gould et al., 2018), and can be observed in the local gastrointestinal environment as well as in the immune system with consequences for general host health (Kinross et al., 2011). Further, recent studies have begun to suggest that these interactions may have significant impacts on the nervous system and consequently on sensation, cognition, behaviour, and disorders of that system (Cryan and Dinan, 2012). Corroboration and characterization of those observed impacts is ongoing, including attempts to extricate the specific role of the microbiome from those of its above-mentioned interacting systems and factors, and to establish the power and potential of the microbiome to influence the nervous system in given circumstances.

One such circumstance is that of the epileptic or seizing brain. Although gut microbiome-brain influence has primarily been explored in neuropsychiatric, rather than neurological, conditions (Cryan and Dinan, 2012) epilepsy is something of an exception to this rule. Perhaps stemming from the long-established role of diet in epilepsy treatment, interest in the potential of direct or indirect microbial roles in the development and treatment of epilepsy has been robust (Dahlin and Prast-Nielsen, 2019; Lum et al., 2020). Notwithstanding this, progress has mostly been observed since 2010 and this is therefore a field in its relative infancy. A specific and targeted microbiome therapeutic intervention for epilepsy could potentially achieve the efficacy of a dietary intervention without its challenging compliance (Martin et al., 2016). Consequently, identifying and characterizing causal influences of microbiome perturbations on seizure-relevant neural properties is a vital avenue of investigation.

This review takes the approach of focusing on the influence of the microbiome on neuronal excitability and activity. Epilepsy is of course a disorder of neural activity, and has often been characterized as a disorder of increased neuronal excitability. It has been proposed that the susceptibility of the brain to entering a seizure state is due to the ease with which physiological oscillations can become pathological with transient or permanent alterations in the excitatory/inhibitory balance (Olsen and Avoli, 1997). As such, any disruption of the excitability of

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individual neurons or networks by the gut microbiome has, at least, the potential to play a role in epilepsy and seizures. The review therefore centres on studies in which the microbiome is manipulated and the effects on neuronal excitability and/or activity are measured. It also considers studies showing non-causal relationships between these variables of interest. Finally, it uses investigations of the relationship between microbiome-influenced systems and other neural properties to highlight potential microbiome modulation of excitability and activity.

These indirect methods of microbiome modulation of neuronal properties are as varied as the biological systems and pathways with which the microbiome interacts. Current evidence suggests that the microbiome-gut-brain axis consists of several major routes (Foster et al., 2017). An in-depth discussion of these can be found elsewhere (Cryan et al., 2019). Briefly, the gut microbiome has been demonstrated to interact with the host via microbial metabolites (such as short-chain fatty acids, and neurotransmitter production), tryptophan metabolism, gut hormone signalling, host immune/inflammatory systems, and the enteric/vagus nerves.

The influence of the microbiome on its host is studied in various ways. Correlative studies characterize the microbiome in different states and its relationship with the host parameter of interest. Germ-free (GF) studies compare host parameters in control mice and mice completely lacking a gut microbiome, indicating the absolute relevance of the microbiome on those parameters throughout the mouse's lifespan (Turnbaugh et al., 2009). Antibiotic studies disturb or deplete the microbiome at particular timepoints via oral administration of antibiotics, either as a tool to achieve microbiome-level effects or to investigate the consequences of therapeutic antibiotic use (Rakoff-Nahoum et al., 2004; Basolo et al., 2020). Probiotic and prebiotic (sometimes referred to collectively as synbiotic) studies answer the opposite question, "feeding" the microbiome and encouraging its growth before measuring the consequences for the host (Hemarajata and Versalovic, 2013). Again this may be done to achieve specific microbiome states (e.g. recovering a "healthy" microbiome from a depleted condition) or as a direct test of therapeutic potential. Finally, faecal microbiota transplantation (FMT, often used in combination with initial antibiotic depletion) directly transplants a microbiome (via stool) from one host to another, to investigate whether host qualities come with it (Gupta et al., 2016).

2. Gut microbiome effects on enteric nervous system neuronal activity

The enteric nervous system (ENS) consists of a web of nerve fibres innervating the mucosal tissue in the gut, principally regulating digestive processes. Inherently, the ENS is at the front-line of potential microbial-neuron interactions, and thus initial investigations of the impact of the microbiome on neurons tended to concentrate on this system. Shared neurophysiology of ENS and central nervous system (CNS) neurons also suggests that influences of the microbiome seen in the ENS may also have the potential to impact CNS function (Wood, 1984). From the toolkit generally available to study the microbiome (see above), studies on ENS excitability/activity have mostly used supplementation with bacteria or their products, with a few using gnotobiotic animals and/or antibiotics to investigate the consequences of microbiome depletion. See Table 1 for a summary of gut microbiome effects on ENS neuronal activity.

An early investigation of microbiome effects on enteric nerves involved the oral administration of *Lactobacillus reuteri* to healthy rats to monitor its effects on peripheral pain. This bacterial gavage resulted in decreased dorsal root ganglion (DRG) single unit activity in response to colorectal distension, but it should be noted that this effect was irrespective of whether the bacteria were alive, heat killed, or gamma irradiated (Kamiya et al., 2006). As such it might be understood not so much as a direct effect of microbiome constituents but a potential effect of *L. reuteri* cells and/or their products. The same group further investigated DRG neuron properties after a similar preparation (with live *L. reuteri* only) and found that an increase in excitability (lower action potential threshold and more spikes induced by depolarization) was also opposed by the bacterial administration (Ma et al., 2009).

The ability of *Lactobacillus rhamnosus* to modulate enteric nerve activity has been investigated by adding 10^9 colony-forming units directly to the lumen. This was observed to cause both increased baseline and distension-induced firing rates of mesenteric neurons (Perez-Burgos et al., 2013) via a mechanism shown to be dependent on synaptic transmission from these myenteric neurons to the vagus nerve (Perez-Burgos et al., 2014). Another component of the microbiome, *Bifidobacterium longum* NCC3001, was also shown to inhibit the excitability of myenteric neurons in a pair of studies. Following up on an initial demonstration of behavioural and neural biochemical changes after

Table 1
Microbiome effects on Enteric Nervous System excitability and activity.

Study	Microbiome Manipulation	Neuronal Parameter	Direction of Change
Kamiya et al., 2006	Bacterial gavage	Stimulus-induced DRG unit activity	Decreased
Ma et al., 2009	Bacterial gavage	DRG neuron excitability	Decreased
Perez-Burgos et al., 2013	Bacterial direct application	Mesenteric neuron firing rates	Increased
Bercik et al., 2011	Bacterial direct application	Myenteric neuron excitability	Decreased
Sessenwein et al., 2017	Bacterial direct application	DRG neuron excitability	Decreased
Pradhananga et al., 2020	Bacterial direct application	Nodose ganglion neuron excitability	Increased
McVey Neufeld et al., 2013	Germ free	Jejunum neuron excitability	Decreased
McVey Neufeld et al., 2015	Germ free	Mesenteric neuron firing rates	Decreased

Green and orange indicate additive and subtractive modulations of the microbiome, red and blue indicate increases and decreases of neuronal parameters.

chronic gastrointestinal inflammation in mice and their mitigation by *B. longum* administration (Bercik et al., 2010), it was demonstrated that perfusion of the same species was associated with decreased excitability (number of action potentials elicited by a stimulus) of myenteric plexus neurons (Bercik et al., 2011). This mechanism was expanded upon in a demonstration that a similar perfusate of *B. longum* reduced input resistance and decreased the hyperpolarization-activated cation current I_h in a myenteric plexus preparation. Because these *L. rhamnosus* and *B. longum* studies use direct application to the cells in question instead of pre-mortem gavage they are further distanced from the likely physiological role of the bacteria in question.

The study of peripheral pain provided some insights into potential modulation of neuronal excitability by the microbiome when Sessenwein et al. (Sessenwein et al., 2017) administered a mix of gastrointestinal microbial products from a human donor to mouse DRG neuron ex vivo cultures by overnight incubation. They found that rheobase (the minimum current capable of reaching depolarization threshold) was increased in a concentration-dependent manner, resulting in decreased excitability and spontaneous action potential discharge without changing input resistance, capacitance, resting membrane potential, action potential duration and threshold, or after-hyperpolarization amplitude. These effects were not present when the bacterial products were heat-killed. In subsequent experiments the same mix had the opposite effect on nodose ganglion neurons, increasing their excitability (Pradhananga et al., 2020). Both of these effects were apparently via the activity of proteases, secretory products of the microbiome.

All of these studies involved the administration of bacteria, either in vivo or ex vivo, and as such demonstrate a potential rather than a defined role of the microbiome on excitability. McVey Neufeld et al. performed whole-cell patch-clamp electrophysiology of jejunum neurons, and found that adolescent GF mice had reduced excitability, through a reduced resting membrane potential, compared to SPF mice function (McVey Neufeld et al., 2013). The same group also found that GF mice had a suppressed response to a K^+ channel blocker (TRAM-34) in the mesenteric nerve bundle of the jejunum, measured as the multi-unit rate of action potential firing (McVey Neufeld et al., 2015). Re-colonizing GF mice with a conventional microbiome was able to rescue both excitability and activity. This suggests that the microbiome effects on local neurons could take place in adulthood, rather than being entirely attributable to developmental changes (see below for more on the microbiome in neural development and its consequences for activity and excitability).

In summary, peripheral nervous system studies demonstrate that the microbial population of the gastrointestinal system are capable of influencing neuronal excitability and activity at the direct interface between the microbiome and neurons. Effects can be in either direction, with the majority so far being suppressive of action potential firing. *L. reuteri* suppresses stimulus-induced DRG firing, *B. longum* decreases myenteric plexus excitability, a mix of microbial products decreases DRG excitability, and GF mice have reduced jejunal neuron excitability and response to K^+ blockers. In opposition, *L. rhamnosus* increases baseline and distension-induced myenteric neuron firing, and the above-mentioned mix of bacterial products increases the excitability of nodose ganglion neurons. While the studies involving direct application of bacteria may have limited implications for the CNS and epilepsy, some of the mechanisms involved (including neuroactivity of microbial secretions) may be relevant. Given the vagus nerve's extensive secondary connectivity to CNS regions via the nucleus tractus solitarius, chronic modulation of vagal activity also likely has the potential to elicit long-lasting changes to CNS circuitry and activity.

3. Gut microbiome effects on CNS excitability

Direct examination of microbiome/gut influences on CNS activity is still relatively understudied. However, extensive evidence indicates that resultant behaviours, particularly in relation to mood and emotional cognition, can be modified by both dietary changes and direct microbiome manipulation (through microbial transplant, pre/probiotic, or antibiotic depletion) (Cryan et al., 2019). It logically follows then that the neural activity underlying such behaviours can change as a result of the dietary/microbial interventions. Nevertheless, it is still an open question as to what forms of neural activity (spanning the levels of single synapse through to whole network activity) are susceptible to the microbiome-gut-brain axis communication, and why certain behaviours/brain regions appear to be more sensitive to gut-based manipulations. See Table 2 for a summary of gut microbiome effects on CNS neuronal excitability.

The hippocampal CA3-CA1 synapse of male germ-free mice exhibited substantially reduced LTP, without altering the somatic output, suggestive of compensatory changes in the excitability of the CA1 neurons (Darch et al., 2021). The sex-specificity is of interest as many of the behavioural impacts of microbiome interventions, both in humans and pre-clinical models appear to involve sex differences (Holingue et al., 2020). Additionally, the unaltered output of the CA1 neurons and potential compensatory mechanism emphasises the importance of gathering a comprehensive view of the functional consequences of any microbiome interaction. Further, a four-week broad-spectrum antibiotic cocktail given to adult mice was sufficient to reduce synaptic transmission in the CA1 region (Caliskan et al., 2021). Specifically, fast EPSPs at the CA3-to-CA1 synapse were suppressed through a postsynaptic mechanism without affecting synaptic plasticity. It was also found that immune-active microglial cells in the hippocampus CA3 and CA1 was elevated in the antibiotic-treated mice, raising the possibility of involvement of non-neuronal cell types in the underlying mechanisms.

Complementary to these examination of the deprived microbiome, multiple studies have employed microbiome-fortifying pre- or probiotics, either in isolation or as potential protection against other effects. Rats fed a probiotic mixture of *B. longum* exhibited an moderate improvement in performance in a Barnes maze, in concert with increased hippocampal CA1 BDNF protein, dendritic arborisation and spine density, as well as increased LTP in hippocampal slices at 70 min post high-frequency stimulation (Talani et al., 2020). Other probiotics, as well as the prebiotic (food source for the microbiome) inulin have also shown the ability to alter hippocampal electrophysiological properties; specifically elevating resting membrane potential, sag conductance and membrane time constant tau, increasing evoked spike frequency and decreasing rheobase – all contributing to increased hippocampal excitability (Romo-Araiza et al., 2018). A separate prebiotic, when administered postnatally, was found to reduce the fast decay of hippocampal NMDAR currents throughout the life of rats as well as reducing anxiety (Spitzer et al., 2021). The efficacy of this intervention was specific to this early life stage; differential effects throughout the lifespan are a common theme of microbiome-host interactions (Langdon et al., 2016; Boehme et al., 2020).

Probiotic *Bifido-* and *Lactobacteria* administration was able to protect against alterations in Mg^{2+} (used as a marker of neurotoxicity) in whole brain samples after administration of neurotoxic doses of oral propionic acid (El-Ansary et al., 2018). It may be interesting to note that propionic acid (among other short-chain fatty acids) is a natural metabolite of the human gut microbiome (Louis and Flint, 2017), and may be a part of the microbiome-gut-brain axis' signalling. However, although propionate has been found to pass the gut barrier and may have protective influences on blood-brain barrier integrity (Hoyles et al., 2018), it is not yet clear whether potentially neuroactive concentrations of propionate (and other microbial products) cross the blood-brain-barrier. Probiotic supplementation was also able to reverse ethanol-induced memory impairments, but without also reversing fast excitatory post-synaptic potential (EPSP) and long-term potentiation (LTP) deficits in the hippocampus (Hadidi Zavareh et al., 2020). This is a notable example of a microbiome intervention having a behavioural effect without an expected accompanying impact on neuronal excitability and activity.

Table 2
Microbiome effects on Central Nervous System excitability.

Study	Microbiome Manipulation	Neuronal Parameter	Direction of Change
Talani et al., 2020	Oral probiotic	Hippocampal LTP	Increased
Romo-Araiza et al., 2018	Oral pro/prebiotic	Hippocampal excitability	Increased
Spitzer et al., 2021	Oral prebiotic	Hippocampal NMDAR current fast decay	Decreased
El-Ansary et al., 2018	Oral probiotic	Whole-brain Mg2+ neurotoxin response	Decreased
Hadidi Zavareh et al., 2020	Oral probiotic	Hippocampal fast EPSP/LTP	Unchanged
Darch et al., 2021	Germ-free	Hippocampal LTP	Decreased
Caliskan et al., 2021	Oral antibiotic	Hippocampal synaptic transmission & induced gamma	Decreased

Green and orange indicate additive and subtractive modulations of the microbiome, red and blue indicate increases and decreases of neuronal parameters.

4. Gut microbiome effects on CNS activity

The susceptibility of the brain to express seizures depends upon the excitability of individual neurons but more directly on how these neurons act, in terms of action potential firing rate and pattern, within networks (Blumenfeld, 2014). In this section we consider studies of the effect of microbiome interventions on the electrical activity of neurons and networks, again organised by the nature of the microbiome intervention. We will include in this consideration studies in which the outcome measure was seizure occurrence itself, primarily through the

Table 3
Microbiome effects on central nervous system activity.

	Study	Microbiome Manipulation	Neuronal Parameter	Direction of Change
A	Luck et al., 2020	Oral probiotic	Cerebellar pyramidal neuron firing	Increased
	Guida et al., 2018	Oral antibiotics	Hippocampal firing	Decreased
			mPFC firing	Unchanged
	Caliskan et al., 2021	Oral antibiotics	Gamma oscillation frequency	Increased
			Gamma oscillation power	Decreased
	Ogawa et al., 2021	Oral antibiotics	REM episodes	Increased
			Theta oscillation power	Decreased
	Chu et al., 2019	Oral antibiotics	Cue-encoding mPFC neuron activity	Decreased
	Luck et al., 2020	Germ free	Cerebellar pyramidal neuron firing	Decreased
	Aswendt et al., 2021	Germ free	Brain-wide functional connectivity	Increased
B	Allen et al., 2016	Oral probiotic	EEG Fz mobility	Increased
			EEG theta power	Decreased
	Kelly et al., 2017	Oral probiotic	EEG parameters	Unchanged
	Firestein et al., 2019	Perinatal antibiotics	1-3 Hz EEG power	Increased
			Broadband EEG power	Unchanged

Green and orange indicate additive and subtractive modulations of the microbiome, red and blue indicate increases and decreases of neuronal parameters. A: animal studies. B: human studies.

lens of its implication for paroxysmal neuronal activity. See Table 3 for a summary of gut microbiome effects on general CNS neuronal activity, and Table 4 for effects on epilepsy-related activity.

5. Non-epileptic activity of CNS neurons

Interest in the microbiome's potential to modulate CNS activity naturally spans fields of research including developmental and basic neuroscience as well as both gastrointestinal and neural disorders. Antibiotics are of interest both as a tool to deplete the microbiome and to investigate potential consequences of clinical antibiotic use. A mixture of antibiotics (ampicillin, streptomycin and clindamycin) administered orally over 2 weeks to healthy mice drastically decreased the firing (and burst firing) rate of CA3 hippocampal pyramidal neurons, changes which were opposed by probiotic treatment (Guida et al., 2018). Firing in the medial prefrontal cortex (mPFC) in response to amygdala stimulation was not changed in the antibiotic-treated mice. A broader cocktail of antibiotics (ampicillin/sulbactam, vancomycin, ciprofloxacin, imipenem/cilastatin, and metronidazole), referenced above for their effects on hippocampal synaptic transmission, (Caliskan et al., 2021), increased the frequency but decreased the power of gamma oscillations in CA3. A similar cocktail and dosing regime has been associated with altered sleep patterns: more frequent REM with reduced power in the theta range (Ogawa et al., 2020). Gamma oscillations have been associated with epileptiform neural activity (Ren et al., 2015) while the relationship between sleep and epilepsy is well established if not characterized (Kostopoulos, 2000; Derry and Duncan, 2013). Interestingly, hippocampal theta inversely correlates with the occurrence of epileptiform events during sleep (Nazer and Dickson, 2009).

Antibiotic-treated mice were found to have reduced activity (as measured by a genetically encoded calcium sensor) in certain medial prefrontal cortex (mPFC) neurons that responded to a conditioned stimulus (Chu et al., 2019). These animals (treated with ampicillin, gentamicin, metronidazole, neomycin, and vancomycin over 2 weeks) shared an impaired fear extinction profile with GF mice, but the latter's neural activity was not measured. Two studies that did investigate neural activity in GF mice found decreased firing (and increased synaptic density) in the cerebellum (Luck et al., 2020) and a general increase in functional connectivity (Aswendt et al., 2021). In the former case, the changes were reversible (or preventable) by re-colonization of the animals as neonates either with a full murine microbiome or with four human-derived *Bifidobacterium* species only, demonstrating a postnatal role for the microbiome in guiding neural development. In the latter study, the GF fMRI-derived global increases in functional connectivity were reversed after ischaemia, such that the GF values decreased and the SPF increased to similar levels. This active influence of the microbiome on the response to neural insult may be relevant to epilepsy-induced cell damage (Henshall and Meldrum, 2012).

Some studies have also measured brain activity in people with altered microbiomes. Two potential psychobiotics (psychoactive probiotics) in *B. longum* and *L. rhamnosus* were tested in healthy volunteers. The former showed EEG increases in Fz mobility and decreases in theta power (accompanying modulation of stress and cognition) (Allen et al., 2016) while the latter demonstrated no electroencephalographic (or behavioural) effects (Kelly et al., 2017). The increase in theta power associated with a microbiome boosted with *B. longum* is compatible with

Table 4
Microbiome effects on epileptic neuronal activity.

Study	Microbiome Manipulation	Neuronal Parameter	Direction of Change
Bagheri et al., 2019	Oral probiotics	PTZ kindling efficacy & severity	Decreased
Tahmasebi et al., 2020	Oral probiotics	PTZ kindling	Decreased
		Hippocampal pop spike amplitude post-kindling	Increased
Kilinc et al., 2021	Oral probiotics	PTZ seizure time to onset and duration	Decreased
Sabouri et al., 2021	Oral probiotics	PTZ seizure duration or severity	Unchanged
Gallucci et al., 2021	Metabolite direct application	Entorhinal cortex neuron hyperexcitability	Decreased
De Caro et al., 2019	Oral metabolite	Colitis-aggravated PTZ seizure susceptibility	Decreased
Olson et al., 2018	Dietary (ketogenic)	Seizure susceptibility & spontaneous seizures	Decreased
Mengoni et al., 2021	Faecal microbial transplant	Pilocarpine seizure susceptibility	Transferred
Medel-Matus et al., 2018	Faecal microbial transplant	Stress effect on amygdalar kindling susceptibility	Transferred

Green and orange indicate additive and subtractive modulations of the microbiome, red and blue indicate increases and decreases of neuronal parameters.

the decrease in hippocampal theta after antibiotic microbiome depletion, and may hint at coherent effects of the microbiome on oscillation, but one must bear in mind the difference in brain regions, species, and developmental stage. Indeed, the failure of the *L. rhamnosus* study to translate behavioural results from mice is another reminder of the dangers of positing mechanistic hypotheses when it comes to the microbiome and the brain. Finally, infants who were exposed to perinatal antibiotics and received standard care in the neonatal intensive care unit were shown to have higher EEG power at 1–3 Hz (but not at higher frequencies) than those who were not so exposed (Firestein et al., 2019).

Once again the evidence suggests a clear possibility for microbiome manipulations, whether additive or subtractive, to influence normal CNS activity. Furthermore, the degree and nature of these influences in specific circumstances must be determined by expanding and corroborating studies. For now we have suggestions that microbiome depletion may overall decrease neuronal activity and power at certain frequencies, at least in the hippocampus, cortex and cerebellum. Microbiome supplementation is less studied but has, if anything, an opposite effect. It must also be remembered that while epilepsy is a disorder of hyperexcitation a disruption of the excitation/inhibition balance in either direction can cause paroxysmal oscillations (Bauer, 1996; McCafferty et al., 2018).

6. Epileptic activity in animals

Perhaps because neuronal activity is considered more frequently in research of neurological than of neuropsychiatric disorders, and epilepsy is the neurological disorder most concretely linked with the microbiome via dietary therapies, a significant number of studies measuring microbiome effects on neuronal activity do so in the specific case of epileptic seizures. Studies of probiotics as a potential epilepsy therapy have been informative. It was demonstrated that a mixture of L. *rhamnosus*, *L. reuteri*, and *B. infantis* administered either before or during chemical pentylenetetrazol (PTZ) kindling in Wistar rats reduced both the efficacy of the kindling and the severity of seizures (Bagheri et al., 2019). A separate study showed that pre-kindling administration of an *Lactobacillus casei*, *Lactobacillus acidophilus*, and *Bifidobacterium bifidum* mix prevented full kindling in Wistars, as well as boosting dentate gyrus population spike amplitude after kindling (but not in un-kindled rats) (Tahmasebi et al., 2020). Probiotics have also been tested on individual PTZ-induced seizures, with an unspecified probiotic mix apparently delaying the time to onset and duration of generalized tonic-clonic seizures in rats (Kilinc et al., 2021), while another *L. casei*, *L. acidophilus*, *B. bifidum* mix did not reduce PTZ seizure duration or severity in mice (but did potentiate the protective effect of diazepam) (Sabouri et al., 2021).

There is also interest in the ability of microbial products, rather than microbes themselves, to modulate epileptic neural activity. Again these can be considered both as potential treatment options and as potential mechanisms of epileptic activity, but both concepts are limited by the ability and tendency of the products to reach their putative sites of action in the brain (whether physiologically in order to influence seizure development, or therapeutically). S-equol, a gut metabolite that is reduced in the Theiler murine encephalomyelitis model of virus-induced seizures, is capable of ameliorating the hyperexcitability of entorhinal cortex neurons from this model when directly applied (Gallucci et al., 2021). Sodium butyrate, a short-chain fatty acid produced by the microbiota, was seen to reduce susceptibility to PTZ-induced seizures (De Caro et al., 2019). Interestingly, this was via oral administration and was only effective in mice with colitis-aggravated seizure susceptibility.

A study by Olson et al. broke new ground in its implication of the microbiome in the anti-seizure effects of the ketogenic diet (Olson et al., 2018). While their results indicated that the diet increased GABA and glutamate levels in the hippocampus via changes in microbiome diversity and the prevalence of particular species, it did not demonstrate a particular mechanistic alteration of neuronal excitability or activity. However, the reduction in seizure susceptibility and spontaneous seizure occurrence both require a decrease in paroxysmal activity. Other studies have probed potential mechanisms of seizure activity modulation via the microbiome-specific intervention of FMT. FMT from donor mice in which pilocarpine had been used to induce status epilepticus both increased baseline EEG spiking (high-voltage deflections of <50 ms duration) in recipient mice, and caused 50% of recipients to enter status epilepticus within 50 min of a single subclinical pilocarpine dose (only 1 control recipient entered status) (Mengoni et al., 2021). FMT was also employed in an interesting study of the intersection between the microbiome, epilepsy, and stress in Sprague Dawley rats. Stressed rats and non-stressed recipients of a stressed donor FMT could be kindled to full seizure in about half the number of amygdala electrical stimulations compared to baseline, while stressed recipients of a non-stressed donor saw the opposite effect. Stress exacerbation on seizure duration was also apparently transmissible by FMT (Medel-Matus et al., 2018). Absence seizures also are manipulable via FMT, in that transplants from non-seizing Wistars and pharmacologically seizure-suppressed WAG/Rij rats reduces their expression in recipient untreated WAG/Rij (Citraro et al., 2021).

The scale and number of trials on probiotic effects on seizure will have to increase considerably to deliver any conclusive and consistent evidence of a potential therapeutic role. Currently, differences between species, seizure type, and probiotic composition limit the interpretability and translational relevance of such studies. On the other hand, well-designed FMT experiments do argue strongly for a microbiome-specific effect while the implication of gut composition in such an established and valuable therapy as the ketogenic diet makes it more probable that such an effect might be relevant, in one form or another, in humans.

7. Epilepsy in humans

Unsurprisingly, we are at a very early stage when it comes to establishing this exact relevance and fully translating microbiome-based therapies to people with epilepsy. The microbe product propionate has been observed to reduce the frequency of epileptiform discharges in human middle temporal gyrus neurons (in slices resected from children with epilepsy) (Bonnet et al., 2018), but the degree to which propionate might actually reach those neurons based on microbiome manipulations is unclear. A single case study demonstrated a complete recovery from seizures in a 17 year old girl with Crohn's disease after FMT from a healthy donor (He et al., 2017), while a pilot study of the potential effects of a probiotic (containing five *Lactobacilli*, two *Bifidobacteria* and one *Streptococcus* species) on drug-resistant epilepsy found that 28.9% of 43 patients had 50% or greater reduction in seizures after 4 months (Gomez-Eguilaz et al., 2018). Notably, this study did not have a control group and so stands more as an indication of the interest in probiotics as an epilepsy treatment than as evidence of their efficacy.

8. Secondary evidence of microbiome effects on neuronal activity

The microbiome is known to influence multiple biological systems that can in turn affect epilepsy-relevant neuronal excitability and activity. Foremost among these potential connections may be the neuroactive activities of certain microbial metabolites. Studies of the interaction between diet and the nervous system also strongly imply at least a partial role for the gut microbiome. Less directly, the microbiome may mediate some part of effects of the immune system, inflammation, and drug metabolism on the nervous system. In this section we will briefly highlight some relevant studies indirectly implicating the gut microbiome in neuronal excitability and activity, and also mention some purely correlational studies that measure microbiome and neuronal properties and their co-variation. The bi-directional nature of the

relationship between the microbiome and the brain, with brain-governed behaviours clearly able to change the composition of the gut flora, limits the conclusions we can draw from such observations and so we give just a few examples.

The gut-microbiome has been shown to have a role in the normal development of primary afferent neurons of the ENS. GF mice have been shown to decrease structural development of ENS neurons, with decreased nerve-fibre density and resultant deficits in contractility of the jejunum tissue and ileum (though not duodenum) compared to specific pathogen-free (SPF) mice (selecting out known pathogenic species in the microbiota) (Collins et al., 2014). A recent study has identified the aryl hydrocarbon receptor (AHR) transcription factor as a biosensor in the ENS under partial microbial control, acting as a link between the microbiome state and neuronal function in the distal myenteric neurons, potentially regulating neuronal excitability through downstream targets of AHR such as *Kcnj12* (Obata et al., 2020). Specifically, the authors found AHR expression was positively correlated with microbial load (along the longitudinal axis of the gut), reduced in GF and antibiotic treated animals, and reinstated in SPF colonization.

CNS neurodevelopment also appears to be at least partially dependent on the microbiome. Adult GF C57/Bl6 mice have been shown to exhibit several morphological differences of primary neurons and interneuron dendritic structures in the hippocampus and basao-lateral amygdala, (Luczynski et al., 2016). The offspring of both GF and antibiotic-treated mice have altered expression of axonogenesis-related genes, and consequently stunted and growth-factor-insensitive thalamocortical axons (Vuong et al., 2020). A failure of the thalamocortical system to properly develop and connect would of course have implications for epilepsy, due to its particular involvement in absence seizures and epileptic loss of consciousness (Crunelli et al., 2020). Another study demonstrated that depleted microbiomes (in GF and antibiotic-treated animals) were associated with increased cFos expression in extrinsic enteric-associated neurons and also with activation of brainstem sensory nuclei, highlighting a potential mechanism of microbiome to brain communication by which the former might modulate CNS activity (Muller et al., 2020).

Microbiome composition in people with epilepsy has been linked to a variety of interacting factors. Firstly, there may be a distinctive epilepsy-associated microbiome profile featuring increased proteobacteria and fusobacteria (Safak et al., 2020). Notably, drug-resistant patients with epilepsy have fewer bifidobacterial and lactobacilli compared to drug-responsive patients and to healthy controls (Peng et al., 2018). Unsurprisingly the ketogenic diet is associated with an altered microbiome in mice (Olson et al., 2018), and it also changes the human microbiome in a manner distinct from that of other high-fat diets (Ang et al., 2020), including in infants with refractory epilepsy (Zhang et al., 2018). The observation that the ketogenic diet decreases kynurenine (but not tryptophan or 3-OH-kynurenine) levels in blood may also reflect the role of the microbiome in the effects of this diet, because of the links between tryptophan metabolism and gut flora (O'Mahony et al., 2015). If these links should lead to causal microbiome effects on epilepsy expression, then it will be important to tease out the mechanisms by which they modulate neuronal excitability and activity en route to influencing seizures.

9. Conclusions & implications for epilepsy

In the burgeoning field of gut microbiome influence on the brain and CNS, there have now been multiple examples of such an influence impacting neuronal excitability and firing in distinct situations. Likewise, multiple possible routes or pathways by which this influence might be exerted have now been demonstrated. What remains is to join the dots: that is, to comprehensively establish in specific physiological or pathological conditions the role that the microbiome *does* play, and the mechanism by which it does so. This is certainly true in the case of epileptic excitability and activity. Microbiota influence on nearby enteric neurons and more distant CNS neurons can include both increasing and decreasing excitability and activity, as demonstrated with both positive and negative modulations of the microbiome. One must also consider that any perturbation of the normal microbiome, whether by depleting or supplementing, might have similar effects on its nervous system influence.

We may draw a few tentative conclusions from the aggregate of relevant studies. There may be a common theme of microbiome depletions (antibiotic, GF) decreasing neuronal firing and power in certain frequency bands (Table 3). Positive microbiome modulations (probiotics, prebiotics, other bacterial additions) seem roughly equally likely to increase or decrease a given parameter of neuronal excitability or activity (Tables 1–3). In the specific case of epilepsy a surprising variety of positive microbiome modulations have proved effective in decreasing seizure susceptibility and/or severity in animal models, which is promising for translatability but carries limited mechanistic information (Table 4). The only examples of the microbiome increasing seizure susceptibility come from FMT studies, perhaps because most seizure studies will understandably be seeking to suppress their occurrence. Human trials of direct microbiome effects are also very much in their infancy, with no demonstrations at scale, apart from the ketogenic diet itself.

In summary, the microbiome holds great promise for the management of epilepsy by modulation of neuronal excitability and activity. In the best case scenario a targeted microbiome therapy could be more potent and easier to administer than a dietary intervention, with fewer side effects than an anti-epileptic drug. To get there, however, we are likely to need both a thorough mechanistic understanding of how the microbiome achieves these modulations and a complete picture of their consequences for epilepsy symptoms and behaviour in a variety of contexts.

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